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Familial C4B deficiency and immune complex glomerulonephritis

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Membranoproliferative glomerulonephritis

Abstract Homozygous complement C4B deficiency is described in a Southern European young female patient with Membranoproliferative Glomerulonephritis (MPGN) type III characterized by renal biopsies with strong complement C4 and IgG deposits. Low C4 levels were independent of clinical evolution or type of immunosuppression and were found in three other family members without renal disease or infections. HLA typing revealed that the patient has homozygous *A*02*, *Cw*06*, *B*50* at the class I region, and *DRB1*08* and *DQB1*03* at the class II region. Genotypic and phenotypic studies demonstrated that the patient has homozygous monomodular RCCX in the HLA class III region, with single long *C4A* genes coding for C4A3 and complete C4B deficiency. Her father, mother, son and niece have heterozygous C4B deficiency. The patient's deceased brother had a history of Henoch–Schönlein Purpura (HSP), an immune complex-mediated proliferative glomerulonephritis. These findings challenge the putative pathophysiological roles of C4A and C4B and underscore the need to perform functional assays, C4 allotyping and genotyping on patients with persistently low serum levels of a classical pathway complement component and glomerulopathy associated with immune deposits.

Introduction

The complement system is an essential element of the innate immune system [1]. The lack of any complement component may severely disturb host defenses. Complement component C4 plays a central role in classical and lectin complement activation pathways. C4 has two isotypic forms, C4A and C4B, encoded by loci in the MHC class III region on chromosome 6. The number of *C4* genes varies between two and eight in a diploid genome among different individuals [2]. A *C4* gene duplication is always concurrent with its neighboring genes *RP* at the 5′ region and *CYP21* and *TNX* at the 3′ region [3]. This unit is known as an RCCX module (Fig. 1). Low *C4A* and *C4B* copy-numbers (i.e., 0 or 1 copy) have a combined frequency of 31.6% and are associated with a variety of autoimmune or infectious diseases [4]. A complete

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deficiency of both C4A and C4B is nevertheless rare but strongly associated with impaired immune complex clearance and systemic lupus erythematous (SLE) [5]. On the other hand, homozygous complement C4B deficiency has a frequency of 3% in the American Caucasian population [6]. There have been a few case reports regarding homozygous C4B deficiency and non-SLE glomerulonephritis [7–11].

Idiopathic MPGN represents only 6.4–7.3% of all primary glomerulopathies [12–15] and only 0.4% of all patients receive renal replacement therapy (USRDS). The renal outcome is poor, with a 10-year survival of 32–40%, and a remission rate of 5.0–7.6% [13,16]. MPGN type III is a rare renal disease of unknown cause, usually sporadic, representing less than 1% of glomerulonephritis in URSD registry data [17]. There are a few cases with MPGN and C4 deficiency reported in the literature [18–21] and even fewer cases associated with C4B*Q0 [19,20].

Here we report a family with two siblings who were inflicted with proliferative glomerulonephritis, one of whom had confirmed complete C4B-deficiency. Four family members had heterozygous C4B deficiency. The dosage of the *C4A* and *C4B* genes, three other constituent genes of the RCCX modules, and C4 protein phenotypes were investigated. The clinical presentation of MPGN type III for this C4B deficient patient is described.

Materials and methods

Blood donors

Informed consents were obtained from blood donors according to IRB-approved protocol. Peripheral blood in EDTA-tubes was used for isolation of genomic DNA and plasma samples, as described [2].

Real-time PCR assays of C4 gene copy-numbers

A TaqMan-based quantitative real-time PCR strategy was applied to determine the gene copy-numbers of total C4, C4A and C4B in DNA samples rapidly, as described [22].

Southern blot analysis

Genomic DNA samples were digested with *Taq*I restriction enzyme, resolved by agarose gel electrophoresis, Southern blotted, hybridized with probes that annealed to genomic regions for *RP* and *C4*, *CYP21*, and 3′ region of *TNX*. B. *Psh*AI–*Pvu*II RFLP for *C4A* and *C4B*. Genomic DNA samples were digested with *Psh*AI that differentiates between the *C4A* and *C4B* isotypic sequences, and with *Pvu*II to improve separation of genomic DNA fragments specific for *C4A* and *C4B*. After agarose gel electrophoresis and Southern blotting to nylon membrane, the membrane was hybridized to a C4d specific probe labeled by ^{32}P-dCTP [2].

Allotyping of C4 proteins

EDTA-plasma samples were digested with neuraminidase and carboxylpeptidase B, resolved with high-voltage agarose gel electrophoresis, immunofixed with goat anti-human C4 antisera, blotted to remove diffusible proteins and stained [2,23].

