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Complement 4 phenotypes and genotypes in Brazilian patients with classical 21-hydroxylase deficiency

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Summary

The aim of this work was to analyse C4 genotypes, C4 protein levels, phenotypes and genotypes in patients with the classical form of 21-hydroxylase deficiency. Fifty-four patients from 46 families (36 female, 18 male; mean age 10·8 years) with different clinical manifestations (31 salt-wasting; 23 simple-virilizing) were studied. *Taq* I Southern blotting was used to perform molecular analysis of the C4/CYP21 gene cluster and the genotypes were defined according to gene organization within RCCX modules. Serum C4 isotypes were assayed by enzyme-linked immunosorbent assay. The results revealed 12 different haplotypes of the *C4/CYP21* gene cluster. Total functional activity of the classical pathway (CH50) was reduced in individuals carrying different genotypes because of low C4 concentrations (43% of all patients) to complete or partial C4 allotype deficiency. Thirteen of 54 patients presented recurrent infections affecting the respiratory and/or the urinary tracts, none of them with severe infections. Low C4A or C4B correlated well with RCCX monomodular gene organization, but no association between C4 haplotypes and recurrent infections or autoimmunity was observed. Considering this redundant gene cluster, C4 seems to be a well-protected gene segment along the evolutionary process.

Keywords: adrenal disease, complement, endocrine immunology disease, MHC, steroids

Introduction

The incidence of primary immunodeficiencies in the Caucasian population is approximately 1 : 3000, excluding cases of selective immunoglobulin (Ig)A deficiency [1]. Deficiencies of the complement system represent 2–6% of all primary immunodeficiency cases [2,3]. Complement plays a role in the innate and adaptative immune system. It interacts and modulates the specific immune response using specific receptors and molecular interactions [4,5].

C4 is an essential component of the classical and lectin activation pathways [6]. The genetics of human complement C4 is complex. The high polymorphic C4 proteins are categorized into two isotypes, C4A and C4B, with multiple allotypes [7,8].

An interindividual variation in the C4 gene copy number and dichotomies of C4 gene size and C4 protein isotypes were observed [9]. Theoretically, two to eight copies of C4 genes can be present in a diploid genome of an individual. The C4 gene size has two forms: long and short. The long and short genes are 21 kb and 14·6 kb in length respectively [10]. The duplication or multiplication of C4 genes also includes three neighbouring genes: the nuclear protein kinase RP1, the steroid cytochrome P450 21-hydroxylase (CYP21) and extracellular matrix protein tenascin-X (*RCCX* = *R*P, *C*4, *C*YP21, TN*X* genes) [11].

The C4-CYP21 complex is located about 400 kb from the DR locus of the major histocompatibility complex (MHC) class II region [8]. There are two copies of the 21-hydroxylase gene, a functional gene termed *CYP21A2* and a pseudogene called *CYP21A1P*. The pseudogene is intercalated and arranged in tandem together with the *C4A* and *C4B* genes [12]. The *CYP21A2* gene is located 3′ to *C4B*, and genetic alterations in the *C4* genes are associated frequently with congenital adrenal hyperplasia (CAH) because of 21-hydroxylase deficiency (21-OHD) [13]. There is a continuous spectrum of manifestations of this disease, ranging from classical and severe forms such as

'salt-losing' and 'simple-virilizing' to non-classical forms which present with mild to moderate clinical manifestations (late onset form) or may occur even without phenotypic manifestations (cryptic form) [12–14].

Total C4 deficiency is rare but, on the other hand, heterozygous or partial deficiencies of C4A (C4AQ0) or C4B (C4QB0) affects approximately 35% of all individuals and about 1% express only a single C4 allele. C4A and C4B null alleles have been associated with systemic lupus erythematosus, insulin-dependent diabetes mellitus, IgA nephropathy, Schönlein–Henoch purpura, subacute sclerosing panencephalitis, autoimmune chronic active hepatitis, membranoproliferative glomerulonephritis, rapid progressive human immunodeficiency virus disease and other disorders [9,15–18]. These associations may be due to either C4 deficiency itself or to the linkage with other MHC III (6p21) genes or both. Individuals with total deficiency of C4B (homozygous C4BQ0) have a higher risk for bacterial meningitis [19]. On the contrary, excessive C4 or overactivation of C4 could aggravate an inflammatory response and render an individual more vulnerable to tissue injuries [9].

