CYP21A2 polymorphisms in patients with autoimmune Addison's disease, and linkage disequilibrium to HLA risk alleles

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Abstract

Objective: Steroid 21-hydroxylase, encoded by CYP21A2, is the major autoantigen in autoimmune Addison's disease (AAD). CYP21A2 is located in the region of the HLA complex on chromosome 6p21.3, which harbours several risk alleles for AAD. The objective was to investigate whether CYP21A2 gene variants confer risk of AAD independently of other risk alleles in the HLA loci.

Design: DNA samples from 381 Norwegian patients with AAD and 340 healthy controls (HC) previously genotyped for the HLA-A, -B, -DRB1, and -DQB1 and MICA loci were used for genotyping of CYP21A2.

Methods: Genotyping of CYP21A2 was carried out by direct sequencing. Linkage of CYP21A2 to the HLA loci was assessed using UNPHASED version 3.0.10 and PHASE version 2.1.

Results: Heterozygotes of the single-nucleotide polymorphisms (SNPs) rs397515394, rs6467, rs6474, rs76565726 and rs6473 were detected significantly more frequently in AAD patients compared with HC ($P < 0.005$), but all SNPs were in a linkage disequilibrium (LD) with high-risk HLA-DRB1 haplotypes. rs6472C protected against AAD (odds ratio = 0.15, 95% CI (0.08–0.30), $P = 3.8 \times 10^{-10}$). This SNP was not in an LD with HLA loci ($P = 0.02$), but did not increase protection when considering the effect of HLA-DRB1 alleles. Mutations causing congenital adrenal hyperplasia were found in heterozygosity in <1.5% of the cases in both groups.

Conclusion: Genetic variants of CYP21A2 associated to AAD are in LD with the main AAD risk locus HLA-DRB1, and CYP21A2 does not constitute an independent susceptibility locus.

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Introduction

Autoimmune Addison's disease (AAD) is caused by the destruction of hormone-producing cells in the adrenal cortex by autoreactive immunological mechanisms. AAD often occurs together with other organ-specific autoimmune disorders, as part of autoimmune polyendocrine syndromes types 1 and 2 (APS1 and APS2 respectively) (1, 2). APS1 is a rare monogenic autosomal recessive disease caused by mutations in the autoimmune regulator (AIRE) gene (3). Isolated AAD and APS2, however, are

thought to be caused by susceptibility variants of multiple genes interacting with environmental triggers. To date, the HLA class II haplotypes DRB1*03:01 and DRB1*04:04 have been shown to be the strongest predisposing genetic factors for AAD with odds ratio (OR) of 2.9 and 3.3 (4) respectively. Risk is particularly high when these haplotypes are combined (OR = 32) (2). The HLA-class I genes HLA-A and HLA-B (OR = 2.6 of HLA-B*08) and MHC-class I-related chain A (MICA) also harbour the AAD risk alleles

(4, 5, 6, 7). In addition, several other genes related to the immune system have been associated with AAD: cytotoxic T lymphocyte antigen-4 (*CTLA-4*) (8, 9), protein tyrosine phosphatase non-receptor type 22 (*PTPN22*) (10, 11), MHC class II transactivator (*CIITA*), C-type lectin domain family 16, member A (*CLEC16A*) (12), cytochrome P450, family 27, subfamily B, polypeptide 1 (*CYP27B1*) (13, 14) and programmed death ligand (*PD-L1*) (15).

The major autoantigen in AAD is the enzyme steroid 21-hydroxylase (21OH), which is expressed mainly in the adrenal cortex (16), and circulating autoantibodies (Ab) against 21OH are present in more than 84% of patients with AAD in various European cohorts (2, 17, 18, 19). 21OH is encoded by *CYP21A2*, and mutations in this gene are the main cause of congenital adrenal hyperplasia (CAH) (20). Polymorphisms and mutations in *CYP21A2* are common, mainly due to the presence of the structurally related pseudogene *CYP21A1P* (21). The variants in *CYP21A2* may potentially have an impact on the immunogenicity of 21OH and thereby influence the chance of an autoreactive response being directed towards it. The examples of such effects are seen in type 1 diabetes where autoantibody frequencies towards the zinc transporter eight (*SLC30A8*) are dependent on which isoform is expressed (22). Moreover, different isoforms of the thyroid-stimulating hormone receptor (TSHR) vary in their immunogenicity (23, 24). The expression of *CYP21A2* in thymic medullary cells could potentially be dependent on polymorphisms, thereby influencing negative selection of 21OH-specific T cells.