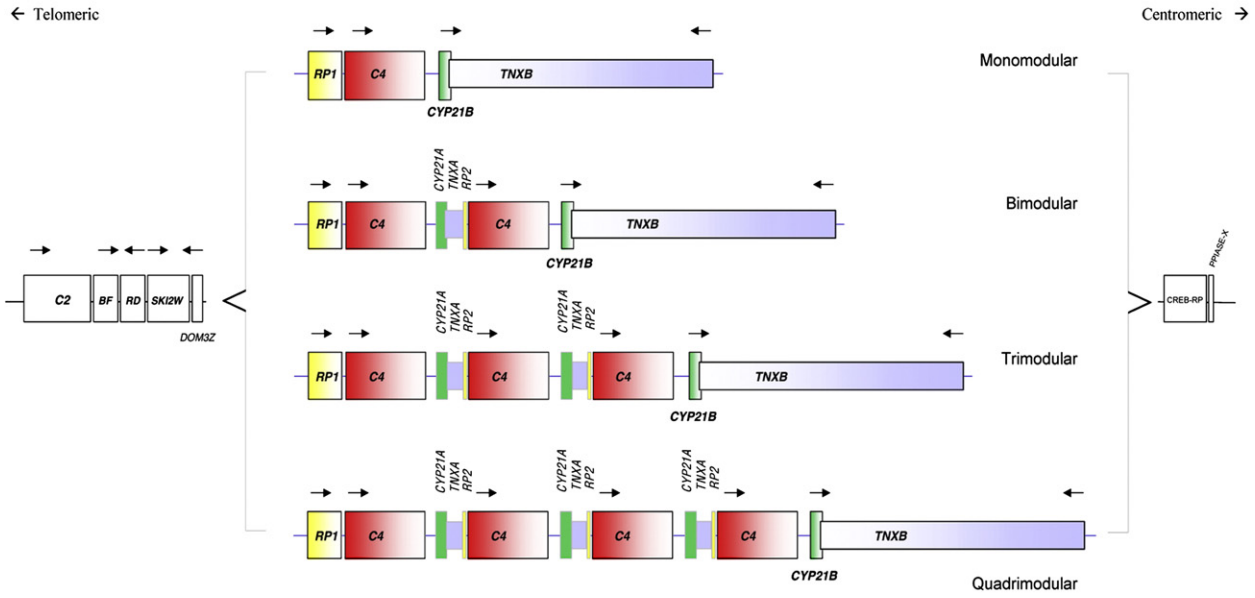
Results

Index case

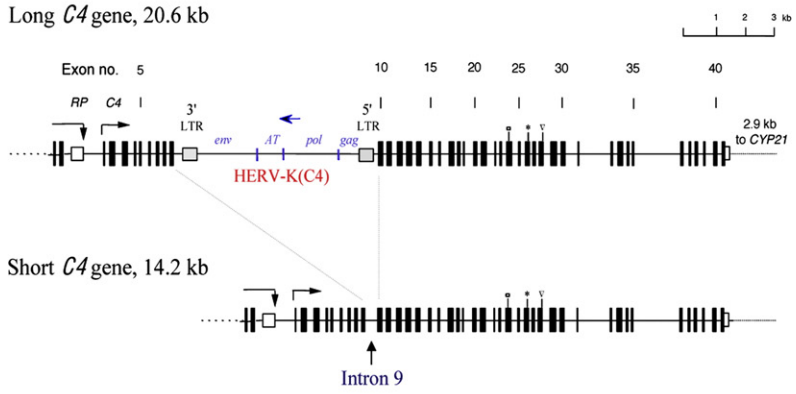
A 28-year old white female first presented with anasarca because of nephrotic syndrome. She was hypertensive; urine protein excretion was 7 g/d and serum albumin 1.7 g/dL, associated with microscopic hematuria, red cell casts and normal renal function. Serum C3 protein concentration was normal, C4 level was persistently low (5–7 mg/dL; normal range 17.4–52.2 mg/dL), IgG was low (247 mg/dL; normal range 690–1400 mg/dL). Autoantibodies (antinuclear, anti-DNA, anti-Sm, anti-RNP, anti-Ro/SSA, and anticardiolipin), MPO-ANCA and cryoglobulins were absent. Bacterial cultures, and viral (HIV, HCV, and HBV) serology were negative. She did not meet diagnostic criteria for SLE. Light microscopy of kidney biopsy showed a probable type III MPGN with extensive deposits (Fig. 2A). The most prominent anomaly was a general thickening of the capillary walls which sometimes assumed a broad glassy eosinophilic appearance and reduced capillary lumens. Hyperlobularity was not apparent. Inflammatory cells were not conspicuous and there were neither tuft necrosis nor crescents. Interstitial fibrosis or tubular atrophy was mild. The presence of frequent double contours in most loops but a lack of spikes was demonstrated with Jones's silver stain. Any existing deposits within the loops were not stained with silver (Fig. 2B). Immunofluorescence (IF) showed intense (3+) C4 and IgG, moderate (2+) C1 and IgM, and weak (1+) IgA and C3, globally distributed large confluent granular deposits, forming a pseudolinear pattern within capillary walls (Fig. 2C). The more intense and larger deposits had globular or semilunar profiles. There was some granular mesangial staining with weaker intensity. These IF results suggested immune complex deposition. Electron microscopy was consistent with a Strife and Anders type of type III MPGN [24,25]. In most capillaries the profile of the normal lamina densa was not visible. In its place were numerous moderately dense deposits, usually uniformly textured aggregates of finely granular material, sometimes less well-defined irregular aggregates. These deposits predominated on the epithelial side, but many extended from epithelium to endothelium. Mesangial deposits were noted more in the paramesangial region. A thin reactive type continuous lamina densa closely hugged and separated the dense deposits from the extensively thinned and effaced podocytes and, discontinuously, from endothelial cells. These inner and outer dense laminas corresponded to the double contours. Mesangial interposition into the capillary was confirmed. Spikes were not identified. In the glomerulus with a few less affected loops the lamina densa was single and normal; scattered fairly large and uniform subendothelial dense deposits were occasionally observed on these loops. No tubuloreticular structures were noted. (Fig. 2D). Lupus nephritis was considered to be the most likely differential diagnosis.

