CLINICAL STUDY

Cytotoxic T lymphocyte antigen-4 Ala17 polymorphism is a genetic marker of autoimmune adrenal insufficiency: Italian association study and meta-analysis of European studies

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Abstract

Objective: Cytotoxic T lymphocyte antigen-4 (*CTLA4*) gene polymorphism has been associated with human autoimmune diseases, but discordant data are available on its association with autoimmune Addison's disease (AAD). We tested the human leukocyte antigen (HLA)-independent association of *CTLA4* +49 (A/G) (Ala 17) and/or *CTLA4* CT60 (A/G) polymorphism with AAD.

Design: DNA samples from 180 AAD patients and 394 healthy control subjects from continental Italy were analyzed, and association statistical analyses and meta-analysis of published studies were performed.

Methods: TaqMan minor groove binder chemistry assays and PCR fragment length polymorphism assays were used.

Results: Frequency of allele G of *CTLA4* +49 was significantly increased among AAD patients (40% alleles) than among healthy controls (27% alleles; $P < 0.0001$). *CTLA4* CT60 polymorphism was associated with AAD only in the heterozygous A/G individuals. The frequency of +49 AG+GG genotypes was significantly higher among AAD patients than among healthy control subjects, in both a co-dominant ($P < 0.0001$) and G dominant model ($P < 0.0001$). *CTLA4* +49 allele G was significantly associated with disease risk in both patients with isolated AAD and in patients with autoimmune polyendocrine syndrome. Multivariate logistic regression analysis showed that *CTLA4* +49 allele G was positively associated with AAD ($P < 0.0001$, odds ratio (OR) = 2.43, 95% confidence interval = 1.54–3.86) also after correction for *DRB1**03-*DQA1**0501-*DQB1**0201, *DRB1**04-*DQA1**0301-*DQB1**0302, and sex. Meta-analysis of five studies revealed a significant association of *CTLA4* +49 allele G with AAD ($P < 0.0001$) with an overall OR of 1.48 (1.28–1.71).

Conclusions: The *CTLA4* +49 polymorphism is strongly associated with genetic risk for AAD, independently from the well-known association with HLA class II genes.

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Introduction

Autoimmune Addison's disease (AAD) is the most frequent cause of primary adrenal insufficiency in western countries and Japan (1, 2), and is caused by an immunologically mediated destruction of the adrenocortical cells (3). Autoreactive T-cells are thought to play a critical role in the adrenal autoimmune process (3), and the disease is made evident by the appearance of circulating adrenal autoantibodies, mainly directed against the steroidogenic enzyme 21- β -hydroxylase

autoantibodies (21OHAb) (4–8). Human leukocyte antigen (HLA) class II genes *DRB1*, *DQA1*, and *DQB1* are the main genetic factors influencing the risk of disease (9). Similarly to other endocrine autoimmune diseases, HLA-*DRB1**03-*DQA1**0501-*DQB1**0201 (DR3-DQ2) and *DRB1**04-*DQA1**0301-*DQB1**0302 (DR4-DQ8) are positively and *DRB1**0403 is negatively associated with risk for AAD (10–12). Other predisposing genetic factors include the *CIITA* gene, the *PTPN22* gene, the vitamin D receptor gene, and the *CYP27B1* gene (reviewed in (13)). However, the prevalence of

AAD (14, 15) is much lower than that of other autoimmune diseases that share a similar genetic association with HLA haplotypes, and the precise mechanisms involved in the immune-mediated destruction of adrenocortical cells remain elusive.

T-cell activation requires both a signal provided by antigen-TCR engagement and co-stimulatory signal mainly provided by the interaction of CD28 with its ligand B7.1/B7.2 on antigen-presenting cells. The cytotoxic T lymphocyte antigen-4 (CTLA4) is a co-stimulatory molecule that binds to B7.1/B7.2, but provides negative signal to T-cell activation via transduction of an intracellular signal and modulation of tryptophan catabolism in B7-expressing dendritic cells (16). Several investigations have been carried out to test the hypothesis that *CTLA4* gene polymorphism contributes to genetic risk for autoimmune diseases. *CTLA4* polymorphism has been found to be associated with Graves' disease, Hashimoto's thyroiditis, type 1 diabetes mellitus (T1DM), primary biliary cirrhosis, multiple sclerosis, celiac disease, and rheumatoid arthritis (17).