We therefore investigated whether *CYP21A2* is a susceptibility gene for AAD. Previously, two studies have addressed *CYP21A2* polymorphisms in relationship with the risk of developing AAD without finding any disease-specific variants (25, 26). However, both studies were underpowered; the largest cohort included 24 patients with AAD. In this study, we analysed the much larger well-characterised Norwegian cohort ($n=381$). As *CYP21A2* is located on chromosome 6p21.3 in the *HLA*-III locus, which is in strong linkage disequilibrium (LD) with the *HLA*-I and II loci, such a large sample size is required to determine whether potential *CYP21A2* variants add to the risk of AAD independently of the *HLA* loci.

Subjects and methods

Subjects

A total of 381 patients with AAD (64% females, 36% males; mean age 58 years) were recruited from the National

Norwegian Registry of patients with Addison's disease (2) and 340 anonymous Norwegian blood donors controls (healthy controls (HC)) were available for genetic analyses. 21OH autoantibody index values as determined by RIA were available for all patients (2). AAD was diagnosed in patients with adrenal insufficiency with either a positive 21OH-Ab test, or adrenal insufficiency combined with another endocrine autoimmune disease. Of the 381 AAD patients, 92% were positive for 21OH-Ab, and 58% had APS2 (Addison's disease plus autoimmune thyroid disease and/or type 1 diabetes), while 34% had isolated AAD.

Ethics

All included patients and blood donors signed a written consent form. The study was approved by the Regional Committee for Medical Ethics of Western Norway and carried out according to the Helsinki declaration.

Genetic analyses

DNA was isolated from whole blood using standard commercial kits. The sequence of the whole *CYP21A2* gene, including ten exons and nine introns, were analysed in 85 patients with AAD. As the antigenicity of 21OH most likely would be located in the coding regions, coding polymorphisms and rare variants detected in the 85 patients were sought for in a larger material of AAD patients ($n=381$) and HC ($n=340$). The *CYP21A2* gene was amplified in two different fragments using primers discriminating the pseudogene from the functional *CYP21A2* gene. The primers specific for *CYP21A2* were targeted to the eight base pair (bp) deletion regions in exon 3 and the exon 6 cluster regions, which are the major markers for distinguishing the pseudogene from *CYP21A2*. The first primer pair amplified a fragment starting with the promoter region and ending with the four-point mutation region in exon 6 (21) (fragment 1); the second primer pair amplified the gene from exon 3 spanning the eight bp deletion region (21) to 170 bp of the 3' end from exon 10 (fragment 2). Expand high fidelity PCR system (Roche) was used for amplifying PCR fragment 1; the thermal cycling protocol consisted of an initial denaturation step at 94 °C for 2 min, followed by ten cycles of denaturation at 94 °C for 15 s, annealing at 55 °C for 30 s and elongation at 72 °C for 2.5 min. Then the cycle was repeated 20 times with an increment of 5 s for each cycle. The PCR was terminated by an elongation step at 72 °C for 7 min. AmpliTaq Gold with Gene Amp PCR system (Life Technologies) was used for amplifying PCR fragment 2; the thermal cycling conditions were initial denaturation at

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96 °C for 10 min, followed by 35 cycles of 94 °C for 20 s, 54 °C for 20 s and 72 °C for 2.5 min. The final elongation step was run at 72 °C for 7 min. The variants and single-nucleotide polymorphisms (SNP) of *CYP21A2* were identified by direct sequencing using standard conditions on an ABI 3730 DNA analyser. The primers (purchased from Eurogentec, Seraing, Liège, Belgium) of PCR and sequencing reactions have been described previously (27, 28, 29). Sequencing data were aligned to the *CYP21A2* gene sequence (<http://www.ncbi.nlm.nih.gov/nucore/M12792.1>). As a quality control to check that the pseudogene was not amplified in the PCR product, we checked that no pseudogene variants were present in the sequences. The copy number of the *CYP21A2* gene was determined in AAD patients and HC by duplex real time PCR method (30). The genotyping of *HLA-A*, *-B*, *-DRB1* and *-DQB1*, and *MICA* was described previously by (4).