Considering the relationship of the genes encoding C4 and 21-hydroxylase, the aim of this study was to evaluate C4 isotype levels, the RCCX modules and the occurrence of recurrent infections and/or autoimmune diseases in Brazilian patients with 21-OHD.

Methods

Patients

Fifty-four patients from 46 families with established diagnosis of classical 21-OHD were included in the study. They have been followed at the Pediatric Endocrinology Outpatient Clinic at University of Campinas (UNICAMP) Medical School Hospital, Campinas, Brazil. All patients presented signs and symptoms of virilization, high plasma levels of 17-hydroxyprogesterone and androstenedione, associated or not with salt-losing history. The clinical and laboratory 21-OHD diagnosis were confirmed in all cases by the occurrence of two affected *CYP21A2* alleles. The molecular evaluation included Southern blotting [20] of *Taq* I digested genomic DNA and allele-specific hybridization or allele-specific polymerase chain reaction (PCR) genotyping [21].

Thirty-six of the 54 patients were female, 31 (22 female, nine male) had the salt-wasting form and 23 (14 female, nine male) had the simple-virilizing form of the disease. The mean age was 10·8 years (ranging from 4 to 22 years). An eventual clinical history of recurrent infections and autoimmune disorders was evaluated. The definition for the recurrent infections was at least six upper respiratory infections per year [22] or two pneumonias in 1 year [23] or two urinary tract infections [24]. Autoimmunity was considered only for defined diseases and the isolated finding of antinuclear factor was not a diagnostic criteria. The study was approved by the UNICAMP Medical School Ethics Committee. All individuals gave written informed consent.

Analysis of the complement activation and C4 isotypes

Haemolytic assays for the classical (CH50, normal range: 53–110 IU/ml) and the alternative pathway (AH50, normal range: 34–78 IU/ml) were performed as described by Mayer [25] and Joiner *et al.* [26] respectively. Ethylenediamine tetraacetic acid (EDTA)-plasma samples were used for biochemical analysis of C4 isotypes. C4 isotypes (C4A and C4B) were evaluated by isotype-specific enzyme-linked immunosorbent assay (ELISA), basically described by Chrispeels *et al.* [27]. In brief, microtitre plates (Nunc, Wiesbade, Germany) were coated overnight at 4°C with rabbit anti-human C4 IgG (Dakopatts, Hamburg, Germany). Non-specific binding sites were blocked with phosphate-buffered saline, 1% bovine serum albumin, followed by the application of diluted samples or standards. After incubation with non-specific mouse anti-human IgG, C4A and C4B antibodies (Dianova, Hamburg, Germany) were added to the samples for 60 min respectively. The reaction was visualized by addition of 2·2-azino-bist (3-ethyleneben/thiazoline-6-sulphone) (Sigma, St Louis, MO, USA) and H_2O_2, and stopped with 0·2 M oxalic acid. The normal range (established previously in healthy volunteers) for C4A was 70–200 μg/ml, and for C4B from 200 to 400 μg/ml (M. K., Heidelberg).

