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ORIGINAL ARTICLE

Novel Mutations Causing C5 Deficiency in Three North-African Families

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Abstract The complement system plays a central role in defense to encapsulated bacteria through opsonization and membrane attack complex (MAC) dependent lysis. The three activation pathways (classical, lectin, and alternative) converge in the cleavage of C5, which initiates MAC formation and target lysis. C5 deficiency is associated to recurrent infections by *Neisseria spp*. In the present study, complement deficiency was suspected in three families of North-African origin after one episode of invasive meningitis due to a non-groupable and two uncommon Meningococcal serotypes (E29, Y). Activity of alternative and classical pathways of complement were markedly reduced and the measurement of terminal complement components revealed total C5 absence. *C5* gene analysis revealed two novel mutations as causative of the deficiency: Family A propositus carried a homozygous deletion of two adenines in the exon 21 of *C5* gene, resulting in a frameshift and a truncated protein (c.2607_2608del/p.Ser870ProfsX3 mutation). Families B and C probands carried the same homozygous deletion of three consecutive nucleotides (CAA) in exon 9 of the *C5* gene, leading to the deletion of asparagine 320 (c.960_962del/p.Asn320del mutation). Family studies confirmed an autosomal recessive inheritance pattern. Although sharing the same geographical origin, families B and C were unrelated. This prompted us to investigate this mutation prevalence in a cohort of 768 North-African healthy individuals. We identified one heterozygous carrier of the p.Asn320del mutation (allelic frequency = 0.065 %), indicating that this mutation is present at low frequency in North-African population.

Keywords Complement system · complement 5 deficiency · meningococcal disease · mutation · African continental ancestry group

Roger Colobran and Clara Franco-Jarava contributed equally to this work.

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Introduction

The complement system is a complex network of plasma and membrane-associated serum proteins that plays a central role in the innate immunity against microorganisms and in removal of waste material such as dying host cells and immune complexes [1]. Recent work has found that the complement

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system has additional roles in the regulation of the adaptive immune response including T cell activation [2]. It is accepted that complement activation can be initiated, depending on the context, through three main distinct pathways (alternative, lectin and classical) all leading to the common terminal pathway. This terminal pathway requires the generation of the C5 convertase complex, which cleaves C5 into the anaphylatoxin C5a and C5b. Deposition of C5b onto a target initiates membrane attack complex (MAC) formation and target lysis [3].

Human complement component C5 is a plasma glycoprotein composed of two disulfide-bound polypeptide chains (C5α and C5β, 115 and 75 kDa, respectively). C5 is mainly synthesized by hepatocytes, monocytes and lymphocytes as an intracellular single chain precursor of 1976 aa, including an 18 aa leader peptide and an arginine-rich linker region (RPRR) connecting the α and β chains [4, 5].

After activation by the C5 convertase, C5 is cleaved into C5a and C5b. C5a is the most potent complement-derived pro-inflammatory peptide [6]. It serves not only as a chemoattractant for neutrophils, eosinophils, monocytes, and T lymphocytes but also triggers multiple defense mechanisms in leukocytes. Through its multiple binding sites C5b initiates and directs the assembly of the membrane attack complex [7].

The *C5* coding gene is located on chromosome 9q34.1 spanning a genomic region of 124 kb. Its canonical open reading frame is composed of 41 exons coding for C5α (exons 1–16) and C5β (exons 17–41). When transcribed, leads to a 6 kb mRNA translated into a pre-C5 protein which is processed into the mature, two-chain C5 form by the removal of the RPRR linker region. Two truncated transcripts using alternative splicing and polyadenylation signals have been reported in mouse but are poorly characterized in humans [8].

C5 deficiency (C5D, OMIM #609536) is a rare autosomal recessive disease associated with recurrent infection episodes, particularly meningitis and extragenital gonorrhoea by *Neisseriae* species, which are the most frequent microorganisms isolated in these patients as well as in patients with other terminal complement component deficiencies [9, 10]. Clinically, meningococcal vaccination and prophylactic antibiotics should be considered for the treatment of C5-deficient patients. The administration of fresh frozen plasma during active infection to replace C5 can also be beneficial.