Statistical analyses

χ^2 and Fisher's exact tests for the determination of significant differences between genotype and allele frequencies of patients and HC, grouped according to age at diagnosis, patients with or without type 1 diabetes or thyroid disease and gender; and calculations of OR with 95% CIs were performed by IBM SPSS Statistics 20 using significance level of $P < 0.05$. The calculation of Hardy–Weinberg equilibrium ($P > 0.001$ cutoff) and LD map of the *CYP21A2* gene SNPs

were performed by Haploview version 4.2 (31). Global association tests with conditional analysis for allele main effects were performed by UNPHASED version 3.0.10 (32) to determine whether the associations with *CYP21A2* were independent ($P < 0.05$) of the *HLA* loci or not. Haplotype frequencies were generated by PHASE version 2.1 (33). D'Agostino and Pearson omnibus normality test was used to test whether the values of 21OH index and age at diagnosis were distributed in a Gaussian manner, and Kruskal–Wallis test with Dunn's multiple comparison was used for testing differences between *CYP21A2/HLA* genotypes or haplotypes and 21OH antibody index values and mean age at diagnosis, using the Graph Pad Prism version 5.02 for Windows (GraphPad Software Inc., La Jolla, CA, USA).

Results

SNPs and haplotypes of *CYP21A2* in AAD patients and HC

The common exon variants p.9_10insL/g.1705_1706 insCTG (rs397515394), p.K102R/g.2361A>G (rs6474), p.N493S/g.4377A>G (rs6473), the intron 2 variant g.2333C>A (rs6467) and the intron 3 variant g.2538 C>T (rs76565726) were frequently detected in both AAD and HC (Table 1). Conversely, p.S268T/g.3323 G>C (rs6472) appeared rarely (Table 1). Several other silent and intron variants were also detected in both AAD patients and HC

Table 1 Differences in genotype frequencies (%) of the most common variants in *CYP21A2* detected in patients with autoimmune Addison's disease (AAD) and healthy controls (HC).

<i>CYP21A2</i> SNP	Genotype			P value	OR	95% CI	P value
rs397515394	+/+	+/-	-/-			+/+ vs +/-	
AAD	129 (35)	204 (55.3)	36 (9.8)	0.001	1.79	1.31–2.46	0.0003
HC	161 (48.3)	142 (42.6)	30 (9.0)				
rs6467	AA	AC	CC			AA vs AC	
AAD	72 (19.6)	198 (54)	97 (26.4)	0.0005	1.92	1.33–2.77	0.0004
HC	107 (32.5)	153 (46.5)	69 (21.0)				
rs6474	GG	GA	AA			GG vs GA	
AAD	130 (35.2)	204 (55.3)	35 (9.5)	0.005	1.69	1.23 – 2.32	0.001
HC	155 (47.0)	144 (43.6)	31 (9.4)				
rs76565726	CC	CT	TT			CC vs CT	
AAD	118 (32.0)	211 (57.2)	40 (10.8)	0.00003	2.10	1.52–2.89	6.1×10^{-6}
HC	157 (47.6)	134 (40.6)	39 (11.8)				
rs6472	GG	GC	CC			G vs C	
AAD	370 (97.4)	10 (2.6)	0 (0)	$3.3 \times 10^{-9*}$	0.15	0.08–0.30	3.8×10^{-10}
HC	277 (85.0)	45 (13.8)	4 (1.2)				
rs6473	GG	GA	AA			GG vs GA	
AAD	131 (34.6)	214 (56.5)	34 (9.0)	0.001	1.83	1.34–2.51	0.0002
HC	156 (48.3)	139 (43.0)	28 (8.7)				

P values were calculated by χ^2 test for 2×3 tables and Fisher's exact test on 2×2 tables.