Discordant results have been generated for the genetic association of *CTLA4* polymorphism with AAD. A first study of *CTLA4* exon 1+49 (A/G) (Ala17) polymorphism reported no statistically significant association with AAD in the German population (18), though AAD patients carrying the *DQA1**0501 allele were significantly more *CTLA4*+49 G allele-positive than controls with the same *DQA1* allele (18). Subsequent studies showed significant associations of *CTLA4* microsatellite polymorphism (variant lengths of a dinucleotide (AT)*n* repeat) within exon 3 (19) and of *CTLA4*+49 (Ala17) polymorphism (20) in English AAD patients. However, two more recent studies (21, 22) in two different European populations did not demonstrate a significant association between *CTLA4*+49 polymorphism and AAD. An association with a series of polymorphisms different from Ala17 was observed in Norwegian and UK patients (22). The sequencing of the 300-kb region of chromosome 2q33 enabled Ueda *et al.* (23) to demonstrate that the CT60 SNP was the most associated marker with some endocrine autoimmune diseases. Accordingly, the discordant results generated by the studies of *CTLA4* polymorphism in AAD patients have not answered conclusively the question whether the *CTLA4* gene contributes to the genetic risk for AAD or not.

A complicating factor is the frequent association of AAD with other autoimmune diseases – more often thyroid diseases, T1DM, or premature ovarian insufficiency – which configures the so-called autoimmune polyendocrine syndromes (APS) (3). Approximately two-thirds of AAD patients show clinical or subclinical manifestations of at least another autoimmune disease (3), thus making the identification of an AAD-specific genetic background somehow problematic. Furthermore, the frequency of at-risk HLA haplotypes in the general population varies greatly among countries (24),

and the interplay between genetic and environmental factors may be profoundly different in different ethnic groups or geographic areas.

In our present study, we analyzed a large set of genomic DNA samples from AAD patients and healthy control subjects enrolled by the Italian Addison Network (IAN), to test the hypothesis that *CTLA4*+49 polymorphism is associated with the genetic risk for AAD in Italy. We also analyzed the *CTLA4* CT60 polymorphism that was found to be positively associated with AAD in a previous study (22). The AAD patients were identified using a comprehensive flowchart that takes into consideration immunological, clinical, and biochemical data (25), so to minimize the possibility to include nonautoimmune cases in the studied population. The data were analyzed separately in APS patients and in patients with isolated AAD, and the interaction between HLA class II polymorphism and *CTLA4* gene polymorphism was tested by stratification analysis and multivariate logistic regression analysis. Finally, to better analyze the genetic contribution of *CTLA4* polymorphism for AAD, we also performed meta-analysis of published results on *CTLA4* exon 1+49 (A/G) polymorphism.

Materials and methods

Patients and control subjects

Genomic DNA was available from 180 AAD patients and 394 healthy control subjects from continental Italy for the *CTLA4*+49 (A/G), and from 153 AAD patients and 242 healthy control subjects for the *CTLA4* CT60 (A/G) genotyping.

IAN includes 14 endocrinological centers located in nine regions of continental Italy (24). Between January 1998 and April 2009, IAN has enrolled 372 patients with primary adrenal insufficiency. According to a recent update of diagnostic criteria for the etiological classification of primary adrenal insufficiency (25) and based on the combined use of immunological, biochemical, and clinical data, 260 cases (70%) were reclassified as AAD. Classification of AAD was based on the presence of 21OHAb and/or adrenal cortex autoantibodies (ACA) in the absence of clinical, biochemical, or imaging signs of a nonautoimmune cause of adrenal insufficiency. Of the 260 AAD patients, genomic DNA was available for the present study from 180 AAD patients (median age at diagnosis: 36 years, range 8–73 years; F/M ratio: 1.78). A total of 111 (62%) cases had other clinical, subclinical, or latent autoimmune diseases and were classified as 'APS'. The remaining 69 AAD patients were classified as 'isolated AAD'. APS I was excluded in all cases on the basis of clinical manifestations, and no APS I patient was included in our study.

