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Clinical and molecular characterization of Factor I and C5 complement deficiencies: from diagnosis to population studies

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Clinical and molecular characterization of Factor I and C5 complement deficiencies: from diagnosis to population studies

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Certify that:

This thesis, titled **"Clinical and molecular characterization of Factor I and C5 complement deficiencies: from diagnosis to population studies"** has been done under their supervision by Clara Franco Jarava and is approved to be presented and defended to qualify for the degree of Doctor in Immunology by the Universitat Autònoma de Barcelona.

Barcelona, 11th May 2017

Dr. Manuel Hernández González

Dr. Roger Colobran Oriol

A mis abuelos

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PREFACE

One striking issue regarding the complement system has always been its nomenclature.

The initial identified components were named with a C, followed by a prime symbol (‘) and the number of molecule identified. An “a” after the component meant that the protein was in an active state (ex: C’1a). That was in 1963, but only five years later, in 1968, the nomenclature was revisited, removing the use of the prime symbol. To give more importance to the products derived from the proteolytic activation, it was accorded to name the sub-products with small letters (a, b, c...) following its formation. However, that obliged to change the way to designate an activated molecule, and it was changed to a bar over the letters (¯).

In 1981, after discovery of the alternative pathway, it was agreed that the newly identified molecules within that pathway would be designated with the term Factor, followed by a letter (ex: Factor B). The only exception was C3, since it was recognized to be the same molecule as in the classical pathway.

Research on complement system was increasing, and consequently, so did the numbers of molecules involved on this network. The lack of consensus resulted in a wide variety of names given to the same proteins, making difficult to compare results among research centres, and making tedious and unattractive to learn about the complement system. For that reason, in 2014 the first update of 1981’s nomenclature was published by the Complement Nomenclature Committee, which was established under the auspices of the International Complement Society and European Complement Network boards[1]. On that publication, authors urge the scientific community to use the recommended abbreviations, in order to keep it –and I will write it literally as they say it- as simple, uniform and unambiguous as possible.

For that reason, we have reproduced in **Table 1** the results from the recommended nomenclature, and it is presented here to serve as a glossary and a guide on how to name the different complement molecules from now on.

Table 1 Nomenclature of Complement Components (old names are indicated in parenthesis)

Recommended Name	Comments
Pathways	
CP	Classical pathway
AP	Alternative pathway
LP	Lectin pathway
TP	Terminal pathway
Proteins	
C1	Complex of C1q, 2C1r, 2C1s
C1q	
C1r	
C1s	
C1-INH	C1 Inhibitor (C1 esterase inhibitor)
C2	
C3	
C3(H2O)	Thioester-hydrolyzed form of C3
C3a	Anaphylatoxin from C3
C3a-desArg	C3a without C-terminal Arginine
C3b	
iC3b	Inactivated C3b
C3dg	
C3d	
C4	
C4a	
C4a-desArg	C4a without C-terminal Arginine
C4b	
C4d	
C4BP	C4 binding protein
C5	
C5a	Anaphylatoxin from C5
C5b	
C6	
C7	
C8	
C9	
Vn	Vitronectin (S protein, S40)
FB	Factor B
FD	Factor D
FH	Factor H
FI	Factor I
MBL	Mannose binding Lectine
Ficolin-1	(M-Ficolin)
Ficolin-2	(L-Ficolin)
Ficolin-3	(H-Ficolin)
MASP-1	MBL-associated serine protease 1
MASP-2	MBL-associated serine protease 2
MASP-3	MBL-associated serine protease 3
FHL-1	Factor H-like protein 1
FHR-1	Factor H-related protein 2
FHR-3	Factor H-related protein 3
FHR-4	Factor H-related protein 4
FHR-5	Factor H-related protein 5
CD59	(Protectin, Homologous restriction factor)
Cn	Clusterin (Apolipoprotein J, SP-40,40)
Protein complexes	
C5b6	Terminal pathway complex of C5b+C6
C5b-7	Terminal pathway complex of C5b6 + C7
C5b-8	Terminal pathway complex of C5b7 + C8
C5b-9	Terminal pathway complete complex
sC5b-9	Soluble C5b-9 with Vn or Cn bound
C3bBb	AP C3 convertase
C3bBbP	AP C3 convertase with Properdin*
C3bBbC3b	AP C3/C5 convertase
C4BP-Protein S	C4BP bound to protein S
Receptors	

CR1	CD35 (C3b/C4b receptor)
CR2	CD21 (C3d receptor)
CR3	CD11b/CD18 complex
CR4	CD11c/CD18 complex
C3aR	Requesting CD number
C5aR1	CD88 (C5aR)
C5aR2	(C5L2) Requesting CD number
CRIg	Complement receptor of the Ig family; receptor of C3b and iC3b
C1qR	C1q receptor
gC1qR	Recognizes globular C1q domains
cC1qR	Calreticulin. Recognizes collagen domain
LHR	Long homologous repeat [in CR1]

Notes

A consensus was not reached for the designation of properdin (where it was to be decided whether to retain the designation properdin or to change the name to factor P (FP)). The membrane-bound protein that inhibits the lytic action of C4b-9 shall be referred to only by its CD59 designation. The issue of the nomenclature for the activation fragments of C2 remains unresolved. Originally, C2a indicated the activated C2 fragment in the C3/C5 convertase of the CP. Since this fragment turned out to be the larger fragment of C2 this nomenclature was no longer consistent with the convention of naming the smaller fragments with earlier letters. An attempt to reverse these “a” and “b” designations was not universally accepted by experts in the complement field.

1 INTRODUCTION

1.1 THE COMPLEMENT SYSTEM

1.1.1 History of the complement system

In the latter 1800s, medical research was focused on elucidating the mechanisms of host immunity in the fight against infectious disease agents. While Metchnikoff established the *cellular theory*, demonstrating that blood cells could phagocytize microorganisms, studies from Fodor, Nuttall and Buchner revealed the concept of *humoral immunity*. In 1889, Hans Ernst August Buchner observed that blood serum contained a heat-labile "factor" that was capable of killing bacteria, and called it *alexin* (from Greek, meaning "to ward off", to protect). This discovery was extended by Jules Bordet, who in 1895 demonstrated that the bactericidal action of *alexin* was destroyed after heating the serum at 55ºC, and that this activity was recovered after addition of fresh serum. Bordet completed the humoral theory identifying two necessary components for the bactericidal activity; one that was heat-labile (*alexin*) and a heat-stable factor, which he called *sensitizer* (now called antibodies). Paul Ehrlich, in 1899, observed that the activity of the heat-labile component completed the action of the antibodies, and thus he coined the term of *Complement*, replacing the historical term alexin [2]. At the beginning of 20th century the action of the complement system was an important point of investigation at the laboratories of Bordet and Ehrlich. Some of the most important conclusions from those studies were i) that the complement system comprised several components with different physical-chemical properties ii) that these components acted in a specific order and iii) that the absence of one of the components inactivated the lytic activity of the whole system. The first components of the complement system were identified by 1920 and were named after the order of discovery and not after their position at the activation sequence. Electrophoretic and ultracentrifugation techniques' improvement during the next decades allowed to characterize complement components as proteins, contradicting the general opinion that they were lipoid complexes [2]. Out-standing eminences like Nilsson or Muller-Eberhard contributed during years to the isolation and characterization of many of the other proteins involved in the complement system. Their experiments also focused on revealing the sequence of the different catalytic pathways [3,4,5]. The antibody-dependent pathway of complement activation was named *classical pathway*. In 1954,

Pillemer discovered a complement activation cascade independent of antibodies, and called it *properdin pathway*, (currently known as *alternative pathway*) [6]. The latest way of complement activation, the "lectine activation pathway" was revealed by Super *et al* in 1989, after the identification in 1978 of the mannose-binding lectin (MBL) [7].

1.1.2 Activation of the complement system

Complement proteins mostly circulate as zymogens that have to be sequentially cleaved in order to be activated. This activation occurs in a cascade fashion, similarly to the coagulation, kinin and fibrinolysis pathways. Activation through cascades allows signal amplification and offers more steps to establish regulatory check-points. Three different activation pathways of the complement system have been described **(see Figure 1)**.

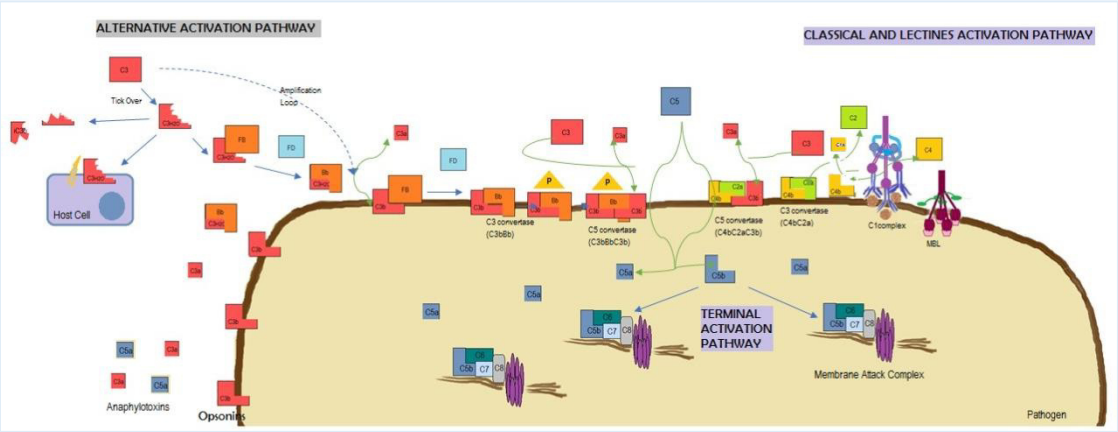

Figure 1 Complement system activation pathways. Upon recognition of different molecular patterns on host and pathogen surfaces, all three pathways lead to the cleavage of C3, situation that triggers the activation of a common terminal pathway. Alternative pathway tick over is a surveillance mechanism. Spontaneously hydrolysed C3 ($C3_{H2O}$) has to recognize and attach to a surface, or it will be degraded. Activation of the complement system in host-cells is avoided due to the expression of membrane regulatory molecules. C3b binds to cell surface and opsonizes the pathogen to enhance phagocytosis. Anaphylatoxins generated from C3 and C5 proteolysis spread into circulation and recruit inflammatory cells to the infection focus. The terminal pathway will finally build up the membrane attack complex (C5bC9n), a lipophilic complex that penetrate the cell membranes and provoke pathogen lysis.

1.1.2.1 Alternative pathway tick-over

Under physiological circumstances there is a constitutive low rate activation of the alternative pathway in a process known as *tick-over*, which serves the host as a surveillance mechanism. C3 molecule has a thioester bond that is spontaneously hydrolysed at a low rate, to form the bioactive molecule C3(H_2O) [8,9]. This molecule

exposes a binding site for another component of the alternative pathway, called Factor B. Once Factor B is bound to C3(H20), it is cleaved by the serine protease Factor D. The newly formed C3(H2O)Bb is a fluid phase C3 convertase, which is able to cleave native C3 molecules into C3a and C3b. C3b molecule can bind to any adjacent surface containing a hydroxyl group. However, C3b does not bind equally efficiently to all the hydroxyl groups and therefore, the particular surface composition will determine the efficacy of complement activation. C3b thioester has a short half-life (60μs) and has to bind covalently to any surface located within 60nm from the convertase. If it remains soluble, regulatory factors like Factor I and Factor H will rapidly inactivate it. C3b can bind to apoptotic cells tagging them for immunological clearance by phagocytes. Host cells can avoid complement activation thanks to different surface molecules like Membrane Cofactor Protein (MCP) or Complement receptor type 1 (CR1), which bind to C3b and prevent the assembly of C3b with Factor B. Pathogens do not have such regulatory molecules on their surface. Therefore, C3b can interact with Factor B and Factor D to form a surface-bound C3 convertase; which will cleave more C3 molecules to C3b and C3a. This amplification loop leads to a pro-inflammatory environment that contributes to generate an adaptive immune response and to a rapid elimination of the pathogen [10].

The alternative pathway membrane-bound C3 convertase, C3bBb, has a short half-life of around 90s. Thus, it has to be stabilized by properdin, a protein secreted by monocytes and lymphocytes (also stored in granules of neutrophils and mast cells) [11]. In association to properdin, the alternative pathway C3 convertase increases its stability 5 to 10-fold. Moreover, the structural changes that occur after the binding of properdin to C3 convertase alter the binding site for regulator Factor H, thus making C3 convertase more resistant to this inhibitor.