*Fisher's exact test.

(listed in [Supplementary Table 1](#), see section on [supplementary data](#) given at the end of this article), but were not further investigated in the large cohort.

For the rs397515394, rs6474 and rs6473 SNPs, the heterozygote genotype occurred significantly ($P \leq 0.001$) more often in patients with AAD than in HC, compared with the homozygote genotype of the major alleles ([Table 1](#)). Haplotype reconstruction of *CYP21A* revealed that ÷AA and +GG (in the order rs397515394/rs6474/rs6473, from now denoted as the ÷AA and +GG haplotypes) mainly occurred together ([Supplementary Table 2](#), see section on [supplementary data](#) given at the end of this article), with a strong LD ($r^2 > 0.87$, $D' > 0.94$, [Fig. 1a](#) and [b](#)). The frequencies of these haplotypes were significantly different between AAD patients and HC ($P = 0.006$), where 35.7% AAD patients and 28.2% HC had the haplotype ÷AA, and 61.6% AAD patients and 67.7% HC had the haplotype +GG. Other combinations of these SNPs were seen in 2.8% of AAD patients and 4.1% of HC.

Regarding the intron variants rs6467 and rs76565726, the heterozygote genotype and the minor alleles were also more common in AAD than in HC ([Table 1](#)). The frequency of rs6467C was 53.4% in the AAD group, compared with 44.2% in HC (OR=1.44, 95% CI (1.17–1.79), $P = 0.007$). The allele frequencies of rs76565726T were 39.4% in AAD and 32.1% in HC (OR=1.38, 95% CI (1.10–1.72), $P = 0.005$). The majority of the rs76565726T alleles were seen together with the +GG haplotype, but never with the ÷AA haplotype ([Supplementary Table 2](#)), and was almost in complete LD with all these three coding SNPs ($D' > 0.97$, [Fig. 1b](#)).

The rs6472C seemed to be a protective variant to AAD, where the allele frequency of the C allele was 8.1% in HC

compared with 1.3% in AAD patients (OR=0.15, 95% CI (0.08–0.30), $P = 3.8 \times 10^{-10}$, [Table 1](#)). The rs6472C allele was only seen together with the +GG variant of the rs397515394/rs6474/rs6473 haplotype ([Supplementary Table 2](#)), which was in complete LD ($D' = 1$, [Fig. 1b](#)). The rs6472C allele in the AAD patients was mainly seen (nine of ten alleles) in the haplotype +CGTCG (in order rs397515394/rs6476/rs6474/rs76565726/rs6472/rs6473). In the control group, the haplotypes +AGTCG and +AGCCG were more frequent (22 and 23 of 53 rs6472C alleles, respectively, [Supplementary Table 2](#)).

Are the *CYP21A2* associations independent of *HLA I* and *II* loci?

To this end, a global regression analysis was performed to determine whether AAD associations of *CYP21A2* were independent ($P < 0.05$) of high-risk *HLA* loci. [Table 2](#) gives that all the *CYP21A2* SNPs associations, except rs6472 ($P = 0.02$), were lost when *HLA-DRB1* was set as conditional marker, suggesting that the *HLA*-type determines the AAD-risk. The SNPs rs397515394, rs6472 and rs6473 showed independence from the *HLA-DQB1* locus ($P = 0.02$, $P = 0.0002$ and $P = 0.04$, respectively), while the rs6467, rs6474 and rs76565726 SNPs were in LD with *HLA-DQB1* ([Table 2](#)). All the exon variants of *CYP21A2* further showed independency to the *HLA-A*, *HLA-B* and *MICA*, while the two intron variants were both in LD with *HLA-A* and *MICA*. The rs6467 was in LD with *HLA-B*, whereas rs76565726 was not ($P = 0.02$) ([Table 2](#)).