Blood samples collected during routine analyses, between March 1994 and December 2003, were available from 394 unrelated healthy control subjects (median age: 30 years, range 4–70 years; F/M ratio:1.02) with no family history of endocrine autoimmune diseases. All patients and healthy individuals gave their informed consent for the study, and the study was approved by the local ethics committee.

HLA-DR and -DQ genotyping

HLA-DR and -DQ genotyping was performed by SSO-dot blot analysis with modifications of a previously described method (26), and using sequence-specific oligonucleotides, 3'-end labeled with digoxigenin (Roche Diagnostics S.p.A). A chemiluminescent signal was generated by using alkaline phosphatase-labeled anti-digoxigenin (Roche) and CSPD (Roche). Chemiluminescence was measured in a microplate scintillation and luminescence counter (TopCount NXT, Packard Instrument Company, Meriden, CT, USA). Each membrane contained ten control samples with known HLA genotype.

HLA-DRB1*04 subtyping was performed by PCR-SSP with sequence-specific primers, according to the method described by Zetterquist & Olerup (27).

Genotyping of CTLA4

The *CTLA4* exon 1+49 (A/G) and the *CTLA4* CT60 (A/G) SNPs were analyzed with TaqMan SNP Genotyping Assays (Applied Biosystems, Foster City, CA, USA) using a Stratagene MX3000P QPCR System and MXPro QPCR Software. In addition, PCR restriction fragment length polymorphism assays (28) were also used for both SNPs to confirm genotyping results.

Adrenal autoantibody assays

21OHAb were tested using a previously published radio-binding assay with *in vitro* translated recombinant human ³⁵S-21OH (6). Antibody levels were expressed as a relative index (21OHAb index), calculated as: (mean c.p.m. sample – mean c.p.m. of two negative standards)/(mean c.p.m. positive standard – mean c.p.m. of two negative standards), with the upper limit of normal values defined as the mean + 3 S.D. of 200 healthy control subjects.

ACA were determined using indirect immunofluorescence on cryostatic sections of human adrenal glands (29). Levels of ACA were expressed as the reciprocal of the end point dilution titer.

Statistical analysis of Italian data

The odds ratio (OR) was calculated according to Woolf (30) and Miettinen (31). Differences in frequencies between patients and healthy control subjects were

tested by the χ^2 method with Yates' correction or the Fisher's exact test; the *P* values were corrected for multiple testing. For the HLA haplotyping, the probability values were corrected for the number of comparisons, according to the number of HLA-DRB1, -DQA1, and -DQB1 alleles or haplotypes observed. For the *CTLA4* polymorphism analysis, both a co-dominant model and G dominant model were applied. Differences in autoantibody levels (21OHAb index) were tested by the nonparametric Mann–Whitney test.

The association of the dichotomous variables presence or absence of *DQA1**0501-*DQB1**0201, *DQA1**0301-*DQB1**0302, and *CTLA4*+49 G allele with AAD, and its dependence on sex, was tested by multivariate logistic regression analysis. Results for logistic regression were expressed in terms of adjusted ORs and 95% confidence interval (CI).

Absolute risk values for the development of AAD of combinations of HLA and *CTLA4*+49 haplotypes/alleles were estimated according to the available prevalence data of primary adrenal insufficiency in continental Italy (14) (117 cases per million, with 97% ascertainment degree, making an estimated ratio of 120 cases per million) and the estimated frequency of AAD among Italian patients with primary adrenal insufficiency (minimum: 70%, according to (32) and IAN data; maximum: 83%, according to (3)). Accordingly, the estimated prevalence of AAD (non-APS I) in continental Italy is 84–100 cases per million.

Linkage disequilibrium between the two *CTLA4* SNPs studied was measured using the software MIDAS rel. 1.0 (33).

Deviations from the Hardy–Weinberg equilibrium (HWE) were tested by comparison of observed and expected genotype frequencies. A *P* value <0.05 was considered significant in all tests. All data analyses were performed using SPSS for Windows (release 13.0.; SPSS Inc., Chicago, IL, USA).