Therapy was started with methylprednisolone pulses, followed by oral prednisolone (1 mg/kg) and six 1 g

A. Copy number variation of complement C4 and RCCX

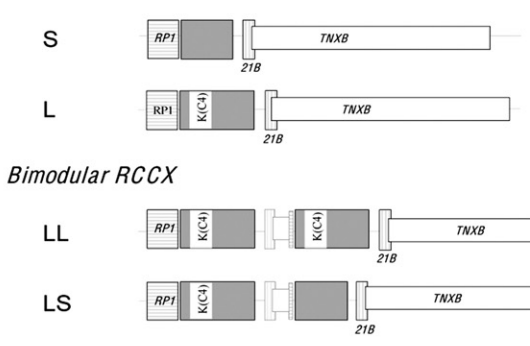


B. Size variation of complement C4 genes



HERV-K(C4), human endogenous retrovirus with lysine-primer binding site located in *C4* gene, 6.36 kb in size.

C. Common RCCX modules in Europeans



	*Taq*I genomic RFLP (kb)		
	RP-C4	*CYP21*	*TNX*
S	6.4	3.7	2.5
L	7.0	3.7	2.5
LL	7.0, 6.0	3.7, 3.2	2.5, 2.4
LS	7.0, 5.4	3.7, 3.2	2.5, 2.4