Although around 50 cases of C5 deficiency have been published, the molecular defect has only been characterized in a limited number of families and so far only 15 different mutations along the *C5* gene have been reported [11–19]. Most of these mutations have been described in only one family, being the most striking exception the C5 p.A252T. This mutation has been recently reported to be responsible for C5 deficiency which is found in approximately 7 % of Black African meningococcal disease cases in the Western Cape (South Africa) [17].

The aim of the present work is to characterize the genetic defects underlying complement C5 deficiency in three unrelated North-African families whose index cases developed invasive meningococcal disease and to determine the frequency of this newly identified mutations in North-African populations.

Methods

Patients

Families A and B attended the Pediatric Infectious Diseases and Immunodeficiencies Unit (UPIIP) of Hospital Universitari Vall d'Hebron (HUVH, Barcelona, Spain). Family C attended and was diagnosed at the Pediatric Unit of Hospital Parc Taulí (Sabadell, Spain) and was later referred to HUVH to perform the genetic study.

Family A

The index case in family A was a girl who at the age of 17 months developed invasive meningococcal disease by an uncommon and relatively benign serotype (E29). The family came originally from Morocco, although the patient was born in Barcelona (Spain). There was consanguinity in the family and there was no relevant familial history of disease. The patient was healthy before the meningococcal sepsis. At the time of the study the mother was 5-month pregnant.

Family B

The index case in family B was a boy, who at the age of 34 months developed invasive meningococcal disease by an uncommon serotype in our media (Y). The family did not report consanguinity nor relevant family history. The index case had two older brothers with no history of infectious diseases. The family came from the area of Alnif (Morocco).

Family C

The index case in family C was referred from Parc Taulí hospital for the genetic study of *C5*. The patient, a girl aged 7, had developed two episodes of invasive meningococcal disease at age of 5 and 6 years due to non-groupable *Neisseria meningitidis*. She has seven healthy siblings and her family did not report consanguinity. The family was also from Alnif (Morocco) but denied any connection with family B.

All patients received standard vaccines by the Spanish National Health Service schedule, which includes Meningococcus C but not other meningococcal serotypes nor pneumococcus. Vaccine calendar was subsequently optimized after diagnosis of C5 deficiency.

A written informed consent was obtained from all the patients and relatives for the genetic studies here reported according to the procedures of the Institutional Ethical Review Board of Hospital Universitari Vall d'Hebrons.

Complement Protein Levels and Function

The study followed the recommendations of the European Complement Network. Pathway activation was assessed by means of commercial assays: CH50 Liposome based test (Wako Pure Chemical Industries, Japan) and APH50 test (The Binding Site, UK). To distinguish primary from secondary deficiencies, the activation product SC5b-9 was measured using an ELISA assay (Quidel Corporation, USA). Individual complement components of the terminal pathway (C5-C9) were measured using plates coated with polyclonal specific antibodies (The Binding Site, UK). These studies were performed in all available family members of the affected individuals.

Molecular Study of the *C5* Gene

Due to the large size of the *C5* gene (41 exons), the corresponding cDNA was sequenced instead of genomic DNA. Total blood was collected from the index patients in Tempus™ Blood RNA Tubes (Thermo Fisher Scientific, USA) and total RNA was isolated using Tempus Spin RNA Isolation Kit (Thermo Fisher Scientific, USA). cDNA was generated by reverse transcription using the Transcriptor First Strand cDNA Synthesis Kit (Roche, USA). All 41 exons of the *C5* gene were amplified by PCR using 6 overlapping fragments (primers and conditions are available upon request). The PCR products were subjected to automated fluorescent sequencing using BigDye Terminator V3.1 Cycle sequencing kit (Thermo Fisher Scientific, USA) in accordance with manufacturer's instructions.

The mutations identified were confirmed in genomic DNA of the patients and, subsequently, all available relatives were screened for the *C5* mutation.