Genotype analysis of the C4/CYP21 locus

Molecular analysis of the *C4/CYP21* gene cluster was performed as described elsewhere [21,28]. Briefly, after *Taq* I digestion DNA samples were submitted to electrophoresis in 0·8% agarose gel in Tris-borate-EDTA buffer (0·09 M Tris-HCl; 0·09 M boric acid; 20 mM EDTA, pH 8·0). DNA samples in agarose gels were transferred to Hybond N^+ membranes (Amersham Biosciences, Uppsala, Sweden) by Southern blotting and hybridized separately to radioactively labelled *C4* (pC4B550) and *CYP21A2* (pC21/3c) cDNA probes. On the basis of differences in both *Taq* I restriction fragment length polymorphism and band intensities, the *C4/CYP21* gene organization in RCCX modules was revealed and used to define genotypes for each individual within a family. Band intensitites were obtained in a LKB 222-020 Ultroscan XL Laser Densitometer (Pharmacia, Uppsala, Sweden). Autoradiographies obtained with different exposition times were used to evaluate gene copy number. Figure 1 illustrates how *C4* and *CYP21* genes are organized in monomodular, bimodular and trimodular alleles and their *Taq* I variants. The absence of either *C4* or *CYP21* corresponding fragments indicated homozygous deletion, whereas decreased hybridization signals indicated a heterozygous condition. Increased hybridization signals were considered duplicated genes. Alleles carrying *C4B* long or short gene copies were recognized by 6·0 kb or 5·4 kb *Taq* I fragments respectively. To avoid misinterpretation between duplicated and deleted alleles, parents and

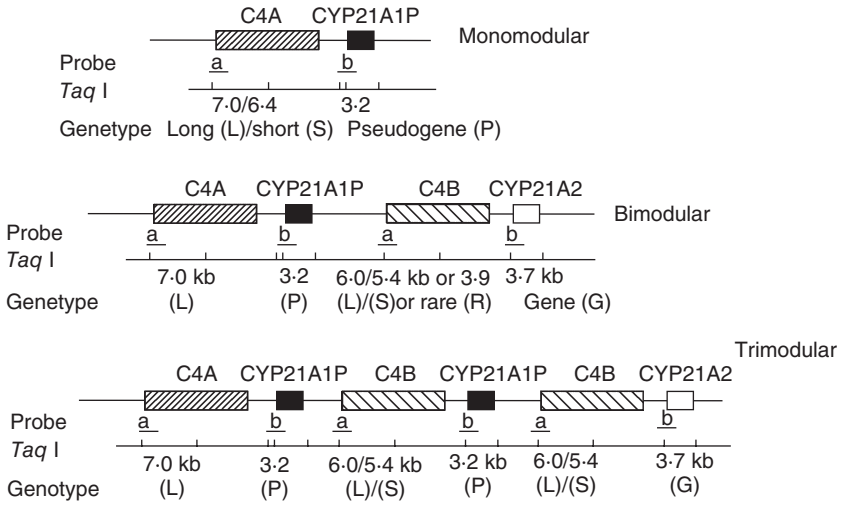

Fig. 1. *C4* and *CYP21* genes organization in modular alleles and their *Taq* I variants.

non-affected siblings were analysed, allowing the verification of correct allelic segregation.

Statistical analysis

Data were presented as mean, standard deviation, minimum and maximum values. Patients were grouped according to clinical history of recurrent infections or autoimmune diseases, the type of CAH and the genotype of C4 modules. The results of complement and C4 allotypes were compared among groups using the Mann–Whitney *U*-test. The *P*-value accepted for statistical significance was 0·05.

Results

Table 1 shows clinical features (sex, age, clinical form of 21-OHD, recurrent infections and autoimmune disorders), biochemical data of the complement pathway (CH50, APH50, C4A, C4B) and molecular data of C4 genotype (*C4A* and *C4B* genes) from the 54 patients evaluated.

Among the 46 families with 21-OHD evaluated, 12 different haplotypes were observed, according *C4+CYP21* gene organization (Table 1, Fig. 2). Bimodular haplotypes were the most frequent and were combined in eight distinct genotypes, in 30 families: LL/LS (a/b; $n = 7$), where LL means $C4A^{7\cdot0}/C4B^{6\cdot0}$ and LS means $C4A^{7\cdot0}/C4B^{5\cdot4}$; LL/LL (a/a; $n = 10$); LS/LS (b/b; $n = 4$), LL/LR (a/c; $n = 1$), LR means $C4A^{7\cdot0}/C4B^{3\cdot9}$; LS/LS (b/f; $n = 2$); LS/LL (b/g; $n = 1$); LL/LL (a/g; $n = 4$; LL/LL (g/g; $n = 1$). Combinations of bi- and trimodular or tri- and trimodular units were observed in eight families: LS/LLL (b/d; $n = 2$); LL/LLL (a/d; $n = 1$); LL/LSS (a/e; $n = 1$); LLL/LSS (d/e; $n = 1$); LSS/LL (e/g; $n = 1$); and LL/LSS (a/h; $n = 1$). In eight families different genotypes with monomodular units were observed: LL/L (a/i; $n = 3$); L/L (i/i; $n = 1$); S/S (j/j; $n = 1$); LL/L (g/i; $n = 1$); LL/S (a/k; $n = 1$), S in this case is $C4A^{6\cdot4}$; L/LSS (i/l; $n = 1$). Distinct *C4* gene organizations were dependent on *CYP21* gene deletions, conversions or mutations.