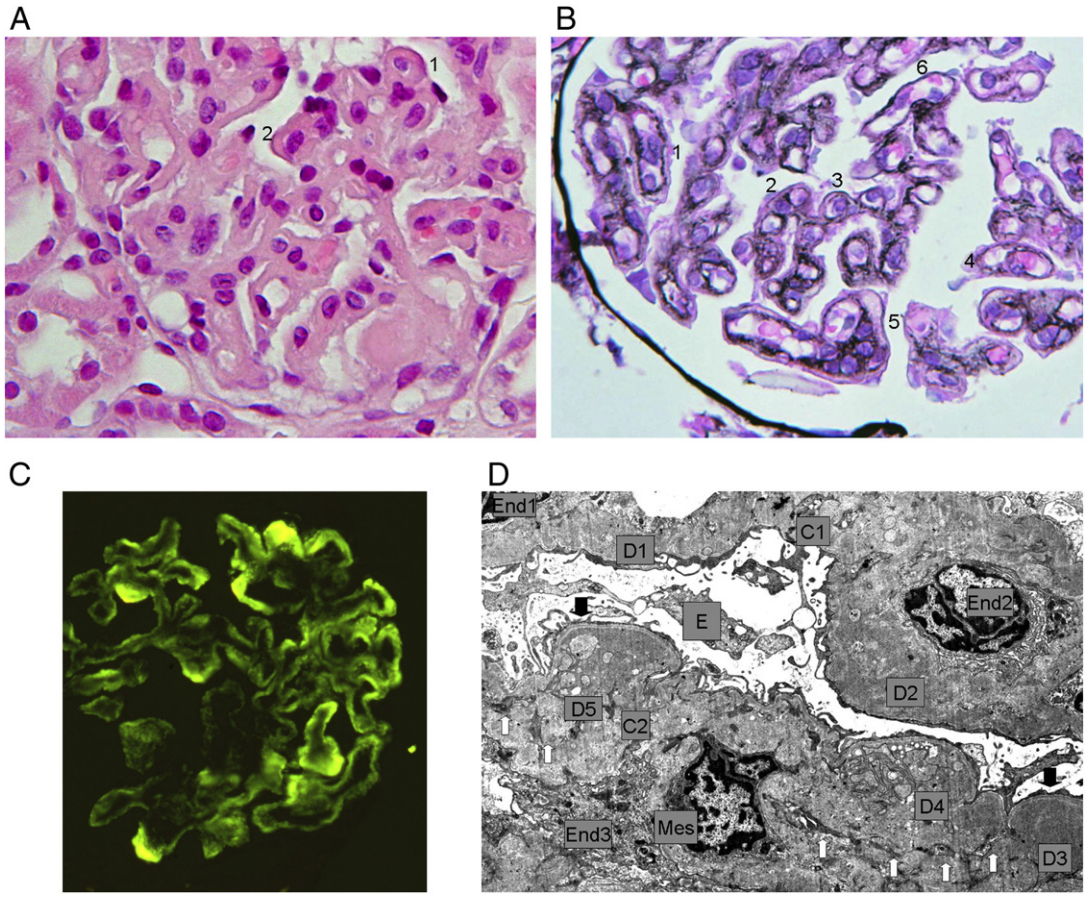


Figure 2 First renal biopsy of the patient: Light microscopy (A) Silver stain (B), immunofluorescence (C), electromicroscopy (D). 2A Detail of the diffuse glomerulonephritis showing prominent generalized thickening of the capillary walls, sometimes with a broad glassy eosinophilic appearance (1, 2). There was slight to moderate increase in mesangial cells and expansion of the mesangial regions. Capillary lumina appear generally reduced. H+E. 2B. Within the thickened capillary walls the basement membrane appears irregular, interrupted or vacuolated (1, 3, 4). There are also several double contours some of which with a fairly thin outer contour (2, 3, 5). Any existing deposits within the wall appeared unstained with silver (5). One capillary wall has relatively normal wall (6). Mesangial matrix was mildly increased (5). Jones's silver. 2C Intense confluent granular deposits of C4 with pseudolinear pattern globally distributed within the capillary walls and less intense mesangial staining. Large intense globular or semilunar collections in the wall were also seen. 2D Low magnification E.M: Capillaries (C1, C2) with endothelium (End1 to End3), Bowman's space and visceral epithelium (E). In the thickened and deformed wall there are large, numerous moderately dense deposits (D1 to D5) more concentrated towards the subepithelial zone, but also extending through the whole wall (D2). Generally the dense deposits have uniform texture (D1 to D3) but some show complex irregular texture which includes less dense vesicular profiles, small clear spaces and groups of convoluted fine plasma membrane like structures (D4, D5). Generally there was no definition of the normal lamina densa of the basement membrane. Reactive type continuous thin lamina densa was seen along the epithelial side effectively separating the dense deposits from the podocytes (between black arrows). Clear cut formation of spikes was not seen. Reactive thin lamina densa on the endothelial side was ill defined and discontinuous. Mesangial cell (Mes) interposition was represented by multiple small cytoplasmic processes (white arrows) within the thickened capillary wall. There was severe thinning of podocytes and extensive effacement of foot processes. Negative magnification 2500×.

Cyclophosphamide monthly pulses. After 16 weeks of therapy the protein/creatinine urinary ratio (PCUR g/g) decreased below 0.6 and prednisone was tapered over eight months. The patient was admitted at the eleven month of follow-up. She had a marked increase of PCUR to 9, and was eight weeks pregnant. Because of the risks for both patient and fetus, a therapeutic abortion was performed and the urine protein excretion declined. For the next 9 months the patient remained without immunosuppressants. The PCUR again increased to almost 5 and therapy with Mycophenolate Mophetil (MMF) 1 g twice-a-day plus prednisolone (PRED) 20 mg every other day, was begun at month 18. There was a

Figure 1 The gene size and polygenic variations of human complement *C4* and *RP-C4-CYP21-TNX* (RCCX modules) in the MHC class III region. A. Copy-number variation of RCCX modules; B. Exon–intron structure and size variation of *C4* gene; HERV-K(C4) is an endogenous virus inserted into intron 9 of long *C4* genes; C. Common RCCX haplotypes and diagnostic *Taq*I restriction fragment size (in kb) for each haplotype (modified from ref. [50]).

quick response, but after six months the PCUR rose progressively to 7, probably related to non-compliance, as admitted by the patient. In the following year and without immunosuppression the PCUR decreased to below 4 and increased again to 8.5 (Fig. 3). A second renal biopsy was performed at the 41st month of follow-up, which confirmed MPGN type III diagnosis, but with fewer immune deposits and without signs of chronicity (Figs. 4 A–C). The glomerular capsule appeared marginally thicker than that observed in the first biopsy. Immunofluorescence showed, in general, weaker reactions for all immune proteins. However there were still extensive moderate (2+) pseudolinear IgG and C4 and slight (1+) C1 and IgM deposits, all predominating within the capillary walls. Large globular or semilunar collections similar to those of the first biopsy remained present. IgA and C3 appeared much reduced, but there were strong segmental capsular and hilar deposits of C3. It was assumed that the evolution was related to the immunosuppressant therapy. MMF/PRED was re-started. During the six years of follow-up the patient received three cycles of MMF/PRED, serum C4 levels remained depressed persistently and the serum albumin and PCUR were never normalized. Renal function remained normal. The patient was under ACE inhibitor treatment from the start.

Family study

The patient's brother died on dialysis at age 28. A diagnosis of Henoch–Schonlein purpura (HSP) with proliferative glomerulonephritis was made at age 14. Neither the biopsy nor C4 protein levels were available for review.

Clinical and laboratory testing of four direct relatives of the patient revealed a normal urinalysis and renal function. Her father, son and niece (sister's daughter) had low C4 levels without disease. Therefore, we proceeded to a genetic analysis and protein allotyping of complement C4.

Genotypic and phenotypic studies of complement C4

The results of the family study for complement C4 haplotypes are shown in Fig. 5. Genomic DNA samples were isolated from peripheral blood mononuclear cells. A TaqMan-based real-time PCR strategy [22] was initially applied to determine the copy-number of *C4A* and *C4B* in each subject. The index patient has a total of two *C4* genes in her diploid genome: two copies of *C4A* but no *C4B*. The father and the niece each has three copies of *C4* genes: two copies of *C4A* and one copy of *C4B*.