Meta-analysis

Identification and eligibility of studies We conducted MEDLINE, Current Contents, and Web of Science searches using 'adrenal insufficiency', 'Addison's disease', 'CTLA4', 'CTLA-4', and 'polymorphism' as keywords for papers published with no date restriction. No additional articles were identified through the references cited in the first series of articles selected. Articles included in meta-analysis were in any language, with human subjects, published in the primary literature and with no obvious overlap of subjects with other studies. Case–control studies were eligible if they had determined the distribution of genotypes in Addison's disease cases and in controls using a molecular method for genotyping. We identified four case–control studies (18, 20–22) that provided information on autoimmune Addison's disease occurrence associated with *CTLA4*+49 A/G polymorphism.

Data extraction and assessment of study quality

For each study, we extracted the following characteristics: authors, year of publication, and characteristics of cases and controls (age at onset, sex ratio, ethnic group, and source of population), number of genotyped cases and controls, frequency of the genotypes, HWE, and ORs estimation. If ORs with 95% CI or HWE assessment were not reported in the publication, we derived them from the raw data presented.

Meta-analysis We combined our data with that coming from literature revision using both fixed effects and random effect models. Heterogeneity among the studies was evaluated by the Cochrane's *Q* test and was considered significant for $P < 0.10$. To test for publication bias, both Begg's and Egger's tests (34, 35) were used. In a sensitivity analysis, we formally assessed the influence of individual studies on pooled estimates according to Tobias' method (36), in which if the point estimate of the pooled estimate with one study omitted lies outside the CI of the overall estimate with all available trials contributing, then the study in question has an excessive influence. Most calculations regarding meta-analysis were performed with computer program MIX Version 1.7 (37) (<http://www.mix-for-meta-analysis.info>).

Results**CTLA4 exon 1 + 49 (A/G) polymorphism is associated with genetic risk for AAD in Italy**

None of the tested genetic variables showed significant deviations from HWE in the studied population.

Allele G of CTLA4 + 49 was significantly more frequent in the group of AAD patients than in the control group, with it being detected in 146/360 (40%) AAD alleles and in 210/688 (27%) healthy control alleles ($P < 0.0001$; Table 1). Thus, allele G was detected

in 45% of healthy control subjects and in 69% of Addison patients (in either heterozygous or homozygous combination). The frequency of the AG + GG genotypes was significantly higher among AAD patients than among healthy control subjects, in both the co-dominant model ($P < 0.0001$) and G dominant model ($P < 0.0001$; OR = 2.66; 95% CI: 1.83–3.86). This association was not influenced by the presence/absence of other autoimmune diseases as allele G frequency was similar in isolated AAD and in AAD plus other autoimmune diseases (APS group; Table 1).

CTLA4 CT60 (A/G) polymorphism in AAD

Neither allele G nor allele A of CTLA4 CT60 polymorphism was significantly more frequent in AAD patients than in the control group, neither in the co-dominant model nor in the G dominant model (Table 2). A significant association with AAD was detected in A/G heterozygous individuals (91/153 (59%) AAD patients compared with 116/242 (48%) healthy control subjects; $P = 0.033$), though this association was no longer significant after correction for a number of comparisons.

A significant linkage disequilibrium was detected between the CTLA4 + 49 SNP and the CTLA4 CT60 SNP ($D' = 0.762$).

CTLA4 exon 1 + 49 (A/G) polymorphism is independently associated with AAD

As expected, the HLA genotyping of the AAD patients revealed that DRB1*03-DQA1*0501-DQB1*0201 (DR3-DQ2; 48% in AAD versus 14% in healthy control subjects, corrected $P = 0.003$) and DRB1*04-DQA1*0301-DQB1*0302 (DR4-DQ8; 23% in AAD versus 6% in healthy control subjects, corrected $P = 0.003$) were positively and significantly associated with AAD.

Table 1 Genotype and allele frequencies for the cytotoxic T lymphocyte antigen-4 (CTLA4) + 49 (A/G) polymorphism in Italian AAD patients.