We next wanted to investigate how *CYP21A2* alleles are linked to the *HLA-DRB1* risk alleles. [Supplementary Table 3](#), see section on [supplementary data](#) given at the end of this article, gives the major *CYP21A2* haplotypes (the SNPs rs397515394/rs6474/rs6472/rs6473 with haplotype ÷AGA and +GGG) in combination with the most common *HLA-DRB1* haplotypes generated by PHASE. The *HLA-DRB1**03:01 mainly occurred together with the +GGG haplotype (i.e. 95.5% and 98.2% of AAD and HC respectively), while the *HLA-DRB1**04:04 haplotype was most commonly seen with the ÷AGA variant (81.8% and 85.3% of AAD and HC cases respectively ([Supplementary Table 3](#)). The *HLA-DRB1**15 and *01 haplotypes also occurred most frequently together with the +GGG *CYP21A2* haplotype, while the *HLA-DRB1**04:01 was most frequently seen together with ÷AGA ([Supplementary Table 3](#)). Furthermore, the individuals with heterozygous *HLA-DRB1**03:01/*04:04 high-risk genotype were also heterozygote for *CYP21A2* ÷AA/+GG in 81.7% AAD patients ($n = 98$) and 83.3% HC ($n = 6$) respectively.

Figure 1

r^2 linkage plot (a) and D' linkage plot (b) of common variants of *CYP21A2*.

Table 2 Global regression analysis of the HLA and CYP21A2 loci for determination of independent associations in autoimmune Addison's disease calculated by UNPHASED.

Test locus	Analysis for conditional locus (P values)										
	A	B	MICA	DRB1	DQB1	rs397515394	rs6467	rs6474	rs76565726	rs6472	rs6473
HLA-A	4.3×10 ⁻⁹ 3.0×10 ⁻⁷ 1.1×10 ⁻³⁵ 2.4×10 ⁻²⁰ 5.5×10 ⁻⁵	0.04 0.0005 1.4×10 ⁻²⁷ 2.4×10 ⁻¹⁷ 1.2×10 ⁻⁹	0.004 7.4×10 ⁻⁸ 7.5×10 ⁻³⁵ 1.7×10 ⁻²¹ 1.7×10 ⁻⁵	0.39	0.03	8.9×10 ⁻⁶	0.002	9.1×10 ⁻⁶	0.002	7.6×10 ⁻⁵	3.0×10 ⁻⁶
HLA-B				0.03	5.9×10 ⁻⁷	6.7×10 ⁻¹⁷	5.0×10 ⁻¹⁰	4.4×10 ⁻¹²	1.2×10 ⁻¹¹	5.0×10 ⁻¹⁰	6.6×10 ⁻¹⁸
MICA				0.006	2.6×10 ⁻⁷	2.5×10 ⁻¹²	9.5×10 ⁻⁹	2.4×10 ⁻¹⁷	1.0×10 ⁻⁸	1.1×10 ⁻⁸	2.8×10 ⁻¹³
HLA-DRB1					8.7×10 ⁻¹⁹	4.2×10 ⁻³⁹	4.8×10 ⁻³⁸	3.6×10 ⁻⁴⁰	9.7×10 ⁻³⁹	4.9×10 ⁻³⁶	3.6×10 ⁻⁴¹
HLA-DQB1							9.3×10 ⁻²²	5.8×10 ⁻²³	9.9×10 ⁻²²	8.0×10 ⁻²⁰	4.2×10 ⁻²⁴
rs397515394				0.09	0.02	5.2×10 ⁻²³	0.002	0.05	3.2×10 ⁻⁷	0.03	0.15
rs6467	0.05	0.74	0.1	0.42	0.10	0.0007		0.0005	0.05	0.003	0.0008
rs6474	0.0005	1.2×10 ⁻⁸	0.0001	0.14	0.08	0.19	0.005		4.1×10 ⁻⁶	0.11	0.83
rs76565726	0.36	0.02	0.29	0.82	0.46	2.8×10 ⁻⁷	0.22	1.2×10 ⁻⁶		0.0002	4.6×10 ⁻⁷
rs6472	1.0×10 ⁻⁹	3.7×10 ⁻⁶	1.3×10 ⁻⁷	0.02	0.0002	1.0×10 ⁻⁸	4.6×10 ⁻⁹	1.2×10 ⁻⁸	6.9×10 ⁻¹¹		1.6×10 ⁻⁸
rs6473	9.0×10 ⁻⁵	3.0×10 ⁻⁹	3.6×10 ⁻⁵	0.12	0.04	0.67	0.003	0.51	8.7×10 ⁻⁷	0.07	