We further investigated the *RP-C4-CYP21-TNX* (RCCX) modular variation by genomic Southern blot analyses of *Taq*I and *Psh*AI–*Pvu*II restriction fragment length polymorphisms (RFLP) and probed for genes in the RCCX module [2]. *Taq*I RFLP identified numbers of long and short *C4* genes and RCCX haplotypes. The index patient has homozygous, monomodular RCCX (L/L) with single copies of long *C4* genes, and single copies of neighboring genes for steroid *CYP21B* and tenascin *TNXB* (Fig. 5A). *Psh*AI–*Pvu*II RFLP showed that the *C4* genes present in the index patient were *C4A* genes, while *C4B* genes were absent (Fig. 5B). Immunofixation using EDTA-plasma revealed the presence of C4A3 allotype and complete absence of C4B protein in the index case (Fig. 5C). The father has bimodular-LL and monomodular-L RCCX (LL/L) haplotypes with three copies of *C4* genes: two *C4A* and one *C4B*. The mother has a total of three *C4* genes with bimodular-LS and monomodular-L; two *C4A* and one *C4B*. The patient inherited the monomodular-L haplotypes with *C4A* (but no *C4B*) from each parent. Immunofixation of EDTA-plasma C4 showed that the mother has C4A3, C4A3 and C4B2 protein allotypes, while the son has C4A3, C4A3 and C4B1 allotypes, which are consistent with the genotypic data.

Complement C4 and the RCCX modules are located in the class III region of the human major histocompatibility complex, or the HLA. Thus, HLA typing of class I and class II genes was performed for the patient and her five direct

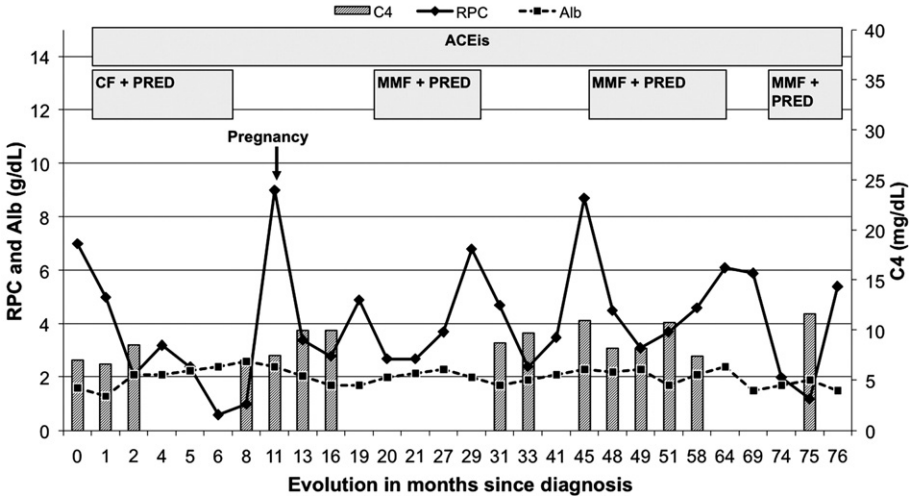


Figure 3 Time course of proteinuria in response to different therapeutic approaches CF: Cyclophosphamide, 6 1 g monthly pulses PRED: prednisolone initially 1 mg/kg four month and then tapering MMF: Mycophenolate Mophetil 0.5–1 g twice-a-day ACEI: angiotensin converting enzyme inhibitors RPC: ratio of urinary protein/creatinine (g/g) Alb: serum albumin (g/dL) C4: Complement component C4 mg/dL (normal range 17.4–52.2).