	Genotype frequencies												<i>n</i>
	Allele frequencies				Co-dominant model				G dominant model				
					AA	AG	GG	<i>P</i> value	AA	AG + GG	OR (95% CI) <i>P</i> value		
	A	G	MAF	OR (95% CI) <i>P</i> value									
Total	214	146	0.405	1.88 (1.44–2.44)	56	102	22	<0.0001	56	124	2.66 (1.83–3.86)	180	
AAD				<0.0001							<0.0001		
Isolated	83	55	0.399	1.82 (1.25–2.66)	24	35	10	0.007	24	45	2.25 (1.32–3.84)	69	
AAD				0.002							0.004		
APS	134	88	0.396	1.81 (1.32–2.47)	35	64	12	<0.0001	35	76	2.61 (1.67–4.08)	111	
				<0.0001							<0.0001		
Healthy controls	578	210	0.266		215	148	31		215	179		394	

MAF, minor allele frequency; OR (95% CI), odds ratio (95% confidence interval); AAD, autoimmune Addison's disease; APS, autoimmune polyendocrine syndrome.

Table 2 Genotype and allele frequencies for the cytotoxic T lymphocyte antigen-4 (CTLA4) CT60 (A/G) polymorphism in Italian AAD patients.

	Allele frequencies				Genotype frequencies								<i>n</i>
	A	G	MAF	OR (95% CI) <i>P</i> value	Co-dominant model				G dominant model				
					AA	AG	GG	<i>P</i> value	AA	AG + GG	OR (95% CI) <i>P</i> value		
Total	155	151	0.493	1.11 (0.84–1.48)	32	91	30	NS	32	121	1.57 (0.97–2.53)	153	
AAD				NS							NS		
Isolated	60	56	0.483	1.07 (0.71–1.6)	12	36	10	NS	12	46	1.59 (0.8–3.18)	58	
AAD				NS							NS		
APS	98	92	0.484	1.07 (0.77–1.50)	21	56	18	NS	21	74	1.46 (0.84–2.56)	95	
				NS							NS		
Healthy controls	258	226	0.467		71	116	55		71	171		242	

MAF, minor allele frequency; OR (95% CI), odds ratio (95% confidence interval); AAD, autoimmune Addison's disease; APS, autoimmune polyendocrine syndrome; NS, not significant.

*DRB1*0403* was absent among the *DRB1*04*-positive AAD patients, but was the most frequent *DRB1*04* allele detected in healthy controls (35% of *DRB1*04*-positive individuals; $P < 0.001$).

Stratification of *CTLA4* results according to HLA status is shown in Table 3. *CTLA4*+49 allele G was detected in 68% of AAD patients negative for both HLA-*DRB1*0301-DQA1*0501-DQB1*0201* (DR3-DQ2) and *DRB1*04-DQA1*0301-DQB1*0302* (DR4-DQ8), in 67% of patients positive for HLA-*DRB1*03-DQA1*0501-DQB1*0201* (DR3-DQ2), and in 77% of patients positive for HLA-*DRB1*04-DQA1*0301-DQB1*0302* (DR4-DQ8) (P =not significant). The highest OR (> 20) was observed for the heterozygous *DRB1*03-DQA1*0501-DQB1*0201* (DR3-DQ2)/*DRB1*04-DQA1*0301-DQB1*0302* (DR4-DQ8) individuals who were also positive for the *CTLA4*+49 allele G, this combination being present in 9.4% of AAD patients and in only 0.5% of healthy control subjects (Table 3). On the other hand, the absence of all the three major genetic markers was significantly and negatively associated with disease risk, with an overall OR lower than 0.2 (Table 3). The presence of the *CTLA4*+49 allele G increased 3.2- to 3.7-fold the OR associated with the combination of at-risk HLA class II haplotypes (Table 3). Based on a prevalence ratio of 84–100 cases per million in continental Italy, estimated absolute risk values for AAD ranged from 1/532–1/633 in Italian subjects positive for all the three major genetic markers to 1/34,531–1/41,109 in subjects negative for all of them (Table 3).

The circulating levels of 21OHAb were not significantly associated with *CTLA4*+49 (A/G) polymorphism, as 21OHAb index was 0.713 (range 0.005–2.244) in allele G-negative AAD patients and 0.648 (range 0–5.332) in allele G-positive AAD patients (P =NS).