Biochemical analysis of the complement pathway and C4 isotypes were expressed as the mean ± standard deviation, minimum and maximum values: CH50 = 53·1 ± 14·4 IU/ml (0–88), AH50 = 46·2 ± 12·7 IU/ml (0–75), C4A = 91·2 ± 38·5 μg/ml (0–162), and C4B = 266·1 ± 124·1 μg/ml (24–517) (Table 1).

Levels of CH50 (nadir 110 IU/ml) and APH50 (nadir 78 IU/ml) were not above the normal range in any patient, but CH50 (nadir 53 IU/ml) was below the normal range in 24 patients and undetectable in one (patient 18: Table 1), and APH50 (nadir 34 IU/ml) was below the normal range in only six patients and undetectable in one (patient 1: Table 1). Fourteen patients presented C4A values below the lower limit for the normal range (nadir 70 μg/ml). They carry in their genotypes either *CYP21A2* gene conversion (LL/LL, $n = 4$; LS/LS, $n = 3$; LS/LL, $n = 4$) or *C4/CYP21* gene deletion (LL/L, $n = 2$; L/L, $n = 1$). The C4B levels were elevated in nine patients (nadir 400 μg/ml) from whom five presented

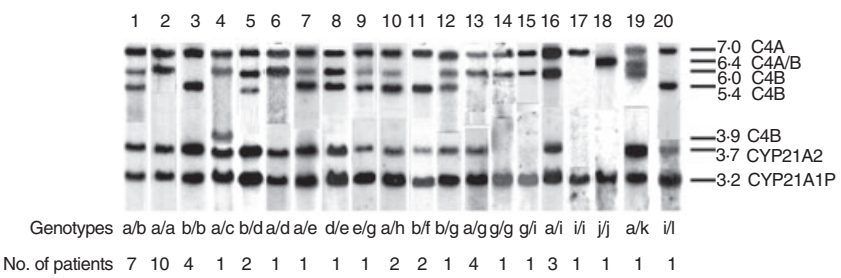

Fig. 2. *C4* and *CYP21* genotypes and 21-hydroxylase deficiency patient numbers of each one.

Table 1. Biochemical data of complement pathway and C4A and C4B haplotypes from 54 patients with classical 21-hydroxylase deficiency.