Detection of *C5* Mutations in Large Cohort of Samples from North-African Healthy Individuals

To study the prevalence of the *C5* mutations in North African individuals, 768 DNA samples from different geographic origins in North Africa were analyzed: individuals from Morocco ($n=254$, including some individuals from the province of Errachidia where the city of Alnif is located), Algeria ($n=96$), Tunisia ($n=247$) and Libya ($n=171$) were analyzed by PCR using sequence specific primers (PCR-SSP). Primers sequence and PCR conditions are given in Supplementary Table 1. PCR products were visualized on 2 % agarose gel using Midori Green Advance (Nippon Genetics Europe GmbH, Germany) (Supplementary Figure 1). Positive samples were confirmed by Sanger sequencing.

Molecular Modelling of C5 p.Asn320del Mutation

The structure of the p.Asn320del mutant was modelled using the standard software MODELLER (version 9.15) [20], with default parameters. As a template, we used the structure of the human complement component 5 [21], with Protein Data Bank identifier 3CU7. The graphical representation in Fig. 3 was obtained with the program PyMOL, from Molecular Graphics System (version 1.7.4 Schrödinger, LLC).

Results

Complement Protein Levels and Function

Classical and alternative pathways activities were undetectable in index patients' sera and were within normal range in all the other family members (Table 1). These data, together with the absence of the activation product SC5b-9, and normal C3 and C4 levels, pointed out to a deficiency in a component of the terminal pathway. Protein C5 levels in sera by radial immunodiffusion revealed a complete absence of C5 in index patients, otherwise presenting normal levels for the rest of terminal complement components. Parents from all patients presented normal C5 sera levels, whereas siblings from patient B presented slightly below normal range levels of the molecule (Table 1). The addition of purified C5 molecule to patients' sera restored both classical and alternative pathways' activation, thus demonstrating that C5 deficiency was the cause of the complement activation defect.

Molecular Study of the *C5* Gene

Family A Carries a Novel Mutation c.2607_2608del/p.Ser870ProfsX3 Associated to C5 Deficiency

C5 cDNA sequencing of the index patient showed a homozygous deletion of two adenines in the exon 21 of *C5* gene, causing a frameshift that leads to the generation of a premature stop codon and a truncated protein of 872 aa (Fig. 1). This mutation had not been reported in the literature or databases, and, following the recommendations of the Human Genome Variation Society (HGVS), we named it c.2607_2608del/p.Ser870ProfsX3.

This mutation was confirmed in gDNA from the patient. Familial studies revealed that parents were heterozygous, confirming the autosomal recessive inheritance of C5 deficiency (Fig. 1). There was consanguinity in the family, thus providing an explanation for the fact that

Table 1 Complement studies in families with C5 deficiency

Individual	Genetic status	CH50 (U/mL)	APH50 (%)	C5 (μg/ml)
A.I.1	wt/mut	56	67.7	96.1
A.I.2	wt/mut	61	79.1	96.1
A.II.1	mut/mut	<1	<1	0
A.II.2	mut/mut	<1	<1	0
B.I.1	wt/mut	56	145,6	101
B.I.2	wt/mut	76	116.7	96.2
B.II.1	wt/mut	57	75.9	25
B.II.2	wt/mut	58	75.9	30.2
B.II.3	mut/mut	<1	<1	0
B.II.4	wt/mut	n.d.	n.d.	n.d.
C.I.1	wt/mut	65	86.8	111
C.I.2	wt/mut	60	87	115
C.II.6	wt/mut	61	82.5	95
C.II.7	wt/mut	56	71.5	68
C.II.8	mut/mut	5	<1	0
Ref. values		34–71	>37 %	43–115

both parents were carriers of the same mutation. Since at the time of the study the mother was pregnant, as soon as the mother gave birth, genetic analysis was performed on the newborn brother, revealing the presence of the mutation in homozygosis (Fig. 1).