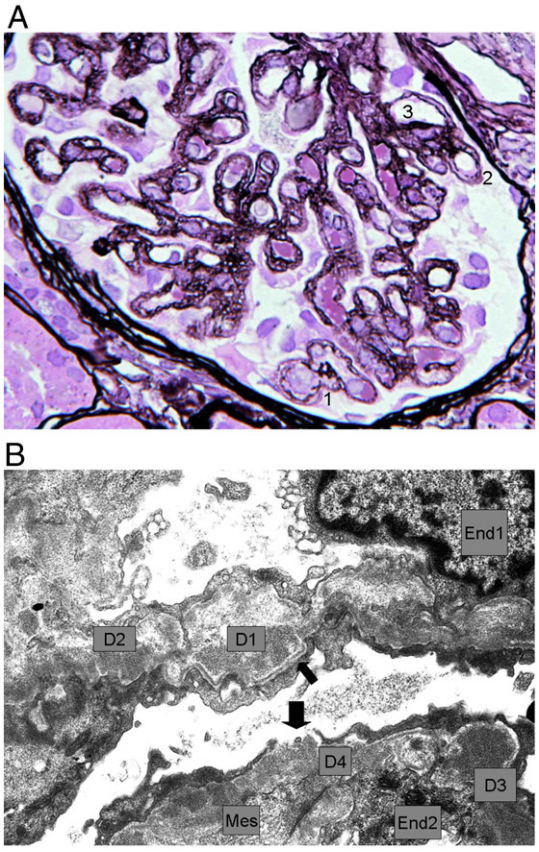


Figure 4 Second Biopsy: silver stain LM (A), EM (B). 4A. The basement membrane was generally irregular, sometimes interrupted (1,2) and double contours were present (3). The vacuolated or moth eaten profile of the walls, seen in the first biopsy, was widespread and also present in the paramesangium. Capillaries with normal single layer basement membrane were not seen. The glomerular capsule appeared marginally thicker than in the first biopsy. 4B. The walls of 2 capillaries with endothelial cells (End1 and End2) contained several dense deposits (examples D1 to D4). D1 appeared rarified and surrounded by thin reactive basement membrane which appeared well-defined and continuous in the subepithelium (thin arrow) and discontinuous in the subendothelium. D2 made direct contact with the podocyte. D3 had fairly uniform high density and was also partly surrounded by subepithelial basement membrane. There was a gap between podocyte processes leaving D4 in direct contact with Bowman's space. Mesangial cell cytoplasm (Mes) interposition was seen in the wall near D4 and other deposits. Negative 6000×.

family members to identify the haplotype associated with C4B deficiency. The patient has homozygous HLA A*02, Cw*06, and B*50 at the telomeric class I region, and DRB1*08 and DQB1*03 at the centromeric class II region. All other family members were heterozygous with this haplotype (Fig. 6).

Discussion

MPGN type III is a rare, sporadic and progressive renal disease of unknown etiology. The finding of immune complexes with cellular inflammatory infiltrate suggests an immune mediated process. In some cases, a genetic basis of the diseases is evident, particularly in families [26] with inherited defects of the complement system [27–29]. Only a few cases have been reported with C4 deficiency [18–21,30] and one with isolated C4B deficiency [19].

Early in her disease presentation, the index patient was treated as if she had lupus nephritis, although she did not fulfill the diagnosis criteria for SLE clinically or serologically [31]. The patient received three alternated cycles of immunosuppression for about six years and spent almost two years without medication. There was a significant reduction of proteinuria during the treatment period but complete remission was never achieved and C4 levels remained depressed throughout the follow-up period.

The brother had reached an end-stage renal disease because of a proliferative immune complex-mediated glomerulonephritis with IgA deposits, and was diagnosed of Henoch–Schonlein purpura (HSP). The histology of IgA nephropathy and HSP is characterized by glomerular IgA, IgG and C3 deposits mainly in the mesangium but also in the capillary wall. Unfortunately, the brother's renal biopsy was not available for review. It is unusual for two siblings to be inflicted with immune complex-mediated glomerular injuries. We speculate that this could be related to the presence of C4B deficiency in the family. In this regard, the coexistence of IgA nephropathy and MPGN in several members of the same family had been reported previously, and it was associated with specific C4 polymorphisms [32].

Complement abnormalities in patients with HSP nephritis have previously been reported [7,11,33–35] and C4 deficiency was suggested as a risk factor for HSP nephritis and other immune complex glomerulonephritis, possibly due to a defective clearance mechanism [36,37]. *C4* null alleles are common in HSP patients and the *C4B*Q0* allele is thought to increase the risk of developing HSP [33]. Other authors have reported HSP patients with familiar C4 deficiency [30]. Low C4 levels in three family members, genotyping and phenotyping of C4 revealed that our patient was homozygous for C4B deficiency with HLA haplotype A*02-Cw*06-B*50-C4A3 (L)-DRB1*08-DQB1*03. Her parents each was heterozygous with this haplotype, and had only one copy of *C4B* gene, similar to the patient's son and niece. Phenotypically, heterozygous deficiency of either C4A or C4B is a common immune protein defect in all human populations as a consequence of the high frequency of the MHC haplotypes with *C4* gene copy-number variations, although homozygous deficiency is rarer [38]. In Caucasians, more than two-thirds of the MHC haplotypes have two copies of *C4* genes; the other haplotypes mainly possess either one *C4* or three copies of *C4* genes. Each of these *C4* genes may be long (L) or short (S). The number of long or short *C4A* and *C4B* genes present in an individual plays a role in determining the basal level of C4 proteins in the peripheral blood plasma. The index patient has only single copy of long *C4* gene coding for the C4A protein on each of her chromosome 6, which is one of the causes for persistently low levels of plasma C4. The varying efficiencies of complement activation that result from differential expression levels and protein polymorphisms of C4A and C4B may have a profound effect on an individual's strengths in innate and adaptive immune systems, and susceptibility to systemic autoimmune