It has been recently discovered that properdin can also trigger the alternative pathway through the recognition of microbial glycosaminoglycan, as well as apoptotic and necrotic cell surfaces, providing a platform for the assembly of C3 convertases [11,12]. Another protein that can also recruit C3b to the cell surface and serve as platform for C3 convertase assembly is Complement Factor H-related protein 4A (FHR-4A), that

binds to certain pathogens such as *Candida albicans* [13], P-selectin [14] and heme group [15].

1.1.2.2 Classical pathway

The classical pathway is initiated through the multimeric complex C1, which is composed of a sensing molecular complex of 18 chains (C1q) and the proteases C1r and C1s [16]. The pattern recognition molecule C1q can bind to more than 100 different target molecules [10], including immune-complexes containing IgG and IgM or the surface bound pentraxin C-reactive protein. Since IgG is a monomer, more than one molecule is needed in order to trigger C1q activation. On the other hand, the pentameric structure of IgM makes a single molecule sufficient to activate C1q. Despite being the most studied, immune complexes are not the only molecules capable to activate C1q. The classical pathway can also be initiated after recognition of pathogen associated molecular patterns such as lipopolysaccharide[17] or bacterial porins[18]. The conformational change induced in C1q after binding to its target surface leads C1r to auto-activate itself and to activate C1s. This active form of C1 allows the complex to interact with molecules C4 and C2, activating them in order to form the C3 convertase of the classical pathway, C4bC2a. The first molecule to be activated is C4 and the bigger fragment, C4b, binds covalently to the same surface where the recognition has occurred. C4b then binds a molecule of C2, which is cleaved by C1s into the serine protease C2a –which remains bound to C4b- and the smaller fragment C2b, which is released to the circulation. The complex C4bC2a forms the C3 convertase of the classical pathway (C4bC2a), which will cleave C3 molecules into the anaphylatoxin C3a and the opsonin C3b. In 1981, enzymatic kinetics experiments performed by Ziccardi and collaborators established that for each C1 activated complex, an average of 35 molecules of C4 and 4.4 molecules of C2 were cleaved [19].

1.1.2.3 Lectin pathway

In a general overview, the lectin pathway is very similar to the classical pathway but differs on the pattern recognition molecules. In the lectin pathway, these recognition molecules are the collectins mannan-binding lectin (MBL), collectin K-1 (CL-K1), and the three ficolins (Ficolin-1, Ficolin-2 and Ficolin-3). Despite their sequences do not share any similarity, these proteins are homologous in topology with C1q [20]. The

collectins (MBL and CL-K1) have a collagen-like region and a carbohydrate recognition domain (CRD), which specifically recognize monosaccharide of glucose, mannose and N-acetyl-glucosamine. In the three human ficolins, the CRD domain is substituted for a fibrinogen-like domain, with affinity for N-acetylated carbohydrates structures, like in N-acetyl-glucosamine. These molecular patterns are expressed in bacteria surfaces like *Escherichia coli* and *Staphylococcus aureus*, on virus like HIV or Influenza A, and on fungi like *Candida albicans* or *Saccharomyces cerevisiae* [21]. Five different types of MBL-associated serine proteases (MASP) have been described so far which associate to collectins and ficolins. The functions of all the MASPs have not yet been fully elucidated. There is an ongoing controversy in the role that MASP-1 and MASP-3 might have on other pathways like the alternative or the coagulation pathways [20]. The best characterized molecule is MASP-2. MASP-2 is auto-activated after MBL recognition of the carbohydrate moieties on the pathogens surface. The auto-activated MASP-2 molecule cleaves then C4 and C2, leading to the formation of the C3 convertase C4bC2a [22]. The lectin pathway is also involved in the clearance of apoptotic cells through recognition of different types of apoptotic cell lines leading to the deposition of C4 and complement activation [23].

1.1.2.4 Terminal pathway

The C3 convertases C4b2a and C3bBb are the precursors of the C5 convertases. A new molecule of C3b binds the C3 convertase, switching its affinity from C3 towards C5. This conversion stops C3b opsonisation, and starts the terminal pathway. Cleavage of C5 by C5 convertases (C3bBbC3b for Alternative and C4bC2aC3b for Classical and Lectin pathways) yields C5a, which is the most potent anaphylatoxin -a potent mediator of inflammation and chemotaxis- and C5b that will initiate the formation of the membrane-attack complex (MAC) [24]. **(C5 molecule is described in detail on 1.1.5)**

Once C5 has been cleaved by the corresponding convertases, C6 interacts with C5b fragment. This interaction induces conformational changes on C6 that allow the C5b6 complex to establish ionic and hydrophobic interactions with the hydrophobic domains of the lipid bilayer. Binding of C7 to the C5b6 complex increases its affinity for lipids and membranes. C5b-7 complexes are anchored to the outer lipid surface of the membrane. The amphiphilic transition that occurs on C8 chains after they are

associated to C5b-7 permits the insertion of hydrophobic peptides into the lipid membrane. The pores formed by C5b-8 complexes have a short life span and a much smaller diameter than the complete membrane attack complex. However a low-rate lytic activity can be observed over erythrocytes and nucleated cells if large numbers of pores are formed [25]. A single C9 molecule binds to C5b-8 complex and alters its globular conformation into an elongated form. C5b-8,9_1 complex is formed rapidly, and followed by slower C9-C9 polymerization up to 16 more C9 molecules. This C5b-9 complex is known as Membrane Attack Complex (MAC), and forms a ring structure with an internal diameter of 5nm and a height of 15nm that fully penetrates the membrane inducing a calcium flux through the pore from the extracellular space that ends up killing the pathogen [10].

1.1.3 Functions of complement system

Knowledge on physiological implications of the complement system has been widely expanded since its early discovery, to which a merely function of antibody "complemental" bactericidal activity was attributed. Nowadays, it is well known that the complement system is implicated in many different key steps of the immune response [26,27].

As stated by Merle *et al*, "the main complement rule is that everything that is not specifically protected has to be attacked"[10]. Regulatory proteins on the membranes of host cells protect them of a complement system attack. On the other hand, any microorganism, cell or debris that lack these molecules will represent an activating surface, and will be potentially tagged by C3b. As it has been previously mentioned, pattern recognition molecules like C1q or MBL also recognize surfaces containing pathogen and danger associated molecular patterns [27].

The most direct functions of the complement system can be summarized as follows:

- **Clearance of apoptotic cells and immune complexes.** After programmed cell death, membrane protein constitution is altered. This involves lower expression of membrane regulatory molecules like CD46, whereas other molecules, which are normally at the inner side of the cell membrane, are now expressed on the surface. Phosphatidylserine, double stranded DNA, GAPDH or calreticulin are some of the

molecules that can be recognized by C1q, thus initiating the classical pathway cascade and leading iC3b deposition on the cell surface, favouring a correct phagocytosis by monocytes [28].

- **Defence against infections.** The membrane attack complex (MAC) can directly lyse the surface of gram negative bacteria. C3b molecules attached to pathogen's surface enhance the phagocytosis and removal of encapsulated bacteria through the interaction of C3b with C3b receptors present on macrophages. On the other hand, the release of anaphylatoxins such as C3a, and especially C5a, leads to chemotactic processes that will guide neutrophils, monocytes and macrophages toward complement activation sites generating a pro-inflammatory state at the infectious focus.
- **Immune regulation.** The complement system also exerts an immune regulatory function, modulating the adaptive immune response through its different components and receptors. It is known that the complement system enhances the humoral response. For instance, C3 degradation products like iC3b and C3dg serve as CR2 ligands for B cell activation and differentiation. Regarding T cells, it has been recently reported that C3 can be activated intracellularly in T lymphocytes by cathepsin L. These studies also show that intracellular C3a binds to intracellular C3aR sustaining basal mTOR activation and T cell survival in resting T cells. Moreover, TCR activation appears to induce C3aR translocation to T cell surface. In collaboration with CD46, this C3aR intracellular signalling contributes to mounting a Th1 response [25,29,29,30]. Regarding C5, recent studies point out a regulated cross-talk between intracellular activated complement components and the NLRP3 inflammasome which is crucial for Th1 induction and regulation. However, these observations require confirmation through independent groups due to the novelty of the results -which include the first description of inflammasome on T cells [30].

As can be observed in **Figure 2**, the complement system also plays very important roles in other systems that are far beyond the immune system. For instance, anaphylatoxins contribute to tissue repair, angiogenesis or cartilage resorption.

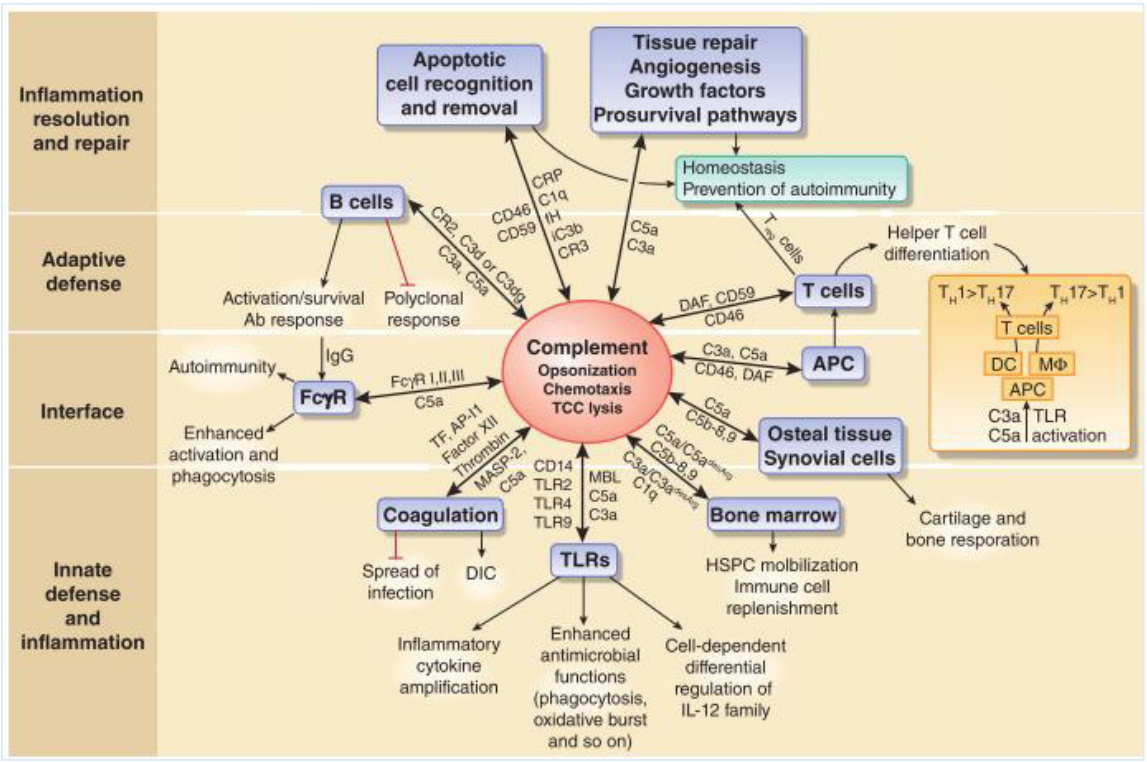

Figure 2 Newly recognized functions place the complement system as a key coordinator in orchestrating immune response and homeostasis. Source: Ricklin D, Hajishengallis G, Yang K, Lambris JD. Complement: a key system for immune surveillance and homeostasis. Nat Immunol. 2010;11(9):785-797.

1.1.4 Regulation of the complement system

The complement system can be a double-edged sword, playing an important role in host defence but also triggering inflammation that can equally damage self-tissues. Therefore, the activation cascades have to be tightly regulated in order to avoid autologous complement-mediated attack. Both the time and the place of activation must be restricted to avoid excessive consumption of complement components and to assure that the reaction is focused on the target and not spreading to the fluid phase or to self-tissues. For this purpose, host tissues express different membrane-bound and soluble regulatory proteins that confine the complement damaging action to the pathogen or damaged cell surface **(Table 2)**. In the complement cascade, the complement regulation can occur in three different ways: (i) spontaneous decay of activated proteins and enzyme complexes (i.e., short half-life), (ii) destabilization and inhibition of activation complexes, and (iii) proteolytic cleavage of "activated" components [33,34].