Families B and C Carry a Novel Mutation c.960_962del/p.Asn320del Associated to C5 Deficiency

In both families B and C index patients the same novel mutation was identified, consisting on a homozygous deletion of three consecutive nucleotides (CAA) in exon 9 of *C5* gene (Fig. 2). The absence of these three nucleotides leads to the deletion of amino acid asparagine in position 320, without modifying the reading frame. This mutation was confirmed in gDNA from the patients. Consequently to the inheritance pattern of the disease, familial studies showed that the parents of both patients were heterozygous for the mutation (Fig. 2). Tested siblings of the index patients were all heterozygous for the mutation, except for C.II.5 who presented the mutation in homozygosis (Fig. 2). Although the parents carried the same mutation, the two families referred no consanguinity. Interestingly, both families were of Berber origin coming from the same region of Morocco (Alnif) and declared that they did not know each other.

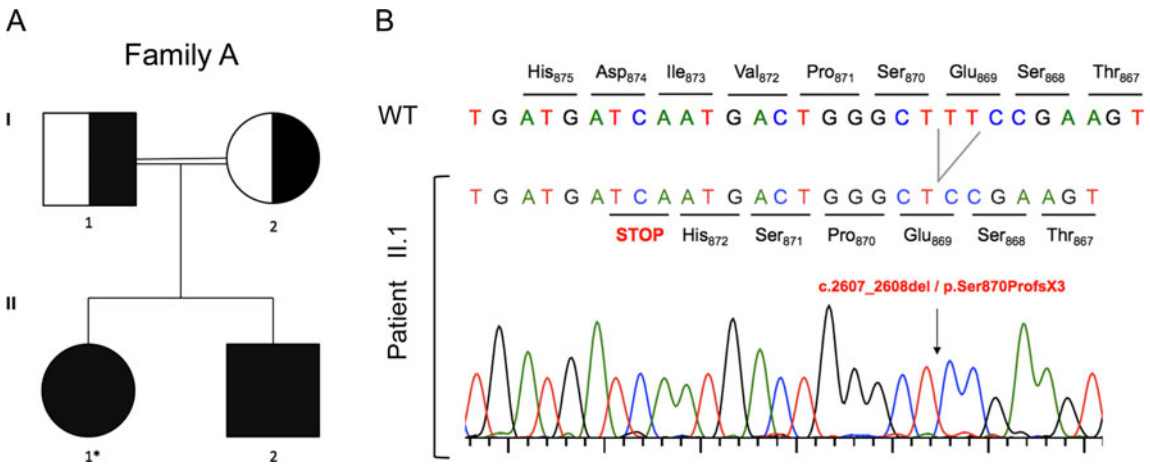

Fig. 1 C5 deficiency in family A. **a** Familial tree showing the segregation of the c.2606_2608del mutation. Both parents (consanguineous) carry the mutation in heterozygous state whereas the index patient (marked with an *asterisk*) and her brother are homozygous for this mutation. **b** The molecular basis of C5 deficiency in family A. Antisense sequence is shown. *Black arrow* indicates the point where the two adenines are deleted leading to a frameshift and a premature Stop codon