For rs6467, the *HLA-DRB1*03:01* haplotype was most frequently reconstructed together with the minor C allele, while the majority of *HLA-DRB1*04:04*, – *15 and – *01 haplotypes were seen together with the A allele. For *HLA-DRB1*04:01*, both rs6467 alleles were more equally distributed (Supplementary Table 3). For rs76565726 SNP, the minor T allele was mainly seen together with the *HLA-DRB1*03:01* haplotype (Supplementary Table 3).

The majority of the rs6472C alleles in AAD (six of ten) were reconstructed by PHASE together with the high-risk *HLA-DRB1*03:01*, while the rest with *HLA-DRB1*07:01* (two of ten) and *HLA-DRB1*13:01* (two of ten). For the HC group, the rs6472C allele was mainly seen together with the haplotypes *HLA-DRB1*13:01* (19 of 53) – *01 (6 of 53) and – *07:01 (11 of 53). The protective effect of rs6472C was lost ($P > 0.05$) when considering the effect of the *HLA-DRB1* haplotypes (Table 3).

CYP21A2 variants related to biochemical and clinical parameters

Although all of the patients in this study were diagnosed with AAD, 8% lacked 21OH-Ab at the time of testing. Our analyses revealed that the frequencies of heterozygous variants of *CYP21A2* were higher in the 21OH-Ab positive group (Supplementary Table 4, see section on supplementary data given at the end of this article). The mean 21OH-Ab levels at diagnosis were also higher in patients with the AAD-associated genotypes of *CYP21A2*, although not statistically significant (Supplementary Table 4).

Furthermore, the *HLA-DRB1*03:01* allele was found in 36.5 and 12.1% of 21OH-Ab positives and negatives respectively. Likewise, for *HLA-DRB1*04:04*, the allele frequency was 18.2 and 8.6% for 21OH-Ab positives and negatives. *HLA-DRB1*04:01* and – *15 were more common in the 21OH-Ab negative group at 19 and 20.7%, respectively, compared with 9.6 and 11.9% in the 21OH-Ab positive group. Finally, the *HLA-DRB1*03:01/*04:04* genotype was found only in 21OH-Ab positives. The mean 21OH-Ab levels at diagnosis were significantly higher in patients with the *HLA-DRB1*03:01* and – *04:04 haplotypes compared with other haplotypes, and similarly for the *HLA-DRB1*03:01/*04:04* genotype ($P < 0.0005$). Patients with the *HLA-B*08* had significantly higher 21OH Ab indices at diagnoses compared with patients with other *HLA-B* haplotypes ($P = 0.028$), while the 21OH Ab indices were significantly higher in patients with the *HLA-A*01* compared with *HLA-A*02* ($P = 0.0031$). The results are summarised in Supplementary Table 4.

The AAD-associated *CYP21A2* variants were more frequent in patients with onset of the disease before

Table 3 Haplotype reconstruction of protective and high risk alleles of *HLA-DRB1* and rs6472.

<i>HLA-DRB1</i>	rs6472	AAD	HC	Odds ratio	95% CI	P value
*1301	C	2	23	0.40	0.073–2.24	0.44
*1301	G	6	19			
*0701	C	2	11	0.70	0.14–3.54	1.0
*0701	G	14	54			
*01	C	0	11	0.11	0.006–2.01	0.06
*01	G	23	61			
*0301	G	259	110	0.18	0.01–3.24	0.18
*0301	C	6	0			

P values were calculated by Fisher's exact test.

40 years of age than in those with later onset. In addition, the protective variant rs6472C was not detected in patients with disease onset before 20 years (Supplementary Table 5, see section on supplementary data given at the end of this article).