Table 2 Complement system regulatory molecules

Soluble regulatory components	
Properdin	Stabilizes alternative pathway C3 and C5 convertase
C1-inhibitor	Inhibits C1r/C1s, MASPs
MASP-3 and Map44	Compete with MASP-1 and MASP-2 for the binding to the recognition molecules
C4-binding protein (C4BP)	Inhibits classical pathway C3 convertase accelerating its decay. Serves as cofactor for C4b cleavage by Factor I.
Factor H	Inhibits alternative pathway C3 convertase accelerating its decay. Serves as cofactor for C3b cleavage by Factor I
Carboxipeptidase	Inactivates C3a and C5a anaphylatoxins generating C3a/C5 desArg.
S protein (vitronectin)	Blocks MAC formation. Inhibits C9 polymerization
SP-40 (Clusterin)	Blocks MAC formation. Clusterin interacts with a structural motif shared by C7, C8 and C9, inhibiting the assembly of C5b-7, C5b-8 and C5b-9 respectively
Membrane regulatory components	
Decay-accelerating factor (CD55)	Inhibition by acceleration of C3 convertases decay both of classical and alternative pathway.
Membrane cofactor protein (CD46)	Serves as cofactor for C4b an C3b cleavage by Factor I
CD59	Blocks MAC formation through C8 and C9 blockade

1.1.4.1 Regulation of the classical and lectin pathway

The initial activation of the classical pathway is regulated by the soluble serine protease C1-inhibitor (C1-INH), which dissociates C1r and C1s from C1q through a blockade of their active sites once these esterases have been activated. The role of C1-INH is to prevent fluid-phase C1 activation by immediate inactivation of any active soluble C1 complexes. Moreover, C1-INH also avoids an excessive complement activation by inactivating at a lower-rate C1 molecules which are already recognizing the antibody on pathogen surfaces [33]. C1-INH can also regulate the lectin pathway via inactivation of MASP1 and MASP2 esterases. But the protease inhibitory action of C1-INH is broad and goes beyond the complement system cascade. C1-INH is a key regulator of the plasma contact system and, it can also inhibit kallikrein, Factor XIIa and Factor XIa of the contact and coagulation pathway's respectively [35]. Proof of the

important role of C1-INH in the regulation of the contact system is given in hereditary angioedema (HAE), a disease in which the absence of C1-INH leads to a massive activation of kallikrein, which in turn leads to an excessive release of bradykinin, causing increased vascular permeability generating the characteristic oedemas of HAE [36].

Apart from C1-INH, other proteins regulating the lectin pathway have been described. MASP-3 and Map44 are alternative splice products from MASP-1 gene which despite sharing structural homology with MASP-1 and similar affinity for MBL and ficolin binding, do not have protease activity and cannot cleave C4 or C2. Therefore, they compete with MASP-1 and MASP-2 for the binding to the recognition molecules, preventing further activation of the lectin pathway cascade [37,38]. A similar regulatory role for the splice product of *MASP-2* gene, MAp19, is still under debate [21,39].

Another regulatory point in the classical and lectin pathway is at C3 convertase (C4b2a) level. Membrane-bound regulatory proteins like decay-accelerating factor (DAF, CD55), membrane cofactor protein (MCP, CD46) or complement receptor type 1 (CR1, CD35) are expressed on host-cell surfaces in order to prevent complement activation. They are part of the gene family known as Regulators of Complement Activation (RCA) [40]. DAF (CD55) inhibits the C3 convertases of both classical and alternative pathways by facilitating subunit dissociation of preformed C3 convertases and by preventing the assembly of new C3 convertases. DAF (CD55) is anchored to the membrane through glycosylphosphatidylinositol (GPI).Deficiencies on GPI cause paroxysmal nocturnal haemoglobinuria (PNH), a disease where patients suffer from complement-mediated anaemia, because DAF cannot be exposed on erythrocytes' surface[41]. MCP (CD46) is a regulatory protein with a wide tissue distribution. It binds to C4b and serves as a cofactor for the serine protease Factor I **(explained in detail in 1.1.6).** Cleavage of C4b will lead to the splicing product C4d that will remain bound to the surface, and C4c that will be released [42]. CR1 is also a glycoprotein that can exert its regulatory activity either by dissociating C3 convertase - or by serving as cofactor for Factor I. Additionally, CR1 is a major immune adherence receptor and plays a role in the clearance of immune complexes [40]. C4 binding protein (C4BP) is also part of the RCA family. It presents an octopus structure and can act on the cell surface as well as in

fluid phase. Due to its structure, C4BP can bind several C4b molecules at the same time. Like CR1, it can directly dissociate the C3 convertase or serve as cofactor for Factor I mediated-cleavage of C4b [10].

1.1.4.2 Regulation of the alternative pathway

One of the most important regulators of the alternative pathway is the soluble glycoprotein Factor H, which is also part of the RCA. This protein binds to C3b in fluid phase to prevent novel convertase formation, thus controlling the fluid-phase tick over and avoiding an uncontrolled amplification of C3 activation. Factor H performs its regulation in three different ways: a) binding to C3b and blocking the binding of Factor B, b) displacing fragment Bb from the C3 convertase C3bBb and c) serving as cofactor for Factor I inactivation of C3b [33].

In addition to Factor H, CR1 and DAF can also bind to C3 convertases of the alternative pathway (C3bBb) on activated surfaces, and dissociate them to avoid cell damage. Remaining C3b will then be cleaved by Factor I in presence of CR1, MCP and Factor H. On resting cells, on the other hand, this inactivation of C3b by Factor I and its cofactors will be immediate. The resulting iC3b will not be able to bind factor B, and therefore there will be no assembly of C3 convertases [10].

1.1.4.3 Regulation of the terminal pathway

The assembly of the membrane attack complex is strictly regulated by several proteins, in order to avoid accidental host cell damage. Two proteins have been described to prevent the fixation of soluble terminal complex to cell surfaces: vitronectin and clusterin. Vitronectin (also known as S-protein) is a glycoprotein that binds to sC5b-9 making the complex water-soluble, and therefore preventing its binding to cell membranes. Moreover, vitronectin inhibits C9 polymerization, thus avoiding the pore formation. Clusterin interacts with a structural motif shared by C7, C8 and C9, inhibiting the assembly of C5b-7, C5b-8 and C5b-9 respectively [25]. In addition to the activity of these proteins, an inhibitory function was reported for C8. In the absence of a cell membrane, the binding of C8 to C5b-7 would induce a conformational change that would impede its capacity to form pores, preventing further attachment of fluid phase C5b-8 to target membranes [43].

The inhibition of pore formation in self-surfaces is regulated by CD59, a GPI anchored protein (like DAF) that is expressed on most cell types. This protein interacts with the C5b-8 and C5b-9 complexes and blocks the membrane perforation. Moreover, within minutes from its deposition on target cells' membrane, the C5b-9 lytic complex can be removed via exocytosis with membrane vesicles or by internalization of the complex [10].

1.1.5 The complement factor C5

1.1.5.1 C5 genetics

The gene encoding C5 molecule (known as C5 or CPAMD4) is located in the large arm of chromosome 9 (9q34.1) and spans 79Kb [44]. Genomic code for *C5* is NG_007364.1 and its open reading frame comprises 41 exons **(see figure 3)**. Exons 1 to 16 codify for C5beta and from 17 to 41 codify for C5alpha chain. Genomic DNA is then transcript to a 6kb mRNA (NM_001735.2). *C5* gene structure is conserved in chimpanzee, Rhesus monkey, dog, cow, mouse, chicken, zebrafish and frog (http://www.ncbi.nlm.nih.gov/gene/727).

1.1.5.2 C5 protein structure

The fifth component of human complement system (C5) was identified and isolated in 1965 by Nilsson and Müller-Eberhard. It was first named β_{1F} globulin due to its electrophoretic mobility [3].

Complement C5 molecule is a 190kDa glycoprotein and its polypeptide chain structure was elucidated in 1979. Total reduction and alkylation of disulphide bonds in C5 molecule revealed a two-polypeptide chain structure: an alpha and a beta chain with molecular weights of 115 and 75kDa, respectively [45]. Like many proteins and polypeptides, C5 is synthesized in a precursor form known as pro-C5 that requires post-translational modifications. The intracellular precursor undergoes a proteolytic cleavage that involves the excision of a signal peptide. Pro-C5 is a 1676 amino acid peptide with a structure consisting on an N-terminal signal peptide (1-18aa) followed by the beta chain (19-673aa), a RPRR linker pro-peptide (674-677aa) and finally the alpha chain (678-1676aa) (http://www.uniprot.org/uniprot/P01031) [46]. The crystal structure was elucidated in 2008, and C5 is registered in Protein Data Bank (PDB) under the code 3CU7. The core of C5 adopts a structure that resembles that of C3 [47].

The major source of serum C5 is the liver. However, minimal expression of *C5* gene has also been demonstrated in many other tissues and cell types like oocytes, visceral pleura, kidney, lung, colon, brain, prostate, testis or lymph nodes. Also monocytes, macrophages and human B and T lymphoblastic cells might be an important source of C5 [44].

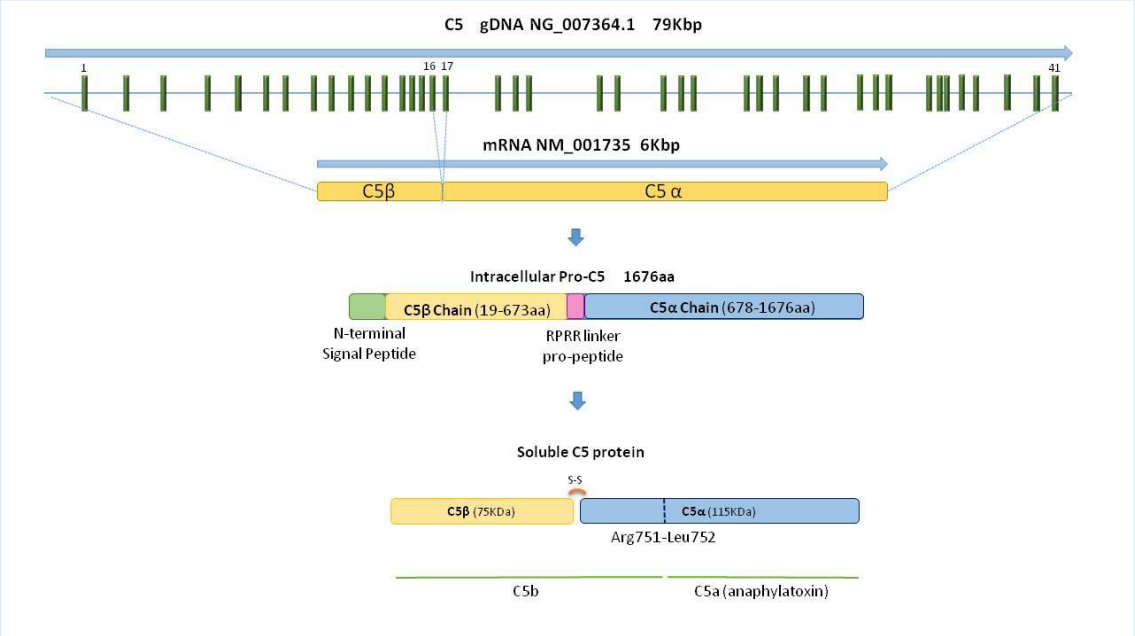

Figure 3 Schematic view of *C5* gene and protein. Genomic DNA (gDNA) contains 41 exons. From 1 to 16 codify for C5β chain and from 17 to 41 to C5α chain. RPRR linker is removed and the two chains are linked by a disulphide bond. C5 convertases cleave C5 between residues Arg751 and Leu752 to generate C5b and C5a active fragments.

1.1.5.3 C5 functions

The proteolytic activation through C5 convertases (C3Bb3b; C4b2a3b) cleaves pro-C5 between residues Arginine751 and Leucine752 of the alpha chain, releasing C5a anaphylatoxin and C5b (beta chain + alpha' chain).

C5b has the transient ability to interact tightly with C6, which will subsequently serve as nucleation site for sequential assembly of C7, C8 and C9 to create the MAC on the activation surface. As it has been previously mentioned, MAC assembly is crucial in the defense of *Neisseria meningitides* infections.

The released small fragment C5a anaphylatoxin is a small peptide of 76 amino acids with a molecular mass of about 10kDa. It is involved in chemotaxis, inflammation and

also in the generation of reactive oxygen species. Moreover, anaphylatoxins induce smooth muscle contraction, histamine release from mast cells and increase vascular permeability [48]. Like all other anaphylatoxins (C3a and C4a), C5a contains a carboxyl-terminal arginine residue, which is rapidly cleaved by carboxipeptidases to generate desargininine C5 (C5 desArg), a molecule with a lower biological activity than C5a.