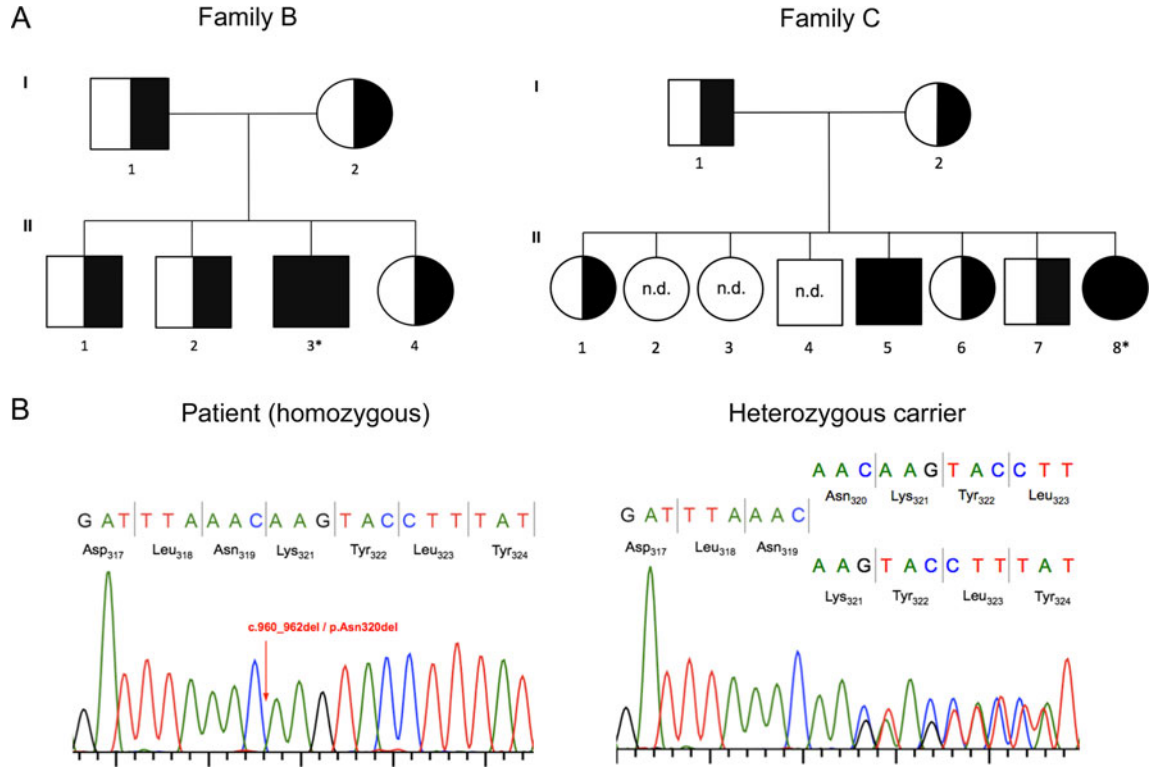

Fig. 2 C5 deficiency in families B and C. **a** Familial trees showing the segregation of c.960_962del mutation in the two families. In both families parents (non-consanguineous) are heterozygous and index patients (marked with an *asterisk*) are homozygous for this mutation. A sibling of family C index case was also identified as homozygous (C.II.5). **b** The molecular basis of C5 deficiency in families B and C. The sequence of a homozygous patient and of a heterozygous carrier is shown. *Red arrow* indicated the point where the three nucleotides are deleted leading to the loss of the residue asparagine 320

This led us to consider the possibility that these two families could share a common ancestor in the area where they come from (Alnif, Morocco).

Frequency of C5 Mutations in North-African Healthy Population

The presence of the p.Asn230del mutation in two unrelated non-consanguineous families of Berber origin coming from the same region in Morocco (Alnif), immediately suggests the possibility that this mutation could be present in a low frequency in the local population. Unfortunately, we were not able to collect samples from this region and we decided to test the presence of this mutation in North-African populations. In this screening we also included the other mutation described (p.Ser870ProfsX3).

Since there were no restriction enzymes that could easily detect these mutations by PCR-RFLP, we designed a PCR-SSP for the screening of 768 samples of healthy donors from North-African populations (Supplementary Fig. 1). One individual from Algeria was found to be heterozygous for the p.Asn230del mutation (Table 2). To confirm the results obtained by PCR-SSP, exon 9 of *C5* was sequenced showing that, indeed, the p.Asn230del mutation was present in heterozygous state. The allelic frequency of p.Asn230del mutation in our Algerian samples was 0.53 % and, globally, 0.065 % in our north-African population. The p.Ser870ProfsX3 mutation was not found in any of the 768 north-African samples.

Effect of Mutation p.Asn320del on C5 Protein Structure

Comparative modelling of the mutant structure shows that Asn320 is located in a loop-rich region, linking the MG3 and MG4 domains of the protein (Fig. 3). At this location, Asn320 is involved in a small network of interactions with nearby residues that stabilizes the local structure in the observed conformation. Deletion of Asn320 is likely to result in a local unfolding of C5, because it implies the loss of a substantial number of interactions that will undermine the interaction network (Fig. 3).