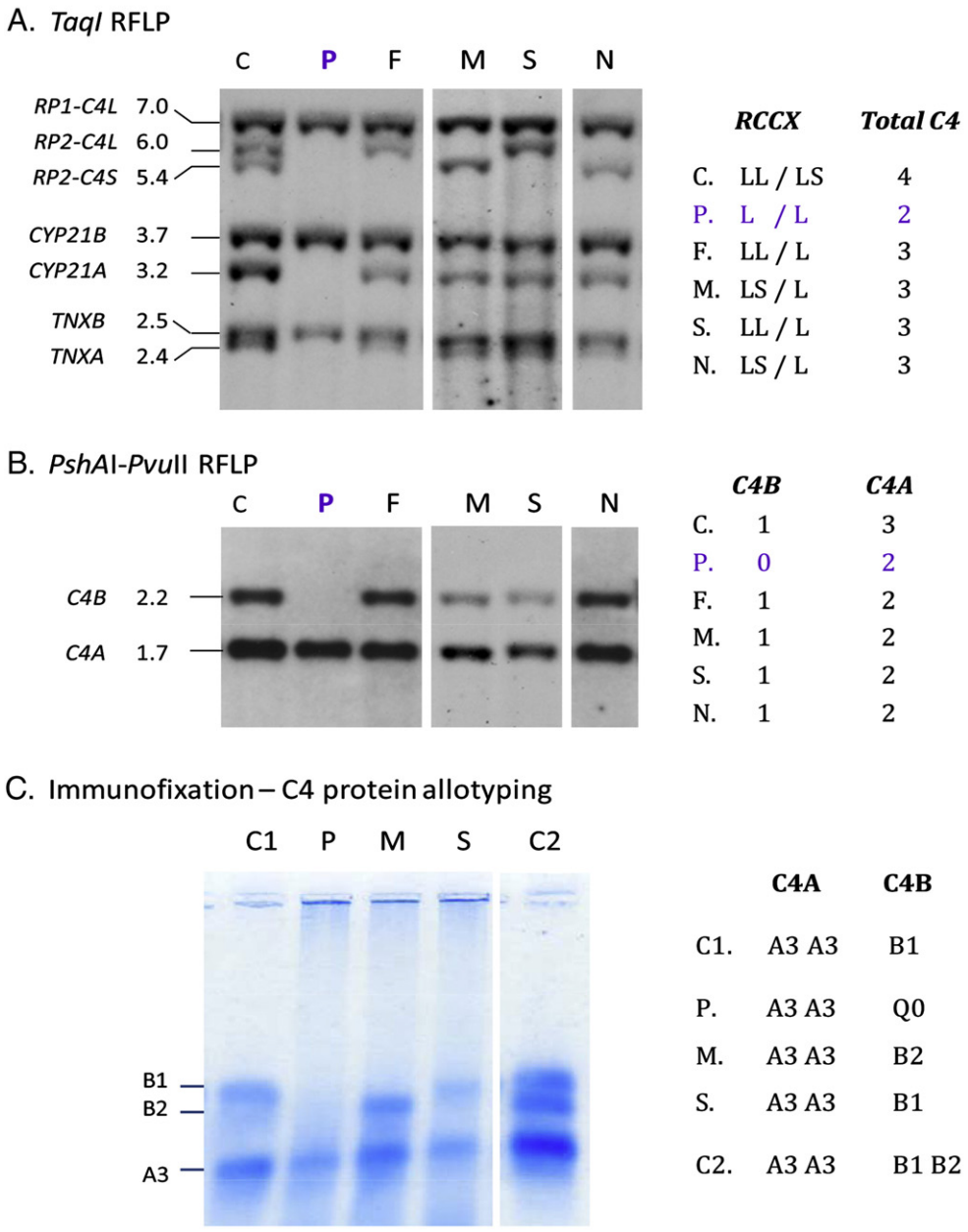


Figure 5 Genotyping and phenotyping of complement C4. A. *Taq*I restriction fragment length polymorphism (RFLP) and Southern blot analyses of RCCX modules. B. *Psh*AI–*Pvu*II RFLP for *C4A* and *C4B*. C. Immunofixation of C4A and C4B proteins. Abbreviations: P, index patient; F, father; M, mother, S, son; N, niece; C, C1 and C2, three different healthy control subjects.

diseases. C4 null alleles represent relevant disease markers and additive genetic risk factors for autoimmune phenomena in multiple races [36]. Cumulative results from more than 35 different studies revealed that heterozygous and homozygous deficiencies of C4A were present in 40–60% of SLE patients among northern and central Europeans, Anglo-Saxons, Caucasians in the US, Afro-Americans, Asian Chinese, Koreans and Japanese [5]. Interestingly, Spanish, Mexican and Australian Aborigine SLE patients had increased frequencies of C4B deficiency instead of C4A deficiency [39,40]. It is possible that different racial and genetic backgrounds could change the thresholds for the requirement of C4A or C4B protein levels in immune tolerance and immune regulation [4]. There is no information on the frequency of C4B deficiency in SLE and lupus-like patients in the Portuguese population. We speculate that the frequency might be similar to that in the Spanish population [40].