1.1.6 Complement Factor I

1.1.6.1 Factor I genetics

Complement Factor I is encoded by *CFI* gene. Other names given to this gene are *FI, C3BINA, IF, ARMD13, KAF, C3b-INA, AHUS3*. *CFI* (NG_007569.1) is located in chromosome 4q25 and encompasses 63kb [49]. Its codifying region contains 13 exons, which is translated into a protein of 583aa. (Ensembl code: ENSG00000205403. www.ensembl.org) **(See figure 4)**

1.1.6.2 Factor I protein structure

Factor I is an 88kDa serum glycoprotein synthesized mainly by hepatocytes [50], although it can also be produced by other cell types like monocytes or fibroblasts [51,52]. Factor I circulates in serum in a concentration around 35µg/mL, however its synthesis can be up-regulated by the inflammatory cytokine IL-6 in hepatocytes [53,54].

Factor I is a multidomain protein synthesized as a single polypeptide chain (http://www.uniprot.org/uniprot/P05156). After the cleavage of linker region amino acids (RRKR), heavy (50kDa) and light (38kDa) chain are linked covalently by a disulphide bridge [50]. Three N-linked glycans are added during the post-translational glycosylation modifications. After being secreted, a signal peptide of 18 residues is cleaved, conferring catalytic activity to the protein. Cristal structure of intact human Factor I was described in 2011 (PDB Code: 2XRC). The mature Factor I protein contains different domains: the heavy chain contains the FI membrane attack complex domain (FIMAC), the Scavenger Receptor Cysteine-Rich domain (SRCR; also known as CD5), two domains of low-density lipoprotein receptors (LDLRa) and a small region without a known homology. The serine protease domain is contained in the light chain of Factor I and comprises the active site composed by the catalytic triad residues His380, Asp429

and Ser525. To notice, at the time of enumerating residue positions, we always include the signal peptide, since it is part of the translated region.

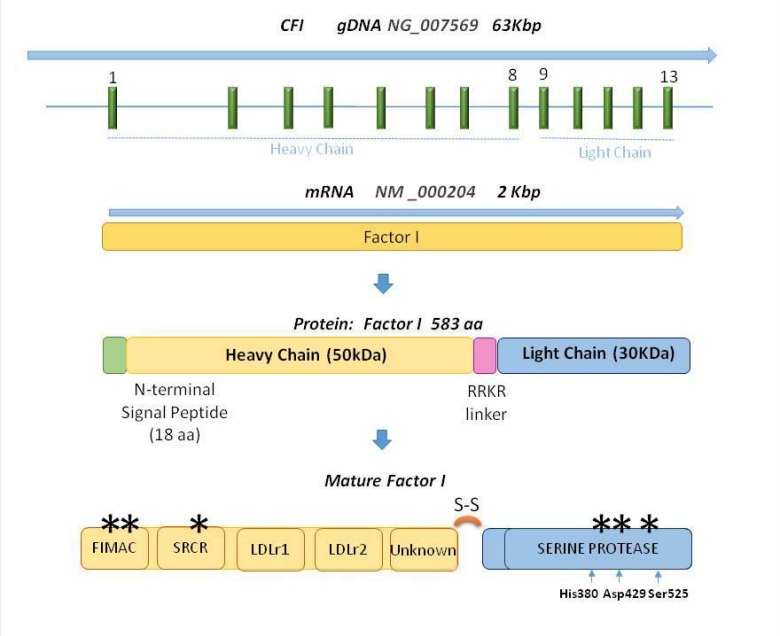

Figure 4 Schematic view of *CFI* gene and Factor I protein structure. Factor I is synthesized as a unique polypeptide that is post-transcriptionally modified. Asterisks (*) represent glycosylation sites. Residues involved in the catalytic triad are represented with an arrow.

1.1.6.3 Factor I functions

The serine protease Factor I is an enzyme with proteolytic activity (Enzymatic code: EC 3.4.21.45). Nearly all serine proteases circulate in an inactive pro-enzyme state and need to be proteolytically activated in order to expose the serine protease domain. Factor I, in contrast, circulates already in a form in which the serine protease domain is exposed. Moreover, Factor I differs from the other serine proteases in the way that it is not regulated by protease inhibitors like alfa2-macroglobulins or serpins. Factor I activity is regulated by the need of different cofactors that bind to Factor I target molecules. The active conformation of Factor I is induced only after formation of large cofactor-target complexes [50].

Factor I cleaves C3b and C4b to their inactive forms iC3b and iC4b in the presence of either soluble (Factor H, C4BP) or membrane-bound (MCP, CR1) cofactors. In pathological conditions in which Factor I or any of its cofactors are dysfunctional, the

amplification loop from the alternative pathway cannot be down-regulated and the generation of more C3 convertase molecules lead to a consumption of serum. As it will be reviewed on the next paragraphs, patients suffering from congenital disorders on CFI gene develop a primary immunodeficiency disease due to an absence of C3.

1.2 PRIMARY IMMUNODEFICIENCY DISEASES

1.2.1 General Overview

Primary immunodeficiency diseases (PID) comprise a group of more than 300 disorders caused by defects in the development and function of the human immune system. Due to this dysfunction of the immune system, patients affected with a PID normally have an increased susceptibility to suffer infections, although they can also present with immune-dysregulation symptoms such as autoimmunity or lymphoproliferation [55].

The latest classification published in 2015 by the International Union of Immunological Societies (IUIS) Expert Committee on Primary Immunodeficiency, comprises 269 different genetic defects affecting diverse components of the immune system [56]. However, for an easier clinical practice, these 269 different conditions are classified into 9 different groups, depending on the branch of immunity affected **(see table 3)**.

Table 3 Classification of primary immunodeficiencies

Group	Name	Example
Group 1	Combined T/B immunodeficiencies	ADA (Adenosine Deaminase) deficiency
Group 2	Combined immunodeficiencies with associated syndromic features	Wiskott-Aldrich Syndrome
Group 3	Predominantly antibody deficiencies	Bruton's disease
Group 4	Diseases of immune dysregulation	ALPS (Autoimmune Lymphoproliferative Syndrome)
Group 5	Defects of phagocytic number, function or both	Chronic granulomatous disease
Group 6	Defects in innate immunity	TLR3 deficiency
Group 7	Autoinflammatory disorders	Familial Mediterranean Fever
Group 8	Complement deficiencies	C2 deficiency
Group 9	Phenocopies of PIDs	Adult-onset immunodeficiency due to auto antibodies against IFNg.

1.2.2 History of PIDs

In 1952, Colonel Ogden Bruton described an 8 year old boy who, since the age of 2, suffered from recurrent and life-threatening infections that included bacterial pneumonia and septicaemia. Thanks to the most modern technique at that time, serum electrophoresis, clinicians could assess that patient's sera lacked immunoglobulins. Moreover, they observed that there was an absence of response to vaccines. Passive immunization with intramuscular gamma globulins dramatically decreased patient's susceptibility to infections [57]. By that time also Rosen and Janeway in Boston, and Good in Florida attended similar patients and could demonstrate that this agammaglobulinemia was almost always inherited in an X-linked pattern, naming the disease X-linked agammaglobulinemia [58]. But it was not until 1993 that Vetrie and Tsukada discovered that the underlying defect in all these patients was due to different mutations in the gene codifying for the B-cell specific tyrosine kinase (BTK).

The complete story of BTK agammaglobulinemia illustrates the importance of recombinant DNA technology for PID's molecular diagnosis. Before this technological revolution, findings on explanatory molecular mechanisms for PIDs occurred due to certain serendipity. For instance, in 1972 a boy with severe combined immunodeficiency (SCID) was found to have a complete absence of adenosine deaminase (ADA) enzyme in its erythrocytes. Since this enzyme is important for lymphocyte development, this was considered the explanation for the immune deficiency [59]. The first molecular studies were performed by linkage analysis and, despite being a tedious technique, it served to identify CYBB as the gene involved in chronic granulomatous disease already in 1986. These first studies were done either in diseases with an X-linked inheritance pattern or autosomal recessive pattern studying consanguineous families.

Undoubtedly, mapping Single Nucleotide Polymorphisms (SNPs) and the identification of the Complete Human Genome in 2003 contributed to the exponential increase of molecularly characterized primary immune defects. Direct Sanger sequencing has been since then extensively used to identify the underlying mutations causing different primary immunodeficiencies. In 2006, already 120 genes had been identified for their

corresponding immune deficiencies; whereas in 2015 the number had an increase of 224% (n=269) [56]. The arrival of high throughput genomics; i.e. genome and exome sequencing, has dramatically elevated the number of potentially disease-related genes. Therefore, the critical point nowadays is to elucidate the role for each of the new genes and their exact contribution to the immunodeficient state of the patient.

Identifying the molecular defect underlying already defined clinical conditions, not only permits a more accurate diagnosis and familiar counselling but also serves for a better understanding of the complex immune system network, potentially leading to new therapeutic strategies.

1.2.3 Frequency of PIDs

In Europe, a disease is considered to be rare when it has an incidence of 1 person per 2000 live births (Orphanet. www.orpha.net Visited 23/08/2016), but this definition varies among different countries. For instance, in the United States, a rare disease is defined as a condition that affects fewer than 200,000 people (NIH web-GARD. https://rarediseases.info.nih.gov/ Visited 24/08/16). Although PIDs when taken as a whole are considered rare entities, the real incidence of each single disease reveals that whereas some are extremely rare, some others should not be considered rare conditions. For instance, IgA deficiency is relatively common, with 1/223-1/1000 individuals suffering from this condition [60]. On the other hand, SCID is rarer, with 1 affected individual for every 100.000 births [61]. Many studies have been carried out in different countries in order to establish the real prevalence and incidence of PIDs, based on their individual registries. In 2013 Abel Laurent, J.L Casanova and colleagues carried out an analysis compiling data from: i) the different continental registries, ii) the most recent survey carried out for the Jeffrey Model Centre network and iii) two telephonic surveys that were carried out on the USA. In this study, they concluded that potentially 1/1200 people worldwide are living with a PID, thus meaning that globally, PIDs could not be considered rare diseases any longer [62].

1.2.4 Evaluation of a patient with a suspected PID

Recurrent infections repeatedly resistant to antibiotic treatment or presenting an unusual severity are the hallmark of PIDs. However, there are other important clinical signs that should raise suspicion for a PID. It is important to remember that alterations

of the immune system can be also secondary to other pathological conditions, and these have to be considered during the differential diagnosis **(see table 4)**.

Table 4 Possible mechanisms explaining secondary immunodeficiencies

Disorders that disrupt usual mucosal clearance mechanisms
Recurrent urinary tract infections (Urethral valve or urethra stenosis)
Recurrent sinusitis/pneumonia/diarrhea (Cystic Fibrosis)
Disorders of natural barriers
Infections due to skin lesions (Eczema, burns)
Hypogammaglobulinemia
Protein loss (Enteropathies, nephropathies)
Secondary to cell metabolism
Malnutrition, Diabetes Mellitus, Sickle cell anemia
Secondary to predictable/idiopathic adverse effects of drugs
Immunosuppressants, Chemotherapy, Corticosteroids, Biological drugs

However, front line clinicians must be aware of the possibility of PID and be able to recognize its clinical symptoms. For that purpose, the Jeffrey Model Foundation (http://www.info4pi.org/) has elaborated a list with 10 PID warning signs to assure an early diagnosis.

These warning signs are the following:

1. Four or more new ear infections within 1 year.
2. Two or more serious sinus infections within 1 year
3. Two or more months on antibiotics with little effect
4. Two or more pneumonias within 1 year
5. Failure of an infant to gain weight or grow normally
6. Recurrent, deep skin or organ abscesses.
7. Persistent thrush in mouth or fungal infection on skin.
8. Need for intravenous antibiotics to clear infections.
9. Two or more deep-seated infections including septicemia.
10. A family history of PID.

If any of these signs are present, a careful evaluation should be started in order to follow or discard the suspicion of PID. Such an initial evaluation must begin with a deep review on patient's and familial history, recording whether there is consanguinity on the family – because many PIDs present as autosomal recessive traits-, a physical examination and a limited initial laboratory testing, which must include a complete blood cell count.

After the initial evaluation, if a PID is still suspected, clinical immunologists recommend a battery of tests to evaluate the main components of the immune system, regardless the severity of the disease. These would include lymphocyte subpopulations, antibody response, T-cell function, phagocyte oxidative burst and initial evaluation of the complement system.