								C4 genotype										C4 genotype	
Patient	21-OHD	Sex	Age	CH50	APH50	C4A	C4B	Father	Mother	Patient	21-OHD	Sex	Age	CH50	APH50	C4A	C4B	Father	Mother
1	SV	M	16	27	0	54	129	LL	LL	28[‡]	SW	M	6	57	67	103	257	LLL	LL
2[†‡]	SV	F	9	46	27	56	138	LL	LL	29	SW	M	5	74	42	112	358	LSS	L
3	SV	M	13	44	31	101	228	LL	LL	30[†]	SW	M	10	88	66	0	517	LS	LS
4	SV	F	9	41	48	81	338	LSS	LL	31[†]	SW	M	12	61	54	0	245	L	L
5	SV	F	9	46	55	83	203	LL	LL	32	SW	M	7	76	52	122	506	LSS	LLL
6	SV	F	17	67	55	114	274	LL	LL	33	SW	F	11	36	35	84	255	LL	LS
7	SV	F	17	53	46	66	193	LL	LL	34	SW	F	10	52	50	133	394	LL	LS
8[†]	SV	F	11	69	58	108	255	LS	LS	35	SW	M	6	51	51	43	126	LL	LS
9	SV	M	10	69	50	109	266	LS	LS	36[†¶]	SW	F	8	47	23	132	27	S	S
10	SV	F	9	77	75	129	24	L	LL	37	SW	F	7	49	47	91	220	LL	LL
11	SV	F	12	49	37	113	130	LL	LL	38	SW	F	15	45	47	80	480	LL	LSS
12	SV	M	7	58	36	97	243	LS	LL	39	SW	F	16	50	52	112	46	L	LL
13	SV	F	22	60	51	53	281	LS	LL	40	SW	F	10	38	34	40	238	LS	LS
14	SV	M	7	51	55	47	235	LS	LL	41	SW	F	12	53	54	123	481	LLL	LS
15	SV	M	11	47	42	46	244	LL	S	42	SW	F	16	72	34	131	400	LS	LS
16	SV	F	8	50	65	154	357	LLL	LL	43[†]	SW	M	14	58	32	98	279	LS	LS
17	SV	M	12	48	36	61	140	LL	LL	44	SW	F	10	54	37	107	260	LL	LS
18	SV	M	14	0	40	23	44	LL	LS	45	SW	M	12	50	36	115	152	LL	L
19	SV	F	10	24	46	39	105	LL	LS	46	SW	F	7	56	43	105	295	LS	LL
20	SV	M	14	57	47	99	190	LL	LL	47[†¶]	SW	F	12	55	46	109	405	LL	LSS
21	SV	F	6	60	51	50	359	LL	L	48[§]	SW	F	6	59	53	106	295	LL	LS
22	SV	F	16	40	32	78	232	LL	LL	49[§]	SW	F	13	54	52	95	145	LL	LL
23	SV	F	5	31	35	42	237	LS	LS	50	SW	F	8	58	53	162	439	LS	LS
24	SW	M	15	64	40	162	413	LS	LL	51	SW	F	4	56	51	120	481	LL	LL
25	SW	F	9	55	58	139	441	LS	LL	52	SW	F	12	46	50	130	217	LL	LLL
26[†‡¶]	SW	F	14	64	53	39	260	LS	LS	53[‡]	SW	F	5	65	61	105	260	LL	LSS
27[†]	SW	F	9	69	65	124	303	LL	LR	54	SW	F	15	43	37	99	331	LL	LL

[†]Urinary tract infections; [‡]upper airway infections; [§]lung infections; [¶]autoimmune disorders (hypothyroidism). Normal ranges: CH50 = 53–110 IU/ml; APH50 = 34–78 IU/ml; C4A = 70–200 μg/ml; C4B = 200–400 μg/ml. SV, simple-virilizing form; SW, salt-wasting form; M, male; F, female; age, years.

genotypes carrying three or four copies of *C4B* gene that combine either bi- and trimodular or tri- and trimodular haplotypes respectively (LS/LLL, $n = 2$; LLL/LSS, $n = 1$; LS/LLL, $n = 1$; LSS/LLL, $n = 1$); and four patients with bi and bimodular (LS/LL, $n = 3$; LS/LS, $n = 1$). One of these patients with high C4B expression and a genotype LS/LS had an undetectable C4A plasma level (patient 30: Table 1). Low levels for both variants C4A and C4B (nadir 200 μg/ml) were observed in six patients; all of them carry two bimodular haplotypes (LL/LL, $n = 4$; LL/LS, $n = 2$). Two patients (siblings) presented low levels of CH50, APH50, C4A and C4B (patients 1 and 2: Table 1). Two other siblings (patients 17 and 18: Table 1) and a non-related patient (patient 35: Table 1) had low levels of CH50, C4A and C4B.

Thirteen of 54 patients presented recurrent infections affecting the urinary tract ($n = 8$; Table 1: patients 2, 26, 27, 30, 31, 36, 43, 47), the upper airways ($n = 5$; Table 1: patients 2, 8, 26, 28, 53), the lungs ($n = 2$; Table 1: patients 48, 49) and urinary tract combined with upper airways infection ($n = 2$; Table 1: patients 2, 26). An additional three patients suffered from autoimmune disorders (hypothyroidism, $n = 3$; Table 1: patients 26, 36, 47), all associated with recurrent urinary infections, one of them combined with upper airway infections (Table 1: patient 26). None of them had severe infections.