Discussion

C5 deficiency is clearly associated to Neisserial infections due to the crucial role of C5 in killing *Neisseria meningitidis* (demonstrated in vitro by Hellerud and collaborators, [22].

Table 2 Frequency of *C5* mutations in North-African population

C5 mutation	North-African populations (number of positive alleles/frequency)				
	Morocco ($n = 508$ alleles)	Algeria ($n = 192$ alleles)	Tunisia ($n = 494$ alleles)	Libya ($n = 342$ alleles)	Total ($n = 1536$ alleles)
c.960_962del (p.Asn320del)	0/0	1/0.53 %	0/0	0/0	1/0.065 %
c.2607_2608del (p.Ser870ProfsX3)	0/0	0/0	0/0	0/0	0/0

In this study we report two novel mutations in *C5* responsible for the complete C5 deficiency in three north-African non-related patients affected by invasive meningococcal disease.

Family A carries a deletion of two nucleotides in C5 exon 21 (c.2607_2608del) causing a frameshift and a premature stop codon that could lead to a truncated protein of 872 aa. This premature stop codon may affect the stability of the mRNA by becoming a substrate for nonsense-mediated mRNA decay (NMD). NMD is one type of mRNA surveillance mechanism during which transcripts with premature termination codons are degraded by a mechanism involving rapid decapping while still fully adenylated [23]. In fact, when we amplified the *C5* from the cDNA of the family A index case, the bands were substantially less intense than those obtained from a control cDNA (data not shown). Conversely, in families B and C (carrying the p.Asn320del mutation), the intensity of the *C5* bands was comparable with the control ones. This supports the idea that much of the *C5* mutant mRNA from the patient homozygous for the c.2607_2608del mutation is degraded by NMD. The rest of mRNA, if translated, would generate a truncated protein that might be unstable, prone to degradation and clearly non-functional.

Families B and C carry a deletion of three nucleotides in *C5* exon 9 leading to the deletion of asparagine 320 (p.Asn320del). The impact of this mutation in the protein structure can be estimated with comparative modelling, which indicates that loss of Asn320 will likely induce a local unfolding in the vicinity of this residue. As in the previous case, this would result in a protein more prone to degradation.

The presence of the same mutation in two families apparently unrelated and non-consanguineous was striking and led us to interview them to get more information about their origin. Both families declared to be of Berber origin and came from the same region in Morocco (Alnif). Since we were not able to obtain samples from this village, we decided to test for the presence of this mutation in 768 DNA samples from different North-African populations. It has to be note that we did not restrict the origin of the control samples to Berbers since it has been demonstrated that all North Africans have a common origin without clear genetic differences between Berbers and Arabs [24, 25]. The fact that we found one individual from Algeria that was heterozygous for the p.Asn230del mutation suggests that this mutation may exist at low frequencies in North-African population. In our case, the allelic frequency of p.Asn230del mutation in Algerian samples was 0.53 % (1 heterozygous out of 96 individuals) and, globally, 0.065 % in North-African population. However, it would be necessary to carry out the screening of this mutation in a larger cohort of individuals to