The type of pathogen causing the infections can also address the diagnostic observation towards a specific immune disorder, and therefore more specific diagnostic tests can be performed. For example, if the patient suffers from recurrent upper respiratory tract infections by encapsulated bacteria, a defect on humoral immunity will be suspected. But if the pathogen causing the pneumonias is *Aspergillus spp.* it will be more likely that the patient presents a phagocyte defect like neutropenia or chronic granulomatous disease. Ideally, functional tests would narrow the suspected PIDs and genetic testing would be performed by sequencing genes of suspicion. Establishing a molecular diagnosis allows doing familiar studies in order to identify other possible affected individuals before the onset of their condition. Since PIDs are normally diagnosed at younger ages, parents are normally young and willing to have more descendants. Therefore, molecular diagnosis can also help to provide these parents of genetic counselling for further pregnancies.

1.3 COMPLEMENT DEFICIENCIES

1.3.1 General overview and epidemiology

The eighth group at the IUIS Classification for PIDs is the one focused on defects of the complement system. Since the complement system is composed of more than 30 proteins with many different functions, the clinical consequences will vary widely depending on the component missing. In general, the complement system plays an

important role in defending against bacterial infections, but is less relevant for the defence against fungi or parasites. Despite the interactions that link viruses and complement proteins [63], increased susceptibility to viral infections is not seen in complement deficiencies.

The activation of the complement system on bacterial surfaces enhances the phagocytosis of the opsonized bacteria, a mechanism of great importance in the defence against encapsulated bacteria. Also the lack of complement molecules can imply a diminished MAC assembly, thus increasing the susceptibility to infections through gram-negative bacteria. Moreover, C5-deficient patients lack the ability to mount an efficient chemotactic response because of the absence of C5a[64].

Complement deficiencies are normally caused by biallelic mutations whereas monoallelic mutations do not usually confer an increased susceptibility for infection. This fact, together with the low frequency of the genetic defects, implies that complement deficiencies are more frequently found in families with consanguinity. But naturally, there are exceptions to the rule. Properdin deficiency has an X-linked inheritance pattern, C2 deficiency in Caucasian populations is generally caused by a slightly common C2 mutation, and deficiency of C1-inhibitor follows an autosomal dominant inheritance pattern.

From the data obtained at the last ESID registry update, in 2014, complement deficiencies represented the 4.89% of registered cases of primary immunodeficiencies, with absolute numbers of 946 cases. However, these statistics included MBL deficient states, which have been reported in 5% of Caucasians and their association to clinical manifestations is still to be resolved [65]. Excluding deficiency of MBL, it has been estimated that the prevalence of an inherited complete complement deficiency is 0.03% in the general population [66].

Like in many other rare diseases, international registries are required in order to have a more accurate idea regarding the real number of cases described for each entity. Moreover, it has to be taken into account that a variation on prevalence has been described among different ethnic origins. For example, C9 deficiency is more frequently seen in individuals from Japan, while C6 deficiency is much more frequently

found in individuals from African descent [67]. But due to the rareness of the mutations, estimates of frequencies are difficult to calculate.

1.3.2 Diagnosis of complement deficiencies

The first and most important thing required for the diagnosis of a complement immunodeficiency is to have the suspicion of the condition. This point, that could seem somehow obvious, is a matter of real awareness. For that reason, Grumach, Kirschfink *et al.* proposed some warning signs that should alert the clinician of a possible complement defect [68]. The alarm signs proposed and the pathways and molecules involved can be read on **table 5**. Differential diagnosis of complement deficiency should consider other humoral immunodeficiencies, like antibody deficiency, or innate defects in MID88, or IRAK molecules.

Table 5 Proposed alarm signs of complement deficiency

Alarm sign	Pathway involved	Molecules affected
Meningococcal meningitis >5 years of age	Alternative Terminal	Factor I, C3, Properdin C5, C6, C7, C8, C9
Other recurrent bacterial infections, esp. Pneumococcus	Alternative Classical	C3, Factor I, Properdin C2
Autoimmune manifestations	Classical	C1q, C4
Angioedema without rash	Classical	C1 inhibitor
Renal and ophthalmic inflammatory disorders	Alternative	Factor I, Factor H

Once a complement deficiency is suspected, like in all other immunodeficiency cases, the laboratory tests following the diagnostic approach must be stratified in different levels[69]:

(I) <u>Global assays identifying lack of pathway function.</u>

Correct functioning of activation pathways is measured either through traditional haemolytic assays (CH50, AP50) or by the recently developed ELISA (Wieslab Complement System; Euro-Diagnostica, Malmö, Sweden), which evaluates all three pathways at once. At this first step, it is recommended to measure also one complement activation product (C3a,

C3d or sC5b9) to distinguish a primary immunodeficiency from an absence of activity due to an excessive consumption of complement molecules. Normally, this first screening step already points out to the candidate defective complement protein/proteins.

(II) Quantification of individual complement components.

Individual complement components, irrespective of their functional activity, can be measured by radial immunodiffusion (RID), nephelometry or ELISA. Once the missing protein has been identified, functional tests can be performed supplementing the patient's serum with an extract of the purified or recombinant protein of interest. This will confirm that the missing protein is responsible for the defective pathway activation.

(III) Molecular analysis to identify genetic alterations.

Direct sequencing of the candidate gene gives the final diagnosis. Apart of being crucial for family studies, molecular characterization of complement defects is important for epidemiologic studies. Reporting the mutations found in different patients from different countries can increase the knowledge about allelic frequencies and perhaps stablish typical mutations that can be more rapidly evaluated in suspicious cases (a prototypical example of this are the mutations affecting the codon 328 of *F12* gene in type III hereditary angioedema).

1.3.3 Treatment of complement deficiencies

Ideally, patients with a known complement deficiency could be treated by replacing of the missing protein. However, this approach cannot usually be followed, and antibiotic prophylaxis, vaccination and hygienic measures must be applied.

The only product that is so far used as replacement therapy is C1-INH; which is used for the treatment of Hereditary Angioedema (HAE) [70]. The use of recombinant C2 and Factor H have been tried in mice experiments but not yet tested in humans [71,72]. Patients with deficiencies at the alternative or terminal pathways, who are primarily at risk for meningococcal infections, are treated with prophylactic / on-demand

antibiotics and vaccination. Vaccination includes the conjugated tetravalent serogroup ACYW-135 meningococcal and MenB vaccine towards B serogroup *N. Meningitidis*. It is also a good clinical practice to vaccinate these patients with the 13-valent and 23-valent pneumococcal vaccines as well as *Haemophilus influenza's*, since both organisms are also encapsulated bacteria and patients with complement deficiencies present an increased susceptibility towards these pathogens. [73]. Moreover, regular infusions of human plasma can be used in cases of C1q and C3 deficiencies with recurrent severe infections [73].

Pathological situations involving excessive complement activation due to defects on regulatory molecules (such as paroxysmal nocturnal hemoglobinuria (PNH) or atypical haemolytic uremic syndrome (aHUS)) are treated with Eculizumab, a monoclonal antibody that specifically blocks C5 molecule. It is important to bear in mind that these patients develop iatrogenic C5 deficiency, thus preventive actions referred above should be also applied to patients treated with Eculizumab.

1.4 COMPLEMENT DEFICIENCIES AND *NEISSERIA MENINGITIDES* INFECTION

Neisseria meningitides is a gram-negative diplococcus member of the bacterial family Neisseriaceae. Its genome comprises around 2 million bases encoding around 200 genes[74]. Meningococci are classified according to differences on several structural components: the serogroup depends on the polysaccharide capsule, whereas the major outer membrane proteins PorB and PorA indicate the serotype and sub-serotype respectively. Finally, differences on lipopolysaccharide (LPS) serve to identify the immunotype. From the 13 different serogroups, the ones isolated on patients with meningococcal disease are serogroups A, B, C, Y, W135 and recently serogroup X. On the other hand, carriage isolates are often non-encapsulated or from serogroups X, Z or 29E [75]. The largest epidemics in Africa have been (with few exceptions) due to serogroup A meningococci. In contrast, meningococci from serogroup B, normally absent in sub-Saharan Africa, are the most commonly found in industrialized countries. Outbreaks of serogroup C occur all over the world, mostly in adolescents and adults. Serogroup Y has emerged for the last 15 years as an important cause of disease in

North America. It is important to remark that rare serogroups like W135 and X were responsible for epidemics in sub-Saharan Africa since 2002 [76].

Neisseria meningitides is the main cause of epidemic meningitis and rapid septic shock. Mortality rates are very high and range from 4-40%. This wide range reflects the wideness of the disease spectrum, which varies from meningitis to sepsis and septic shock. Meningitis has a mortality rate of 4-6%, and in septic shock, mortality rates up to 40% have been reported [77]. Moreover, around 10-20% of the survivors present permanent severe sequelae that include deafness, mental retardation and amputations. On the other hand meningococcal infection can also present in some patients as a cause of transient or mild bacteraemia, leading to minimal clinical signs. It is also remarkable that the majority of individuals carry meningococcus as a commensal microorganism of upper respiratory mucosal surfaces [78]. Nevertheless, the persistence of large epidemics of serogroup A in Africa, the appearance of infections through serogroups Y, X and W-135 in distinct regions during the last decade, and the presence of disease by serogroups B and C in industrialized countries denote the importance of meningococci as a global threat [74].

1.4.1 Epidemiology

The global prevalence of meningococcal infections is of 500.000 cases per year. During epidemic outbreaks in sub-Saharan Africa, the incidence can be dramatically increased up to 1case per 100 individuals [78]. Although the main focus of the epidemics is still found in countries enclosed under the term of the African meningitis belt - which comprise the countries between Ethiopia in the east to Senegal in the west- other counties at the east and south of Africa, present an increased epidemic risk **(Figure 5**). The annual rate of meningococcal disease in many regions of Africa in between epidemic episodes is still several times higher than the rates of endemic causes in industrialized counties, thus emphasizing the need of preventive vaccination strategies on those regions [74].

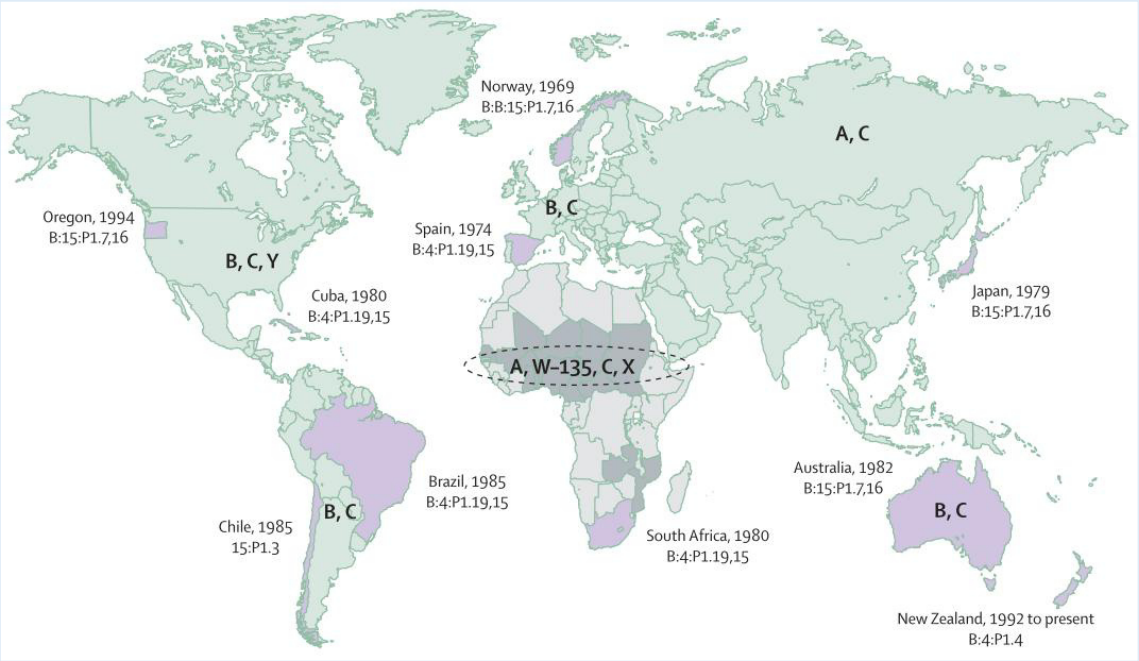

Figure 5 Worldwide distribution of major meningococcal serogroups and of serogroup B outbreaks by serotype (shaded in purple). The meningitis belt (dotted line) of sub-Saharan Africa and other areas of substantial meningococcal disease in Africa are shown. Source: Epidemic meningitis, meningococcaemia and Neisseria meningitidis. Stephens, Greenwood, Brandtzaeg. 2007. The Lancet, 369 (9580) 2196-2210

In Europe and North America meningococcal disease occur usually as sporadic cases and the highest age-specific incidence rates are observed in children younger than 5 years old [76]. *N. meningitides* is a commensal for which humans are the sole reservoir. Around 5-10% of adults out of epidemic period carry meningococcus on the mucosal nasopharynx. This nasopharyngeal colonization is an important immunological process in order to generate protection for future infections. In Europe and North America the carriage rates are low during the first years of life, dramatically increasing during the adolescent age and reaching the maximum peak between 20 and 24 years old. Meningococci are transmitted by direct contact with nasal and oral secretions or by inhalation of respiratory droplets. Close contact with patients with meningococcal disease increases the risk of infection 500 to 2.000 fold [78].