To better define the independent contribution of *CTLA4*+49 polymorphism to genetic susceptibility for

AAD, we then performed multivariate logistic regression analysis. *DRB1*0403* could not be included in this model because of co-linearity problems (with $B = -20.8 \pm 9655$), and the frequency of the *CTLA4*+49 allele G was tested in *DRB1*04*-positive healthy individuals who were positive or negative for the *DRB1*0403* subtype. The *CTLA4*+49 allele G was equally frequent in *DRB1*0403*-positive and *DRB1*0403*-negative healthy control subjects carrying the *DRB1*04-DQA1*0301-DQB1*0302* (DR4-DQ8) haplotype (27% in both groups), which shows that the association of *CTLA4* gene polymorphism with AAD did not depend on *DRB1*0403*. In the multivariate logistic regression model, *DRB1*03-DQA1*0201-DQB1*0501* (DR3-DQ2), *DRB1*04-DQA1*0301-DQB1*0302* (DR4-DQ8), *CTLA4*+49 allele G, and sex were included as independent variables. All the variables found to be associated at the univariate analysis were also found to be independently associated with AAD, after correction for sex (Table 4). Thus, frequency of *CTLA4* allele G continued to be positively associated with genetic risk for the disease ($P < 0.0001$, OR = 2.43, 95% CI = 1.54–3.86), also after correction for *DRB1*03-DQA1*0501-DQB1*0201* (DR3-DQ2) and *DRB1*04-DQA1*0301-DQB1*0302* (DR4-DQ8). In addition, the correction for sex in the multivariate analysis also excludes the possibility that the association of *CTLA4* with AAD was influenced by a different sex distribution among patients and healthy control subjects. When the multivariate logistic regression analysis was repeated for isolated AAD patients and for APS patients, similar results were obtained (Table 4). *CTLA4* allele G continued to be independently associated with disease risk even when the number of G alleles was included in the analysis, instead of presence or absence of G ($P = 0.002$; OR = 1.75, 95% CI = 1.23–2.47 in total AAD patients; $P = 0.037$; OR = 1.70, 95% CI = 1.03–2.79 in isolated AAD patients; and $P = 0.014$; OR = 1.70, 95% CI = 1.11–2.58 in APS patients).

Table 3 Estimated absolute risk values for development of AAD in Italy according to combinations of human leukocyte antigen and cytotoxic T lymphocyte antigen-4 (CTLA4)+49 haplotypes/alleles.

	AAD patients	Healthy controls	P value	P _c value	OR (95% CI)	Estimated absolute risk ^a
DR3-DQ2 pos/DR4-DQ8 pos/CTLA4+G pos	17/180 (9.4%)	2/394 (0.5%)	<0.001	<0.008	20.4 (4.67–89.5)	1/532–1/633
DR3-DQ2 pos/DR4-DQ8 neg/CTLA4+G pos	44/180 (24.4%)	19/394 (4.8%)	<0.001	<0.008	6.39 (3.60–11.3)	1/1967–1/2342
DR3-DQ2 neg/DR4-DQ8 pos/CTLA4+G pos	14/180 (7.8%)	9/394 (2.3%)	0.004	0.032	3.61 (1.53–8.50)	1/2949–1/3510
DR3-DQ2 pos/DR4-DQ8 neg/CTLA4+G neg	24/180 (13.3%)	32/394 (8.1%)	NS	NS	1.74 (0.99–3.05)	1/6090–1/7250
DR3-DQ2 neg/DR4-DQ8 pos/CTLA4+G neg	5/180 (2.8%)	11/394 (2.8%)	NS	NS	0.99 (0.34–2.91)	1/10 000–1/11 905
DR3-DQ2 neg/DR4-DQ8 neg/CTLA4+G pos	48/180 (26.7%)	147/394 (37.3%)	0.016	NS	0.61 (0.41–0.90)	1/13 970–1/16 630
DR3-DQ2 neg/DR4-DQ8 neg/CTLA4+G neg	23/180 (12.8%)	174/394 (44.2%)	<0.001	<0.008	0.19 (0.11–0.30)	1/34 531–1/41 109
DR3-DQ2 pos/DR4-DQ8 pos/CTLA4+G neg	5/180 (2.8%)	0%	0.005	0.04	ND	ND

AAD, autoimmune Addison's disease; NS, not significant; ND, not determined; P_c, corrected P value for number of comparisons (n=8); OR (95% CI), odds ratio (95% confidence interval).
^aCalculated using a 100 per million or a 84 per million prevalence of autoimmune Addison's disease in continental Italy.