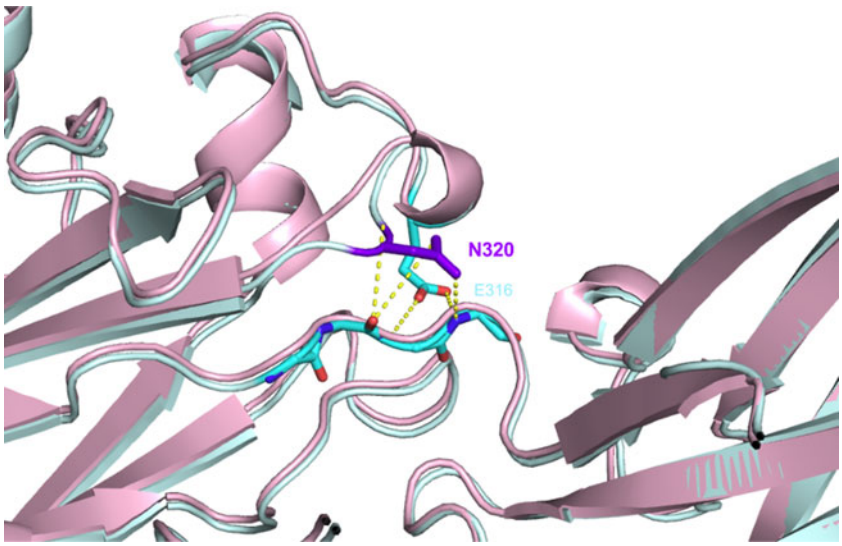

Fig. 3 Structure model of p.Asn320del. To emphasize the impact of this mutation we show the native structure (*light blue*) with the model of the mutant (*pink*). Asn320 (*shown in lilac*) is located in a structurally irregular region, where it interacts with neighboring residues (*dashed yellow lines*). There it forms a small interaction network involving a few other residues, like Glu316 (*shown in light blue*). We can see that absence of Asn320 results in the loss of its native interactions thus undermining the strength of the stabilizing network

Table 3 Reported *C5* mutations in families with C5 deficiency

N°	Location	Mutation (cDNA)	Mutation (protein)	Families	Type of mutation	Protein effect	Origin	Reference
1	Exon 1	c.55C>T	p.Q19X	4	SNV	Stop codon, truncated protein.	USA [African-American (2)]; Saudi Arabia (1); South Africa [Black African (1)]	[17–19]
2	Exon 7	c.754G>A	p.A252T	14	SNV	Amino acid change	South Africa [Black African (12), Cape Coloured (2)]	[17]
3	Exon 9	c.892C>T	p.Q298X	1	SNV	Stop codon, truncated protein.	Italy	[16]
4	Exon 9	c.960_962del	p.N320del	2	INDEL	1 amino acid deletion	Morocco (2)	This study
5	Exon 10	c.1115A>G	p.G335AfsX337	1	SNV	Splicing defect, truncated protein	Turkey	[15]
6	Exon 11	c.1178_81delAAAC	p.T394fsX396	1	INDEL	Frameshift, truncated protein	Netherlands	[27]
7	Exon 14	c.1775T>G	p.M592R	1	SNV	Amino acid change	Denmark	[14]
8	Exon 15	c.1883_84AG>CTCT	p.E628AfsX649	1	INDEL	Frameshift, truncated protein	Spain	[16]
9	Intron 18	c.2348+1G>A	p.Q785YfsX789	1	SNV	Splicing defect, truncated protein	Denmark	[14]
10	Exon 20	c.2536T>C	p.Y846H	1	SNV	Aminoacid Change	Spain	[16]
11	Exon 21	c.2607_2608del	p.S870PfsX873	1	INDEL	Frameshift, truncated protein	Morocco	This study
12	Unknown	deletion exons 26-27	p.W1077X	1	Unknown	Truncated protein	Norway	[13, 14]
13	Intron 27	c.3486+1G>T	p.1131-1162del	1	SNV	Splicing defect, 32 amino acids deletion	Norway	[13, 14]
14	Exon 30	c.4017G>A	p.1289-1339del	1	SNV	Splicing defect, 51 amino acids deletion	Brazil	[12]
15	Exon 36	c.4426C>T	p.R1476X	2	SNV	Stop codon, truncated protein.	USA (African-American); South Africa (Cape Coloured)	[17, 19]
16	Exon 40	c.4871_73CCC>GC	p.A1624fsX1645	1	INDEL	Frameshift, truncated protein	Spain	[11]
17	Exon 41	c.4972C>T	p.Q1658X	1	SNV	Stop codon, truncated protein.	Netherlands	[27]

establish a more reliable frequency of this mutation in North-African population and, more interestingly, in North-African meningococcal disease cases.