1.4.2 Physiopathology

The mechanisms that produce meningococcal sepsis and meningitis involve upper respiratory attachment and invasion, damage of endothelial cells and invasion of the central nervous system, followed by the initiation of an inflammatory cascade [78]. For a

better understanding on physiopathology of invasive meningococcal disease, the infectious process will be now briefly reviewed.

First the meningococcus crosses the upper respiratory mucociliary blanket and colonizes the nasopharynx. After that, it attaches to human epithelial cells and enters into the cells through endocytosis. Meningococci accumulate then in phagocytic vacuoles that will be later released into inter-epithelial spaces, below epithelial cell tight junctions.

Although usually transient, the entry of meningococci into the bloodstream is much more frequent than clinically recognized. However, the capacity of the pathogen to survive and multiply depends directly on its ability to circumvent humoral immune response. Meningococci proliferation leads to a systemic release of inflammatory cytokines (IL-1, IL-6 and TNFα) and therefore, disease severity is promoted by high levels of circulating pro-inflammatory cytokines produced in response to infection [79].

The way the meningococcus crosses the blood brain barrier remains still unsolved. However, it seems that the choroid plexus can be a possible site of entry of meningococci into the cerebral spinal fluid (CSF). Additionally, the inflammatory cytokines released in meningococcal bacteraemia might increase blood brain barrier permeability, thus facilitating the entrance of meningococci into the CSF. Other closed spaces like pericardium and large joints are also susceptible of pathogen's entry. Bacterial survival and proliferation in those locations is the cause of meningococcal meningitis, pericarditis or arthritis. High concentrations of LPS endotoxin in plasma lead to an excessive activation of the coagulation system and a down regulation of the fibrinolytic system, resulting in disseminated intravascular coagulation [80].

1.4.3 Clinical presentations and treatment

As it has been previously mentioned, invasive meningococcal disease can course with a wide range of clinical manifestations.

Haemorrhagic skin lesions are present in 28-77% of the patients with invasive meningococcal disease during admission [74]. Petechiae of meningococcemia are usually bigger than those caused by thrombocytopenia or leucocytic vasculitis induced by other infections.

Meningitis is the most common clinical presentation of invasive meningococcal disease. More than 60% of the patients in industrialised countries develop meningitis without septicaemia. In developing countries, the proportion of patients with meningitis is much higher [74].

Fulminant meningococcal septicaemia is characterised by a rapid proliferation of meningococci in the circulation, resulting in very high levels of bacteria (10^5-10^8/mL) and meningococcal endotoxin (10-10^3 EU/mL) in the bloodstream [74]. There is a destructive intravascular inflammatory response due to the large bacterial growth, which progressively leads to a circulatory collapse and severe coagulopathy. Renal, adrenal and pulmonary dysfunction might present together with thrombotic lesions in the skin, limbs, kidneys, adrenals, choroid plexus and sometimes the lung. Survivors can be severely handicapped due to the vascular complications that can lead to amputation of digits or limbs. It is important to notice that fulminant septicaemia is recorded less frequently during African epidemics than it is for patients in industrialised countries [74].

Early antibiotic treatment should be the primary goal, since effective antibiotics immediately stop the proliferation of *N. meningitidis*. After the emergence of sulpha-resistant meningococci in the 1960s, penicillin or other beta-lactam antibiotics have been used to successfully treat invasive meningococcal disease [78]. The aim of current control strategies is to reduce meningococcal transmission, to prevent invasive disease and to enhance host resistance. In that way, chemoprophylaxis with rifampicin, ciprofloxacin or sulphonamides eradicates nasopharyngeal carriage of meningococci and prevents transmission and invasive disease [78].

The current meningococcal polysaccharide vaccines are protective against serogroups A, C, Y and W135. The vaccine against group B meningococci has been the last one to be developed, due to its poorly immunogenic capsule. Bexsero® (Novartis. Basel, Switzerland) was firstly authorized by the European Medicines Agency (EMA) on January 2013 [81] and one year later by the Spanish medicine agency, AEMPS [82]. Accordingly to the Spanish Paediatrics Association (AEP), infants should receive the monovalent vaccine against C serotype (MenC) at 4 and 12 months, and one final dose

at 11-12 years old. This final dose can be substituted for the tetravalent MenACWY vaccine. Vaccination against meningococci of B serogroup is currently not included on the national health system vaccination calendar for the general population, and it is only indicated on those patients with a higher risk of infection, like immunocompromised patients with humoral defects [83].

1.4.4 Host genetic determinants of *Neisseria meningitidis* infection

The wide spectrum of *N. meningitidis* clinical presentations raises the question of why some patients show very few clinical symptoms while others have a fatal outcome within hours after onset of the symptoms. The underlying basis of such a wide variation would include several factors as genetic variations in the pathogen and in the host, as well as environmental factors. It has been calculated that the overall contribution of host genetic factors to the risk ratio of meningococcal infection is about one third [84]. The contribution of polymorphisms on several molecules, to the susceptibility and/or severity of meningococcal disease has been widely studied. Most of the studies have focused on molecules involved in the pathogen sensing, like Toll-like receptors (TLR4) or LPS Binding Protein. Variants on TLR4 gene have been found more frequently in patients with meningococcal disease than in healthy individuals [85]. This is not unexpected since TLR4 is one of the sensing molecules that firstly recognize LPS on pathogen surfaces. A recent study showed that despite some polymorphisms of LBP did not show correlation with the susceptibility or severity of bacterial sepsis in people, an association was reported with the outcome of bacterial sepsis in men [77]. Angiotensin converting enzyme (ACE), which is associated with pro-inflammatory response, presents an allele (D allele) with a deletion of 284bp that is associated with higher ACE activity. Homozygosity for the D allele has been associated with a higher predicted risk of mortality and longer stay at intensive care unit in children presenting meningococcal disease [86]. Associations between components of the adaptive immune response like FcγR haplotypes and susceptibility or severity of meningococcal disease have also been reported [77]. But among all the factors, the ones that appear to have a more direct contribution are those found on the complement system, underlining the importance of serum bactericidal capacity in the host defence against meningococcal infection.

1.4.5 The complement system and *Neisseria meningitidis* infection

As previously revisited, complement system activation through any of the three pathways leads to: i) generation of anaphylatoxins C5a and C3a, ii) phagocytosis through C3b opsonisation and iii) formation of pores on the pathogen surface. Since the complement system plays a central role on the defence against Neisseria spp. infection, it is easy to understand why polymorphisms and mutations on several components of the complement system cascades have been widely associated with an increased susceptibility to meningococcal infections.

- Mannose binding lectins (MBL): allelic variants on MBL have been described to result in aberrant unstable multimers. In children, this variant alleles were associated with susceptibility to meningococcal infection [87].
- Properdin: mutations on this positive regulator of the alternative pathway are associated with increased susceptibility to infection with *N. meningitidis*, with an increased disease severity and poor outcome.
- Factor D: Complement deficiency on Factor D was seen in a family with severe *N. meningitidis* infections.
- C3: C3 has a central position in complement activation, since all three pathways converge in the activation of C3 to form anaphylatoxin C3a and opsonin C3b. Therefore, deficiencies of C3 result in compromised opsonisation and phagocytosis of bacteria, as well as inability to form the C5 convertase, thus impeding the generation of the membrane attack complex (MAC). Hence, deficiency of C3 results in increased susceptibility to meningococcal and other bacterial infections. Apart of intrinsic genetic defects on C3 gene, C3 deficiencies can also be secondary to defects on regulatory molecules or to the presence of C3 nephritic factor. In any of these cases, the lack of C3 molecule in sera would increase the susceptibility to meningococcal infections.
- Late complement components deficiency (C5-C9): Patients having late complement components deficiency (C5-C9) are known to have recurrent gram-negative bacterial infections, due to their inability to form the membrane attack complex.

However, the complement system plays a dual role in the pathogenesis of *Neisseria meningitides*. Despite the fact that patients lacking components of the membrane attack complex have a risk 1.000-10.000 fold higher than immunocompetent individuals to suffer meningococcal disease, -including recurrent attacks disease during their lifetime - patients with MAC deficiencies seem to present fulminant systemic attacks less frequently than immunocompetent individuals [79]. Stimulation with C5a results in up-regulation of pro-inflammatory cytokines and chemokines and degranulation of polymorphonuclear leukocytes. Lack of serum lytic activity results in a lower release of lipopolysaccharide thus reducing the excessive release of cytokines responsible for fulminant disease [77,64].

Observing the global incidence of meningococcal disease, one could expect complement deficiencies to occur more frequently than reported. The generally low acknowledgement of the complement system itself and the "mild" consequences of its deficiencies have probably contributed to infra-diagnose these conditions. But, how often and in which situations should the complement system be evaluated? This topic was addressed already in 1989, when Fijen and Kuijper reported that complement study was present in half of the 46 patients in whom meningococcal disease due to uncommon serogroups had developed after the age of 10 years [88]. Later, in 2001, the group from Newcastle leaded by Andrew Cant reported –after studying 297 children aged 2 month to 16 years- that it is unnecessary to routinely screen all children following meningococcal disease if caused by groups B or C. However, their study concludes, it is important to assess the previous health of the child and to investigate appropriately if there have been previous suspicious infections, abnormal course of infective illnesses, or if this is a repeated episode of neisserial infection [89]. Very recently, in 2016, a French nationwide study suggests that to avoid late or no diagnosis, any adult displaying first episode of *N. meningitidis* infection should be tested for complement deficiency [90]. Taken the devastating sequelae that meningococcal disease can cause, how would an early diagnosis of complement deficiency benefit patients in risk for this disease? With this project, we aim to answer this and other related questions.

2 HYPOTHESIS AND OBJECTIVES

2.1 HYPOTHESIS

Primary immunodeficiency diseases of the complement system are more frequent than what is reflected on international registries. The implementation of specific techniques for the study of the complement system, on the daily care practice of the clinical laboratories, allows an increase of the number of cases diagnosed. Early diagnosis of complement immunodeficiencies affecting C5 and Factor I components correlate with a better clinical prognosis and less probability to acquire severe and/or recurrent infections.

2.2 OBJECTIVES

1. To expand and improve complement system tests in use in Vall d'Hebron clinical immunology laboratory.
2. To apply improved functional and molecular tests to patients with suspected deficiencies in Factor I and C5 complement components to reach a complete molecular and functional diagnosis.
3. To investigate recurrent mutations causing C5 deficiency at the population level.
4. To assess the performance of an improved diagnostic algorithm to detect complement deficiencies in patients with recurrent bacterial infections.

3 RESULTS

OBJECTIVE 1: TO EXPAND AND IMPROVE COMPLEMENT SYSTEM TESTS IN USE IN VALL D'HEBRON CLINICAL IMMUNOLOGY LABORATORY.

At the Immunology Division of Vall d'Hebron University Hospital only a few techniques were available for the study of the complement system before the onset of this project. Classical activation pathway (CH50) and circulating C3 and C4 levels are commonly performed to assess complement activation in certain systemic autoimmune disorders like systemic lupus erythematosus (LES). Moreover, techniques applied for the diagnosis of Hereditary Angioedema –C1q and C1 inhibitor levels, as well as C1 inhibitor function- were also implemented on the clinical laboratory. Despite C3, C4 and CH50 served also as initial tests for complement deficiencies, these techniques were insufficient for a complete evaluation of possible complement system deficiencies. The lack of hospitals in our region offering more advanced techniques obligated clinicians to send patient's samples to laboratories in other regions. This fact is not only expensive and time consuming, but most importantly, it introduced many incidences regarding the special temperature conditions that serum samples require to avoid spontaneous complement activation during their transport.

Therefore, one of the purposes of this project was to increase the number of techniques available in our laboratory and implement them in the daily routine, with the aim that not only clinicians at our hospital, but also those from hospitals within our region, could benefit from a better and faster diagnosis of defects on the complement system.