There was no association between autoimmune thyroid disease of the APS2 patients and the *CYP21A2* variants. The rs76565726T allele was, however, more frequent in APS2 patients with type 1 diabetes than in AAD patients without type 1 diabetes (OR = 1.517, 95% CI (1.027–2.240), $P = 0.04$), but there was no such association with the other *CYP21A2* variants. There was no association between gender and *CYP21A2* variants.

Rare variants of *CYP21A2* in AAD patients and HC

A rare heterozygous coding variant, p.A159T/g.2637G > A, was found in 3/370 patients with AAD and in 1/330 HC. All four were also heterozygous for the rare haplotype *HLA-B*4801* which was absent in other patients and controls. Known disease causing mutations frequently reported in patients with CAH (34) were also found as heterozygous in both AAD patients and HC. The I2 splice variant (rs6467G) was detected in 2/370 AAD patients and 1/331 HC. The eight bp deletion in exon 3 (rs387906510) was found in one AAD patient and in one HC. The non-classical CAH variant p.V281L (rs6471) was found in 3/380 AAD patients and 5/326 HC, while p.P453S (rs6445) was found in 3/379 AAD patients and 4/322 HC. No copy number variations were detected in AAD patients or HC; they all had two copies of *CYP21A2*.

Discussion

It is not known why certain proteins, such as 21OH, become autoantigens in organ-specific autoimmune

diseases. However, common features are that autoantigens are intracellular enzymes with restricted tissue expression. One possibility is that only altered or modified self-proteins can induce autoreactive immune responses, and that genetic polymorphisms may represent such alterations. By direct sequencing of *CYP21A2* in AAD patients, we did not find any disease-specific risk alleles, but heterozygous genotypes of common *CYP21A2* variants were significantly more frequent in AAD patients than in HC. However, we found that all the *CYP21A2* SNPs associations, except for rs6472, were dependent on the *HLA-DRB1* locus, which is the strongest single genetic risk factor of AAD (2, 4, 35, 36). Therefore, the higher heterozygous genotype frequency of the two main haplotypes of *CYP21A2* alleles, +AA and +GG, merely reflects the high-risk genotype of *HLA-DRB1*03:01/*04:04* (2). The protective rs6472C association could also be explained by the linkage with the HLA protective variants.

The non-coding AAD-associated *CYP21A2* variants rs6467 and rs76565726 were also dependent on *MICA* and *HLA-A* or -B. The rs76565726T allele was further associated with type 1 diabetes of the APS2 patients included in the study. Interestingly, the rs6467C and rs76565726T alleles have both shown to be in LD with components of the 8.1 ancestral haplotype (37).

Our data indicated that all of our patients with the *DRB1*03:01/*04:04* genotype were positive for 21OH Ab and had the highest 21OH Ab indices compared with other genotypes. This is consistent of *HLA-DRB1*03:01/*04:04* genotype association with expression of 21OH Ab (38). The corresponding *CYP21A2* genotype did not add to the high 21OH Ab association of *HLA-DRB1*03:01/*04:04*, which underlines the strength of *HLA-DRB1* risk factors. Similarly, we here found a trend for association between *CYP21A2* genotypes and age of disease onset. This is in agreement with previous findings of a significant lower mean age of onset in patients who carries the *HLA-DRB1*03:01/*04:04* genotype (2).

A hypothesis proposes that low protein expression due to genetic polymorphisms might increase the probability of autoreactive T cells to escape from the thymus during negative selection. This has been suggested for the variable number of tandem repeats polymorphism in the gene encoding insulin and its association with the production of Ab and autoreactive T cells to insulin in type 1 diabetes (39, 40). Although the *HLA-DRB1* locus proves to be the highest predisposing factor of AAD, the significance of *CYP21A2* heterozygote genotypes is not clear because it is not known if the gene is inherited co-dominantly like the *HLA* genes.

In conclusion, sequencing of the exons and introns of *CYP21A2* did not reveal any disease-specific risk variants associated with AAD. Instead, *CYP21A2* polymorphisms are in LD with the high-risk haplotypes of the *HLA-DRB1* loci, and do not independently add to increased risk or protection, but merely reflects the effect of *HLA-DRB1*.

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.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/EJE-12-0891>.

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