Meta-analysis of association studies of CTLA4+49 (A/G) polymorphism in AAD

We identified four case-control studies that provided information on AAD occurrence associated with the CTLA4+49 (A/G) polymorphism (18, 20–22). Thus, five studies were included in the meta-analysis (including our present Italian study; Fig. 1). CTLA4+49 (A/G) data showed no significant deviations from HWE in any of the five studies.

The meta-analysis showed a positive and significant association between the CTLA4+49 (A/G) polymorphism and AAD in the five studies ($P<0.0001$) with a global OR of 1.48 (1.28–1.71; Fig. 1). The Cochrane's *Q* test did not detect any significant heterogeneity of OR ($P=0.3$) among the studied populations. The sensitivity analysis did not result in loss of statistical significance by excluding one study at a time. The random and fixed effects models provided similar results.

Discussion

The cytotoxic T lymphocyte antigen-4 (CTLA4) gene has emerged as an important susceptibility locus for autoimmune endocrinopathies (17). However, controversial data have been published for the association of CTLA4 polymorphism with AAD (18–22). In our present study, we demonstrate that CTLA4 exon 1+49 (A/G) (Ala17) polymorphism is significantly associated with disease risk in AAD patients from continental Italy. In spite of a strong linkage disequilibrium with CTLA4+49 (A/G), the CTLA4 CT60 (A/G) polymorphism was not found to be significantly associated with AAD in our study, contrarily to what was observed in other populations (22). We also show that isolated AAD is associated with CTLA4+49 polymorphism, thus excluding the possibility that the genetic association might be due to the high frequency of other autoimmune diseases in patients with AAD. We performed both stratification analysis and logistic regression analysis to demonstrate that CTLA4+49 (A/G) polymorphism is independently associated with genetic risk for AAD, and that allele G is increasing the risk to develop clinical AAD. Finally, to resolve the controversy generated by previous association studies in AAD (18–22), we performed meta-analysis that included all studies currently available on CTLA4+49 allele G frequency in Addisonian patients. The results of this meta-analysis clearly provide further indication that CTLA4+49 allele G contributes to the genetic background predisposing for AAD.

Several hypotheses can be formulated to explain the discrepancies among different studies of CTLA4+49 (A/G) polymorphism in AAD. First, the number of AAD patients studied in the reference populations ranged from 57 to 134 (18, 20–22), and the low number of

Table 4 Results of multivariate logistic regression analysis for association with autoimmune Addison's disease in Italy.

Factors	Estimate	S.E.M.	Wald χ^2	P	OR (95% CI)
Total AAD patients					
<i>DRB1</i> *03- <i>DQA1</i> *0501- <i>DQB1</i> *0201	1.686	0.243	48.231	<0.0001	5.40 (3.35–8.68)
<i>DRB1</i> *04- <i>DQA1</i> *0301- <i>DQB1</i> *0302	1.238	0.335	13.634	<0.0001	3.45 (1.79–6.65)
<i>CTLA4</i> +49 allele G	0.890	0.235	14.377	<0.0001	2.43 (1.54–3.86)
Isolated AAD patients					
<i>DRB1</i> *03- <i>DQA1</i> *0501- <i>DQB1</i> *0201	1.933	0.340	32.361	<0.0001	6.91 (3.55–13.45)
<i>DRB1</i> *04- <i>DQA1</i> *0301- <i>DQB1</i> *0302	0.980	0.510	3.696	0.048	2.67 (1.02–7.24)
<i>CTLA4</i> +49 allele G	0.687	0.351	3.826	0.049	1.99 (1.01–3.95)
APS patients					
<i>DRB1</i> *03- <i>DQA1</i> *0501- <i>DQB1</i> *0201	1.583	0.303	27.257	<0.0001	4.871 (2.67–8.83)
<i>DRB1</i> *04- <i>DQA1</i> *0301- <i>DQB1</i> *0302	1.514	0.394	14.795	<0.0001	4.543 (2.10–9.82)
<i>CTLA4</i> +49 allele G	0.876	0.299	8.584	0.003	2.402 (1.34–4.32)

OR (95% CI), odds ratio (95% confidence interval).