Table 6 summarizes the number of determinations that have been done during the last three years in our clinical laboratory. It is important to remember that both C3, C4 and to a lesser extent CH50 are indicated in the follow-up of LES. Therefore, the numbers do not correspond to determinations ordered under a complement deficiency suspicion. Besides complement deficiencies causing recurrent or severe bacterial infections, differential diagnosis for atypical uremic haemolytic syndrome is also done by studying circulating levels of Factor H and Factor I.

Table 6 Laboratory tests performed from 2014-2016 in Hospital Universitario Vall d'Hebron

LABORATORY TESTS	2014	2015	2016	Δ (2016 vs. 2015)
Classical pathway activity (CH50)	188	580	607	27
Alternative pathway activity (AP50)		34	186	152
Lectine's pathway activity		3	8	5
Circulating complement factor C2	4	12	41	29
Circulating complement factor C3	3276	4782	5076	294
Circulating complement factor C4	3289	4815	5072	257
Circulating complement factor C5		1	17	16
Circulating complement factor B		5	13	8
Circulating complement factor H		9	14	5
Circulating complement factor I	1	9	12	3
Circulating complement factor properdin	1	1	42	41
C3 nephritic factor	1		4	3
Soluble membrane attack complex (sC5b9)			186	186

OBJECTIVE 2. TO APPLY IMPROVED FUNCTIONAL AND MOLECULAR TESTS TO PATIENTS WITH SUSPECTED DEFICIENCIES IN FACTOR I AND C5 COMPLEMENT COMPONENTS TO REACH A COMPLETE MOLECULAR AND FUNCTIONAL DIAGNOSIS.

For an easier comprehension, results to objective 2 will be presented in two differentiated chapters. Materials, methods and results to this objective have been published and can be read at the corresponding articles.

Chapter 2.1: Clinical, functional and molecular characterization of patients with complement deficiencies affecting Factor I

Clinical Laboratory Standard Capillary Protein Electrophoresis lead to Factor I Deficiency Due to a Novel Homozygous Mutation.

Clara Franco-Jarava*, Roger Colobran*, Jaume Mestre-Torres, Victor Vargas, Ricardo Pujol-Borrell, Manuel Hernández-González.

Immunology Letters. 2016 Jun;174:19-22.

*Early versus late diagnosis of complement Factor I deficiency: Clinical consequences illustrated in two families with novel homozygous CFI mutations***.**

Clara Franco-Jarava, Elena Álvarez de la Campa, Xavier Solanich Moreno, Francisco Morandeira-Rego, Virginia Mas Bosch, Marina García-Prat, Xavier de la Cruz, Andrea Martín-Nalda, Pere Soler-Palacín, Manuel Hernández-González, Roger Colobran.

In preparation

Chapter 2.2: Clinical, functional and molecular characterization of patients with complement deficiencies affecting C5 component

Novel mutations causing C5 Deficiency in Three North-African Families.

Roger Colobran*, Clara Franco-Jarava*, Andrea Martín-Nalda, Neus Baena, Elisabeth Gabau, Natàlia Padilla, Xavier de la Cruz, Ricardo Pujol-Borrell, David Comas, Pere Soler-Palacín, Manuel Hernández-González.

J Clin Immunol. 2016 May;36(4):388-96.

**Both authors equally contributed to this work.*

Chapter 2.1: Clinical, functional and molecular characterization of patients with complement deficiencies affecting Factor I

One of the objectives of this project was to implement routine tests for the diagnosis of complement deficiencies in the clinical laboratory. Although this usually means to develop new and modern technologies, it is not always the case. Capillary electrophoresis, a technique that has been routinely used in clinical laboratories for at least 20 years, is a method of separating proteins based on their physical properties. Serum is placed on a specific medium, and a charge is applied. Various serum protein fractions (usually 5-6 fractions) are differentiated according to the proteins net charge. Since immunoglobulins migrate in beta (IgA) and gamma fractions (IgM, IgG), capillary electrophoresis is mostly used for the diagnosis of monoclonal gammapathies. However, this test also permits clinicians to evaluate the overall levels of other proteins, like alpha1-antitrypsin, ceruloplasmin, apo-lipoproteins or transferrin, being a useful test for evaluation of liver state or malnutrition. Among complement proteins, C3 is the most abundant in serum, and it is also the main representative protein of the beta-2 fraction. Therefore, theoretically, if C3 levels in serum were low, beta-2 fraction should be also decreased.

As it has been mentioned in the introduction, C3 deficiencies can be either primary (due to intrinsic C3 genetic defects), secondary due to a massive consumption (defects on FI, FH, CD46, or gain of function FB mutations [91]) or secondary, due to the presence of C3 nephritic factor. In these studies we report 3 new cases of complement C3 secondary deficiency due to an absence of the C3 negative regulator Factor I. As it will be presented, patients with Factor I deficiency suffered from recurrent upper respiratory tract infections and meningococcal disease, leading to important neurological sequelae in one of the cases. The different ages of the patients described on the second study highlight that early diagnosis of Factor I deficiency has a clear impact on the quality of life of the patients.

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Clinical laboratory standard capillary protein electrophoresis alerted of a low C3 state and lead to the identification of a Factor I deficiency due to a novel homozygous mutation

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ABSTRACT

Complement factor I (CFI) deficiency is typically associated to recurrent infections with encapsulated microorganisms and, less commonly, to autoimmunity. We report a 53-years old male who, in a routine control for non-alcoholic fatty liver disease, presented a flat beta-2 fraction at the capillary protein electropherogram. Patient's clinical records included multiple oropharyngeal infections since infancy and an episode of invasive meningococcal infection. Complement studies revealed reduced C3, low classical pathway activation and undetectable Factor I. *CFI* gene sequencing showed a novel inherited homozygous deletion of 5 nucleotides in exon 12, causing a frameshift leading to a truncated protein. This study points out that capillary protein electrophoresis can alert of possible states of low C3, which, once confirmed and common causes ruled out, can lead to CFI and other complement deficiency diagnosis. This is important since they constitute a still underestimated risk of invasive meningococcemia that can be greatly reduced by vaccination.

1. Introduction

The complement system is an important arm of the immune system. It participates in the host defense against pathogens both in the natural immunity and the adaptive response, in the removal of immune complex and apoptotic cells and in maintaining immune system homeostasis [1]. It comprises more than 30 soluble and membrane-bound proteins that upon recognition of different molecules are proteolytically activated following one of the three amplification pathways described; classical, alternative and lectins. Activation of C3 by any of the pathways will lead to the common terminal pathway, in which C5 is activated and the membrane attack complex (MAC) is assembled. Classical (CP) and lectins' pathway (LP) converge in the activation of C4, which acquires then proteolytic functions and together with C2 constitutes the C3 convertase of these two pathways. On the other hand, the alternative pathway (AP) is initiated at a low rate by spontaneous C3 hydrolysis but only when is stabilized by the binding of Factor B, results in the assembling of the C3 convertase of the AP. This constitutes an amplification loop that must be tightly regulated in order to prevent a massive consumption of C3 [2].

One of the most important regulators is the serine protease Factor I. Since this factor has no endogenous inhibitor neither circulates as an uncleaved proenzyme, it is regulated by the presence of its cofactors. Factor I needs the presence of Factor H/MCP/CR1 in order to cleave C3b into the inactive form iC3b. However, it is also capable of disrupting C4b using C4BP as cofactor. The Factor I gene (*CFI*, OMIM*217030), located in chromosome 4q25, consists of 13 exons which encode a protein of 583 amino acids and 66 kDa. Factor I mature protein is heavily N-glycosylated and circulated as a zymogen at a concentration of 30–40 μg/mL [3]. The protein consists of two polypeptide chains linked by a single disulfide bond. The

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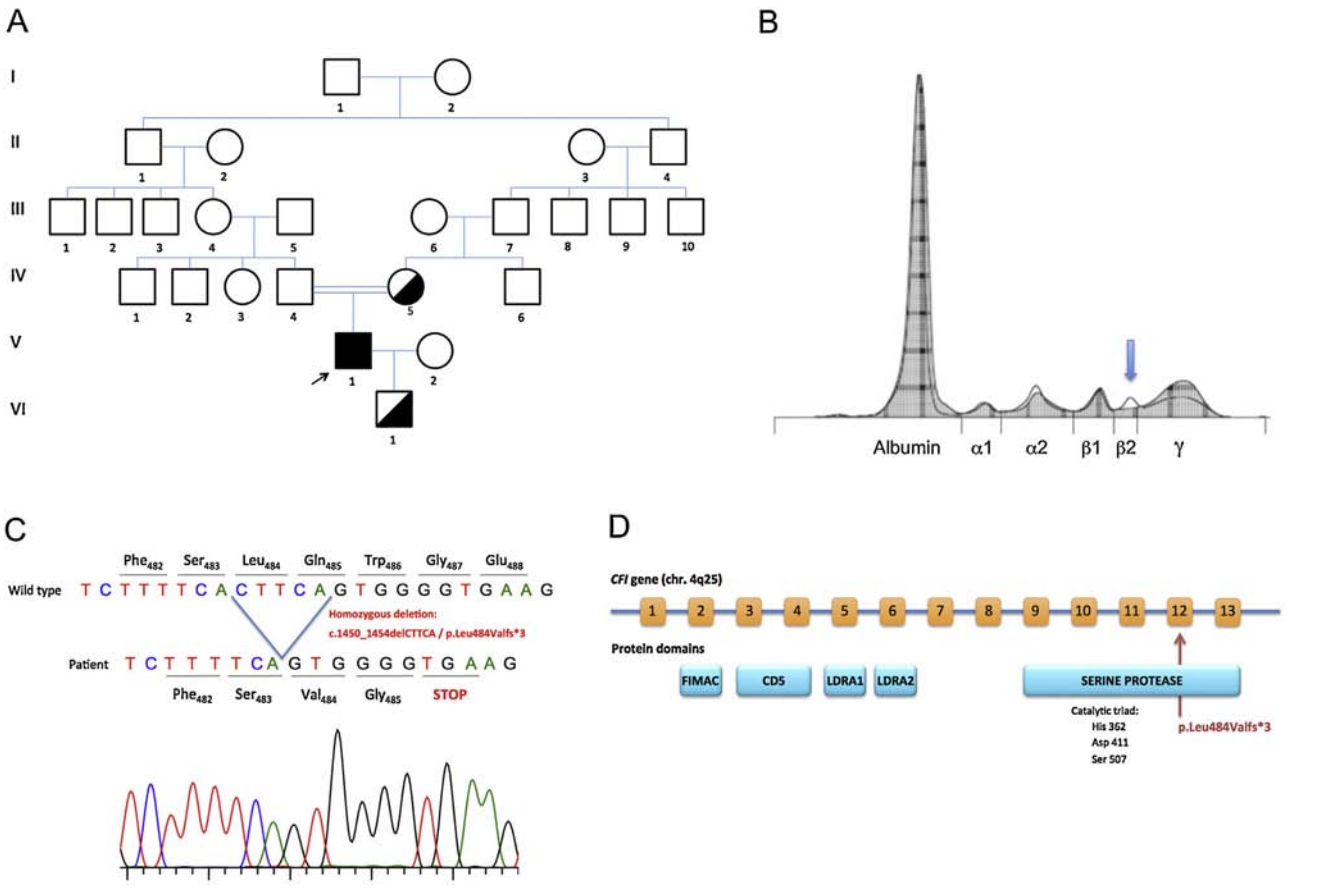

Fig. 1. A. Patient's serum proteins analysis by capillary electrophoresis. Patient (grey) and control (white) profiles are shown. Blue arrow indicates the flat β2 zone of the patient. B. Sequencing the *CFI* gene from the patient. Patient's CFI sequence is compared with the wild type one and the homozygous deletion of five nucleotides is indicated. C. Schematic model of the *CFI* gene and the main protein domains. The mutation found in our patient is depicted (red arrow). D. Pedigree of the patient family. Index patient is denoted with an arrow. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

heavy chain (50 kDa) contains the N-terminal region, a Factor I MAC domain, a scavenger receptor domain, two low-density lipoprotein receptor domains and a C-terminal region that presents a high variability across species [4]. The light chain (38 kDa) contains a serine protease domain with a conserved catalytic residue.