While the coexistence of C4B deficiency and type III MPGN could be coincidental, there are pathophysiological pathways that can explain the association. C4 deficiency is frequently associated with immune complex disease [36]. C4B is important in the formation of the classical pathway C3 covertase, leading to the assembly of the membrane-attack complex against microbes [37]. A C4B deficiency would adversely affect the efficiency and progression of the complement activation, and possibly diminish the capacity of phagocytosis and clearance of pathogenic immune complexes, apoptotic and necrotic cells, predisposing an

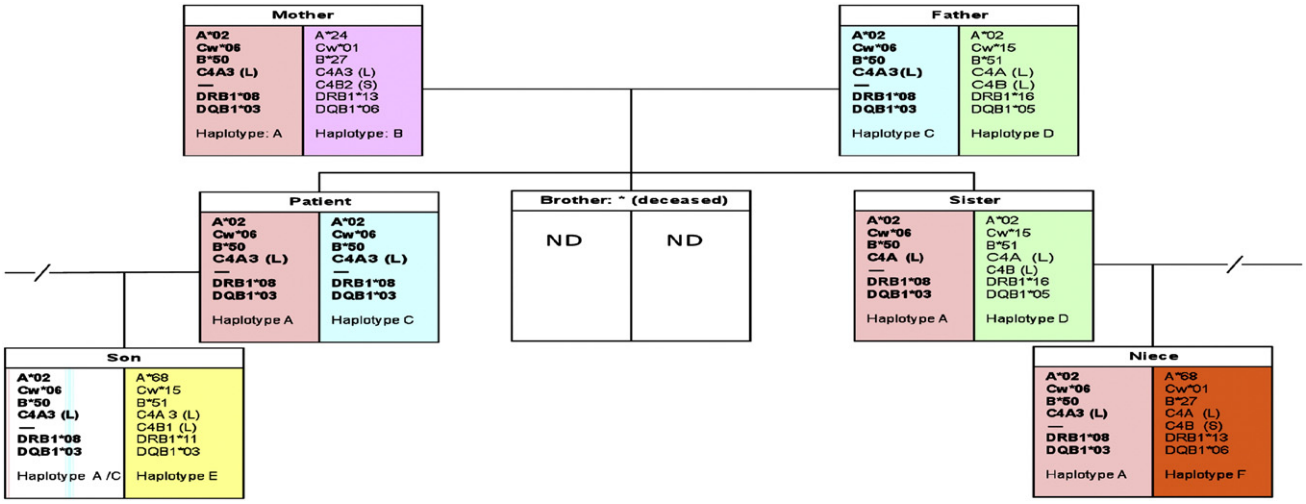


Figure 6 Family tree with HLA and complement *C4* genotypes. One of the patient's siblings was deceased and no genetic data was available.

individual to developing immune complex-mediated diseases such as HSP or MPGN. Human subjects with a genetic predisposition to infection or an impaired protection against autoimmunity would be vulnerable to immune complex glomerulonephritis. In this regard, infection may be complicated by MPGN or HSP/IgA nephropathy. Different reports have showed an association between glomerulonephritis and C4B deficiency in patients with SLE or Lupus-like disease [10,20,37,41]. Tsuda et al. [19] also reported a MPGN-III patient from Japan with null C4B and persistently low C4 levels. Interestingly, the patient's renal biopsy revealed strong positive staining for C3 and weak staining for C4.

We further investigated the status of two other immune effectors genes that exhibit gene copy-number variations and play a role in renal diseases, which were complement factor H-related genes *CFHR3-R1* located at chromosome 1q32 [42,43], and immunoglobulin Fcγ-receptor *FCGR3B* located at chromosome 1q23 [44], in our patient and direct family members. Through genomic Southern blot analyses of *Taq*I digested genomic DNA with gene-specific probes for *CFHR1*, *CFHR2*, *CFH3* and *CFHR4*, and for *FCGR3B* and *FCGR3A*, we did not observe a deletion of *CFHR3—CFHR1*, or low copy-number *FCGR3B*, in all six members of the family (data not shown).

The clinical course of MPGN type III is not clear, long-term kidney survival is poor, and there is little evidence for a specific treatment to improve outcome. Reviews of immunosuppression trials in adults did not reveal any significant benefit [45—48]. The onset with nephrotic syndrome worsens the prognosis. Lhotta [41] described a C4-deficient patient with membranous nephritis who responded well to MMF therapy. We therefore decided to use this option, bearing in mind that MMF inhibits the proliferation of activated B and T lymphocytes and antibody synthesis, and limits immune complex deposition in the glomeruli [49]. We observed that MMF reduced urinary protein excretion by almost 70%. The treatment was well tolerated, without infections. Renal function in our patient has remained normal during more than 6 years of follow-up.

Immunofluorescence staining for C4 is not a routine part of renal biopsy evaluation in the USA. However, in this case, it was the unusual pattern of very strong and widespread C4 capillary wall deposits observed on immunofluorescence that suggested from the outset that we were faced with a rare condition. This finding together with the persistent low C4 in the serum, absence of lupus or infection parameters initiated the search for a possible familial defect. We suggest that routine use of C4 staining could result in identification of further similar cases.

C4B deficiencies have not been well-documented in (and rarely associated with) MPGN-III. Measurement of C4 protein levels may not detect C4A or C4B deficiencies; more elaborate diagnostic tests, such as C4 protein allotyping and genotyping, are required to identify complement C4 deficiencies in familial glomerulopathy. Our understanding of the exact mechanisms by which complement abnormalities lead to specific disease phenotypes is still poor. More in depth analyses are required for a better understanding of the pathogenesis and, thus, the development of specific therapies.

Conflict of interest statement

None

Acknowledgments

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