cases may explain the lack of statistical significance in some studies. Secondly, primary adrenal insufficiency may be caused by a nonautoimmune process (post-tuberculosis, post-paracoccidioidomycosis, adrenoleukodystrophy, adrenal hemorrhage, sepsis, genetic disorders, or others), and only an accurate etiological classification of each patient can ensure that only autoimmune cases are included in the studied population. Given the low number of cases enrolled in some studies, the inclusion of nonautoimmune patients may critically reduce the power of the statistical analysis. For this reason, the IAN has developed a comprehensive flowchart for the accurate classification of primary adrenal insufficiency and the identification of patients with AAD (25), which has enabled us to include in our study only patients with a demonstrated adrenal autoimmune process. Thirdly, the high frequency of the low penetrance disease *CTLA4* alleles in the normal general population (23) could also explain the difficulties in confirming the association in different populations. Finally, the risk of developing primary adrenal insufficiency is not dramatically different among European countries – ranging from 120 cases per million in Italy (14) to 140 cases per million in Norway (15) – but the frequency of at-risk HLA haplotypes is two- to three-fold higher in Scandinavia than in Southern Europe. Accordingly, non-HLA genes, as well as environmental factors, must play a critical role in modulating the risk for AAD. In addition, one cannot

exclude the possibility that the weight of each specific gene might differ in different geographic areas, under the pressure of a specific environment, thus making possible that a gene marker is strongly associated with disease risk in one country, but not in another.

The *CTLA4*+49 allele G is significantly increased in patients with T1DM or thyroid autoimmune diseases, and in some studies, it has been postulated that AAD would be associated with *CTLA4* polymorphism when in the context of APS (18, 20). Our study shows that AAD is associated with this gene marker also in patients with no other manifestation of autoimmunity, thus supporting the hypothesis that *CTLA4* polymorphism contributes to the genetic background for adrenal autoimmunity. Nevertheless, AAD shares the major genetic predisposing markers with other endocrine autoimmune diseases, and at present it is not yet possible to resolve the specific genetic background of each disease component of APS. It had been proposed that the *DRB1**0404 subtype might discriminate AAD patients from T1DM patients (12, 38), but the frequency of *0404 in the general population cannot provide an explanation for the high difference in prevalence between T1DM and AAD. In addition, no selective association with *0404 was found in our previous study in Italian AAD patients, in which only the *0403 subtype was found to be strongly and negatively associated with both T1DM and AAD (11).

Figure 1 Odds ratio meta-analysis plot of the association of *CTLA4*+49 (A/G) polymorphism with AAD.

It is unclear why we could not reproduce the results of other studies (23) regarding association of the *CTLA4* CT60 polymorphism with AAD. One possibility is that the number of subjects studied did not ensure sufficient power to the statistical analysis. So we cannot rule out the possibility that this polymorphism might be associated with adrenal insufficiency also in the Italian population. However, based on our data, we can exclude an OR higher than 1.4, much lower than the one observed with the *CTLA4* +49 polymorphism. The observed *CTLA4* CT60 allele frequency in our Italian control population is similar to that reported in a previous study from a different country (22). On the other hand, the frequency of allele G of *CTLA4* +49 was much lower in our Italian controls than in controls from other European countries in previous studies (18, 22), which may explain why a strong association to AAD for the G allele of *CTLA4* +49 was found in Italy, but not in the other two above-mentioned cohorts.

At present, it is still unclear how gene polymorphism might influence *CTLA4* function. It has been proposed that the predisposing alleles might influence the levels of circulating soluble CTLA-4 (sCTLA-4) (23). A reduction in sCTLA-4 level could determine the blocking of CD80/CD86 molecules, with subsequent T-cell activation through CD28 (23). Alternatively, higher levels of sCTLA-4 could compete with membrane-bound CTLA-4 for CD80/CD86-binding sites, thus causing a reduction of inhibitory signaling.

In conclusion, our study provides data indicating that *CTLA4* polymorphism modulates the genetic risk for AAD. Further studies are needed to unravel the molecular mechanisms of genetic predisposition to AAD and to expand our knowledge of the effects of the predisposing alleles/haplotypes on the development of the adrenal autoimmune process.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

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