Complement and C4 isotypes analysis showed a significant difference only for CH50 between groups with (60·9 ± 10·9 IU/ml) and without (50·7 ± 14·6 IU/ml) recurrent infections ($Z = -2{\cdot}45$; $P = 0{\cdot}01$). No statistical differences between groups with or without autoimmune diseases were detected.

Regarding the C4 modular genotype, significant differences were observed between LL/LL and LL/LLL for C4 (20·8 ± 5·2 mg/dl and 33·5 ± 0·7 mg/dl; $Z = -2{\cdot}24$; $P = 0{\cdot}01$) and for C4A (88·9 ± 21·6 μg/ml and 142 ± 17 μg/ml; $Z = -2{\cdot}23$; $P = 0{\cdot}01$). LL/LSS for C4 (20·8 ± 5·2 mg/dl and 30 ± 3·5 mg/dl; $Z = -2{\cdot}71$; $P = 0{\cdot}004$) and C4B (219 ± 93·3 μg/ml and 370·7 ± 93·9 μg/ml; $Z = -2{\cdot}40$; $P = 0{\cdot}01$) and LS/LS for CH50 (20·8 ± 5·2 IU/ml and 60·8 ± 17·4 IU/ml; $Z = -2{\cdot}71$; $P = 0{\cdot}03$) and C4B (219·4 ± 93·3 μg/ml and 321·2 ± 103·3 μg/ml; $Z = -2{\cdot}18$; $P = 0{\cdot}004$).

Discussion

Genetic recombination is a driving force for diversity and polymorphism. In general, recombination between misaligned homologous chromosomes can result in either deletion or duplication of genes. Furthermore, unequal crossover between duplicated gene regions is responsible for a variety of human genetic diseases. Such duplications have been observed frequently in the human MHC class III. Located on chromosome 6p21, between class I and class II, the MHC class III encodes genes of various functions, including complement components C2, C4, factor B (Bf) and 21-hydroxylase genes (*CYP21A2* and *CYP21A1P*). The order of the genes in the direction of transcription is *C2*, *BF*, *C4A*, *CYP21A1P*, *C4B*, *CYP21A2* and they have mapped between human leucocyte antigen (HLA)-B and HLA DR. Because of the tight linkage, these genes are usually inherited as a single complotype. In some haplotypes, linkage disequilibrium includes the HLA-B and HLA-DR regions forming extended haplotypes [29]. The MHC class III region has been studied because of its association with many autoimmune and genetic diseases. A gene cluster containing the duplicated *C4* and the *CYP21* genes is of particular interest because of its linkage to diseases, such as systemic lupus erythematosous and CAH [30].

C4 is a highly polymorphic protein, which is reflected by a wide concentration range found in the population. There are two distinct classes of C4 protein, C4A and C4B, which have diversified in their predominant functions of opsonization/immune clearance (C4A) and the better-known killing of the pathogens by lyses and neutralization (C4B). C4 isotype levels were evaluated by ELISA using monoclonal antibodies to Rodgers (Rg1) and Chido (Ch1) determinants related to C4A and C4B respectively, so it was a specific binding [9,31].

Although we observed low C4A and C4B protein levels, no direct association with the recurrent respiratory and/or urinary tract infections was significant. Most of our patients had normal CH50 and C4 levels and no complete C4 deficiency. Twenty-five and six patients had, respectively, CH50 and AH50 levels below the normal range (53 IU/ml and 34 IU/ml respectively), reflecting either consumption because of inflammation or (partial) deficiency. Five patients had low levels of both CH50 and AH50.