With the data presented here, the molecular basis of hereditary complement C5 deficiency has been elucidated in 29 families with 17 different mutations described (Table 3). These mutations are randomly distributed along the *C5* gene and most of them (11 out of 17) resulted in truncated proteins either by nonsense mutations, insertion-deletion (indel) mutations or splicing defects. The rest of mutations can be divided in three in-frame deletions, which lead to the synthesis of an abnormal smaller protein, and three amino acid changes. Most of *C5* mutations (13 out of 17) have been described in only one family. There are few exceptions to this general observation: the p.R1476X mutation has been identified in two patients of Black African origin and the p.Q19X in four patients (3 of sub-Saharan Africa and 1 from Saudi Arabia) [17–19]. Therefore, it seems plausible that these two mutations exist at low frequencies in Black African populations. This possibility was recently addressed by Owen and collaborators who did not found the p.Q19X and p.R1476X mutations in any of the 750 control samples from South Africa (500 Black African and 250 Cape Coloured) indicating that these mutations are very rare in the Western Cape [17]. However, data from 1000 genomes Project (http://www.1000genomes.org) indicate that in ASW population (African Ancestry in Southwest USA) the frequency of p.Q19X mutation (rs121909587) is 0.82 % (although the number of individuals tested is only 132), whereas there was no presence of this mutation in the other 25 populations analysed. No data about p.R1476X (rs121909588) mutation was found in 1000 genomes project.

The most prevalent C5 mutation described so far is the p.A252T. Owen and collaborators recently reported that this mutation is responsible for C5 deficiency in approximately 7 % of Black African meningococcal disease cases in the Western Cape in South Africa [17]. This mutation has been included in the SNP database (rs112959008) as part of the 1000 Genomes Project catalogue of human genetic variation [26]. It is present in sub-Saharan African populations at an approximate frequency of 3 % and it is absent in other populations outside Africa. It has been suggested that the mutation may have arisen many years ago in Central Africa and spread by migration [17].

Although C5 deficiency has been diagnosed in many populations, this is the first report identifying the molecular defect in North-African families, coming from Morocco.

Biochemical evaluation of heterozygous carriers showed differences among C5 levels. Remarkably, parents from all three families had normal levels of C5 protein in sera whereas among the siblings we found both normal (C.II.6 and C.II.7) and low (B.II.1 and B.II.2) values. One would expect that heterozygous individuals present values below the normality range, as it has been repeatedly reported [12, 14, 16]. However, Owen and collaborators found several heterozygous individuals with normal C5 values in their cohort and, additionally, they described that the normality range of C5 in Black Africans (22–260 μg/ml) is significantly wider compared with the Caucasians (43–115 μg/ml) [17]. We do not know the normality range of C5 in North-African healthy population and, consequently, our biochemical results have to be interpreted with caution. Despite the differences in levels of C5 molecule, haemolytic assays were normal in all cases of heterozygous individuals.

Clinically, as occurs with other terminal complement component deficiencies, vaccination to most common encapsulated bacteria (*Haemophilus influenzae*, *Streptococcus pneumoniae* and *Neisseria meningitidis B*) and prophylactic antibiotics are considered for the treatment of C5 deficient patients as well as fresh frozen plasma during active infection to replace C5. In our case, the identification of the molecular defect underlying C5 deficiency in the index patients allowed us the very early detection of an undiagnosed C5 deficient newborn in family A (A.II.2). This prompted the clinicians to adopt a very early prophylactic therapeutic attitude towards the patient (including vaccination), with the consequent prevention of suffering from an episode of invasive meningococcal disease.

Since two of our three patients were diagnosed after only one episode of invasive meningococcal infection, perhaps complement screening studies should be evaluated not only on patients suffering from recurrent infections, but also on those presenting only one invasive episode from an uncommon *Neisseria* serotype, specially when consanguinity is present and/or in those patients from African origin.

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Compliance with Ethical Standards

Conflicts of Interest None of the authors has any potential financial conflict of interest related to this manuscript.

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