Heterozygous deficiency of either C4A or C4B is common, with a frequency of approximately 20% each in the Caucasian population; complete deficiencies of both C4A and C4B proteins are extremely rare [31]. However, in patients suffering from infectious disorders and autoimmune diseases, homozygous or heterozygous deficiencies of C4A and/or C4B have been found with a significantly higher frequency. Probably because of the relatively low number of study patients and lack of complete C4 deficiency, we were unable to find a clear disease association with respect to C4 genotypes and C4 levels. Recurrent respiratory infections in our study population might be explained more clearly by the relative immaturity of the immune response system at this age. Urinary tract infections can also be justified by the presence of labial fusion in girls with CAH [32–34]. Although no significant correlation of C4 isotype deficiency was identified clearly in patients with hypothyroidism, it is relevant that three of our young patients presented this autoimmune manifestation. Female patients present hypothyroidism more frequently [35,36]. Recently, Demirbilek *et al.* [36] reported a mean age of 11·4 years for the hypothyroidism diagnosis in a paediatric population. The three patients with hypothyroidism reported by us were female, and were diagnosed at ages that varied from 8 to 14 years. Therefore, it could be concluded that thyroid impairment is relatively

common in females at this age range; however, we evaluated a relatively low number of individuals, finding three of 54 patients affected.

Taq I Southern blot bears information on the dichotomous size variation of C4 genes and the number of RCCX modules – hence, the number of C4 genes – in a diploid genome. Chung *et al.* [9] tried to establish real-time PCR to determine the number of *C4A* and *C4B* genes, but the technique proved to be difficult. In 2000, Blanchong *et al.* [7] had already described the high variability and complexity of *C4* gene size, number and modular variations in 150 healthy Caucasian females and 22 CAH patients. Our findings also indicate a high modular variability among 21-OHD patients.

The specific length variants and the number of RCCX modules are assigned based on presence and absence and relative band intensities. Variation in the number of RCCX modules and the size of the *C4* genes leads to the presence of seven common RCCX length variants: monomodular L (long) and S (short); bimodular LL and LS; and trimodular LLL, LSS and LLS (or LSL) [7,37]. In addition, two rare length variants, bimodular SS and quadrimodular LLLL, have been implicated [38,39]. All the patients except one presented common RCCX length variants described previously. This patient (patient 36: Table 1, genotype j/j or S/S) presented a novel haplotype in which the C4A/B 6·4 kb fragment, associated frequently with the deletion of *CYP21A1P* 3·2 kb fragment, is associated with *CYP21A2* 3·7 kb deletion. This haplotype was observed for the first time in this patient and is probably a result of two different events of unequal cross-overs, the first resulting in the common haplotype bearing the the *C4A/B* 6·4 kb fragment and the *CYP21A1P* 3·2 kb fragment deletion and the second generating the *CYP21A2* 3·7 kb deletion haplotype. Those events must have occurred in at least three previous generations as the patient's parents, who are first cousins, and the maternal grandfather also carry the same haplotype.

The C4B levels were elevated in nine patients. Four presented genotypes carrying three or four copies of the *C4B* gene that combine either bi- and trimodular or tri- and trimodular haplotypes respectively. One of these patients with high C4B expression and homozygous for haplotype b had an undetectable C4A plasma level. Low levels for both variants C4A and C4B were observed in six patients; all of them carry two bimodular haplotypes. Fourteen patients presented C4A values below the lower limit for the normal range. They carry either *CYP21A2* gene conversion or *C4/CYP21* gene deletion. These data indicate that despite the fact that in some cases C4 gene deletion or duplication corresponded to low or high levels of C4 protein respectively, this association was not absolute.

We conclude that in a series of 46 families with 54 patients with CAH because of classical 21-OHD, in spite of the complexity of gene organization found in the cluster C4+CYP21 (12 different haplotypes), laboratory evaluation of complement activation and C4 allotypes were in the normal ranges, and the patients did not present significant recurrent infections or autoimmunity. Considering this redundant gene cluster, C4 seems to be a well-protected gene segment along the evolutionary process. The results show the importance of molecular medicine approach in diagnosing complex diseases and creating conditions for genetic counselling, treatment and prevention of diseases. Definitive and efficient techniques to elucidate the number of *C4A* and *C4B* genes and polymorphism of the protein products are important to determine the role that C4A and C4B play in disease associations in the prognosis and therapeutic intervention of autoimmune and inflammatory diseases.

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