The absence of Factor I leads to a continuous consumption of C3 due to an uncontrolled amplification of C3 cleavage, resulting in an acquired C3-deficiency state [5]. Complete Factor I deficiency (OMIM#610984) is a rare immunodeficiency inherited in an autosomal recessive manner and, so far, around 40 cases have been described, with 16 mutations being described [6–10]. The clinical presentation is remarkably variable among the different patients studied. Like in primary C3 deficiency, these patients are more susceptible to infections by encapsulated bacteria, such as *Haemophilus influenzae*, *Streptococcus pneumonia* or *Neisseria meningitidis*. Defective opsonization in these patients makes them susceptible to recurrent pyogenic infections as well as to aseptic meningitis. Moreover, the deregulation of the cascades can lead to autoimmune diseases, glomerulonephritis or vasculitis [11]. Monoallelic mutations in Factor I have also been identified in atypical hemolytic uremic syndrome (aHUS, OMIM#612923) patients, resulting in only partial deficiency and with a very different clinical presentation comprising the clinical triad of thrombocytopenia, microangiopathic hemolytic anaemia, and acute renal failure [12,13].

In this paper, we describe an adult patient with a complete Factor I deficiency that was detected because of a minor abnormality in the capillary protein electrophoresis (CPE) profile. The clinical record revealed a number of infection episodes that lead to a full investigation of the complement system and the detection of a novel homozygous mutation causing Factor I deficiency.

2. Material and methods

2.1. Plasma analysis

Proteins profile was assessed by CPE (Capillarys2. Sebia, Evry Cedex, France). Complement C3 and C4 levels were measured by nephelometry (BNII. Siemens, Erlangen, Germany) and classical activation pathway, CH50, was measured using a liposome based assay (Wako. Neuss, Germany). Complement factor I was measured by radial immune diffusion (The Binding Site. Birmingham, UK).

2.2. Sequencing of the CFI gene

CFI gene (all exons and flanking regions) was sequenced in the patient. In the rest of the family only exon 12 was sequenced. Genomic DNA was extracted from EDTA-containing whole blood samples using a QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Polymerase chain reaction (PCR) to amplify the 13 exons of *CFI* and their flanking regions was carried out (primers and PCR conditions are available upon request) and purified PCR products were sequenced on an ABI 3100 DNA Sequencer using the BigDye Terminator sequencing kit 3.1 (Applied Biosystems, Foster, VA, USA).

Table 1
Laboratory data.

	Laboratory test	Result	Reference
Serum protein Electrophoresis	Albumin (%)	66.4	55.8–66.1
	α1 (%)	2.9	2.9–4.9
	α1 (%)	7.6	7.1–11.8
	β1 (%)	5.6	4.7–7.2
	β2 (%)	1.8	3.2–6.5
	γ (%)	15.7	11.1–18.8
Immunoglobulins	IgA (mg/dL)	193	70–400
	IgG (mg/dL)	1135	700–1600
	IgM (mg/dL)	81	40–230
Complement System Evaluation	Complement, component C3 (mg/dL)	19.3	85–180
	Complement, component bC4 (mg/dL)	18.5	10–40
	Complement, classical pathway activity (UI/mL)	2	34–71
	Complement, alternative pathway activity (%)	0	40–100
	Complement, component factor I (mg/dL)	0	3–5
	Complement, component factor B (mg/dL)	0.77	20.5–40
	Complement, component factor H (mg/dL)	19.14	12–56

3. Results and discussion

The patient is a 53-year old Caucasian male from Spain. A history of consanguinity was reported (Fig. 1A). The only remarkable antecedent is individual III.8, who apparently died of meningitis. There was no family history of immunodeficiency, autoimmune disorders or other complement-related complications like aHUS. In early childhood the patient suffered recurrent otitis media and pharyngitis, with a moderate reduction in school attendance. Pharyngitis episodes were less frequent after tonsillectomy at the age of 8 years, although otitis episodes still occur every two or three years. At the age of 20 the patient suffered an episode of invasive meningococcal infection leading to a septic shock that was treated with high dose penicillin. The patient also reported an infection in a sacral cyst that required debridement. Since the age of 30 the patient is monitored in the Liver Unit of Vall d'Hebron Hospital because of a moderate elevation of liver enzyme levels, being diagnosed of non-alcoholic fatty liver disease (NAFLD). Twenty years later, during one of the annual reviews for the NAFLD, the complement deficiency was suspected since the patient's serum protein electrophoresis showed a flat beta-2 zone of 1.8% (ref. 3.2–6.5%) (Fig. 1B). Since the most abundant protein in this fraction is complement C3, an initial evaluation of the complement system was performed. The complement study revealed an almost absent activity in classical pathway, normal C4 levels, decreased levels of C3 and a total absence of Factor I (Table 1). Alternative pathway activation and Factor B levels were absent, whereas Factor H levels were within normal range. Immunoglobulin levels and lymphocytes subsets were normal. Common causes of complement dysregulation such as liver cirrhosis or other severe liver diseases were ruled out after proving that liver function tests were normal. Viral serology excluded HCV and HBV infection. An ultrasound elastography (Fibroscan®) showed a low level of liver fibrosis (elastance: 6.7 kpa) and the CT scan and ultrasonography suggested a diagnosis of liver steatosis. A liver biopsy was not considered necessary. Kidney function and nutritional indexes were also normal. On the basis of these results, a primary Factor I deficiency was suspected.

CFI sequencing revealed a homozygous deletion of 5 nucleotides in exon 12, causing a frameshift and leading to a truncated protein of only 485 amino acids (Fig. 1C). The newly generated stop codon is located in the serine protease domain and affects the enzyme catalytic triad. The truncated protein, if translated, would lack the third aminoacid required for the protease activity, Ser507 (Fig. 1D). This mutation had not been reported and, following the recommendations of the Human Genome Variation Society (HGVS), we named it c.1450_1454delCTTCA/p.Leu484Valfs*3. Whilst the patient carried the mutation in homozygosis, his mother and his son carried the mutation in heterozygosis, thus confirming the autosomal recessive inheritance pattern of the mutation (Fig. 1A). In the mother, Factor I levels were low (1.38 mg/dL, ref. 3–5) indicating that carrying this mutation in heterozygosis leads to a partial Factor I deficiency. However, this partial deficiency is not sufficient to impair the alternative pathway regulation, since mother's C3 levels were between the normal ranges (94.7 mg/dL, ref. 85–180). As a result of the diagnosis, the patient was vaccinated against the three most typical encapsulated bacteria (*H. influenzae* B, *S. pneumoniae* and *N. meningitidis*). Effectiveness of vaccination towards Haemophilus and pneumococcus was measured through an increase of 4-fold on antibody titers. Antibody response to *N. meningitidis* could not be determined. Moreover, an early-onset antibiotic treatment for respiratory infections was also strongly recommended.

The spectrum of *CFI* mutations described have been classified in two categories: i) Those resulting in low or absent serum level of Factor I (type I *CFI* mutations), ii) Those presenting with normal serum level of Factor I but decreased activity (type II *CFI* mutations). In our case, complete Factor I deficiency results from a novel homozygous type I mutation that produce a truncated protein not detected in the patient's serum. With this mutation, only 17 mutations in CFI gene have been reported in the literature as responsible for complete Factor I deficiency and clinical presentation of the patients is remarkably variable. As described by Reis et al. [5], increased susceptibility to respiratory tract infections and meningitis are the most common clinical features, followed by kidney impairment and autoimmune diseases. However, the clinical spectrum of complete factor I deficiency might be wider than previously thought, since one case of leucocytoclastic vasculitis was recently described [10]. As in one of the patients from Nilsson's study [6], the severity of the infections in our patient declined with age, and probably for this reason he had never been studied before for complement deficiencies. Accordingly to the other genetic studies previously published, the heterozygous relatives of the index patient did not present any of the complications that are normally associated to partial factor I deficiency (typically aHUS). It has been postulated that either infections or pregnancy trigger aHUS in carriers of monoallelic Factor I mutations, together with other polymorphisms or variants not yet described. However the mother of our patient had not developed aHUS thus reinforcing the idea that a yet unknown specific genetic background is required in order to develop aHUS.

In this paper we want to draw the attention to the fact that the sensitivity of CPE, now routinely used in most clinical laborato-

ries, makes feasible the detection of both primary and secondary C3 defects if beta-2 fraction is reduced. Once C3 reduction is confirmed and common causes such as liver diseases, protein loss or nutritional defects are ruled out, this could lead to Factor I and other complement deficiency diagnosis. This is important since they constitute a still underestimated risk of invasive meningococcemia that can be greatly reduced by vaccination. This case is also a reminder that adult invasive meningococcemia should be investigated for complement and other immune defects.

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Early versus late diagnosis of complement Factor I deficiency: Clinical consequences illustrated in two families with novel homozygous *CFI* mutations.

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ABSTRACT

Complete complement Factor I (CFI) deficiency (#610984) is a rare autosomal recessive immunodeficiency. So far, around 40 cases, with 16 mutations, have been described. The absence of CFI leads to a continuous consumption of C3, resulting in an acquired C3-deficiency state. CFI deficiency is associated to recurrent infections with encapsulated microorganisms. We report 2 families with complete CFI deficiency. The patient in family A is a 60 year-old male who referrers no consanguinity, and a history of multiple respiratory infections as well as an episode of meningitis at age 20. In family B, the index patient is a 3 year-old girl, with consanguineous parents and abdominalgia. Complement studies in this patient were performed after observing a flat beta2 zone in serum protein electrophoresis. Both patients presented low C3 levels, absent levels of Factor B and Factor I, and absent activation of both classical and alternative pathways. The mutation found in the adult patient, p.Gly162Asp, was previously described although in heterozygous state and associated to renal disease. In family B, *CFI* gene sequencing revealed a novel homozygous mutation, p.His380Arg. Familiar studies on family B allowed the diagnosis of the youngest brother (2-months old), who also carried the mutation in homozygosis. This study points out that factor I deficiency can be more prevalent than expected and that an early diagnosis can be very helpful. This is important since they constitute a still underestimated risk of invasive meningococcemia that can be greatly reduced by vaccination.

INTRODUCTION

The complement system can become a double-edge sword: it has to be tightly regulated to deal with the activation on pathogen surfaces while avoiding an excessive consumption or activation on self-surfaces [1]. Therefore, several fluid phase and membrane proteins play an important role in limiting the complement's activation at the different pathway stages [2]. One of these proteins is Factor I (FI), an enzyme with serine-protease activity mainly involved in the regulation of the alternative pathway amplification loop. FI normally circulates in blood in a zymogen-like state at a concentration that ranges between 30-100 µg/mL [3], and in presence of its cofactors (Factor H, MCP, CR1 and C4BP) FI inactivate C3b and C4b thereby inhibiting all pathways of complement [4]. The gene encoding this protein is *CFI* and is located in chromosome 4q25. It consists of 13 exons that encode a protein of 583 amino acids. FI is a heavily N-glycosilated protein synthesized as a single 66-kDa chain, which is processed post-translationally splitting the molecule into two chains linked by a single disulfide bond. The heavy chain (exons 1-8) contains one FI membrane attack complex (FIMAC) domain, one CD5 like-domain or scavenger receptor cysteine-rich (SRCR) domain, and two low-density lipoprotein receptor domains (LDLR). The light chain (exons 9-13) contains the serine protease domain with the conserved catalytic residues [5, 6].

The absence of FI results in a continuous consumption of C3 due to an uncontrolled amplification of C3 cleavage, resulting in an acquired C3-deficiency state [7]. Monoallelic or biallelic mutations in *CFI* lead to partial or complete FI deficiency with different clinical consequences. Partial FI deficiency is due to *CFI* mutations in heterozygous state and it is strong risk factor for atypical haemolytic uremic syndrome (sHUS, OMIM#612923), an uncommon form of thrombotic microangiopathy with a poor renal prognosis [8]. Complete FI deficiency (OMIM#610984) is a rare autosomal recessive primary immunodeficiency resulting in severe and recurrent infections by encapsulated bacteria, such as Haemophilus influenzae, Streptococcus pneumonia or Neisseria meningitides [4]. Although the first case of complete FI deficiency was described in 1971 [9], it was not until 1996 when the first genetic defect was identified [10]. Since then, less than 20 mutations causing complete FI deficiency have been described, the vast majority of them in the last 10 years [11–17]. Although clinical features of the patients described can singularly involve vasculitis or renal involvement [13,16], almost all the cases present with recurrent respiratory infections, and many of them have episodes of meningococcal disease due to *Neisseria meningitidis* infection. Increased awareness of FI deficiency, as well as other complement deficiencies, could allow an early diagnosis and benefit the patients with preventive therapeutic approaches, such as specific vaccination or prophylactic

antibiotics. This would decrease the higher risk of severe infections that can lead to very serious sequelae, if not resulting fatal.

In this article, we illustrate the importance of early versus late diagnosis of FI deficiency in two families with novel homozygous *CFI* mutations affecting critical residues of FI protein. Due to the difference at the age of diagnosis (60 versus 4 years) the clinical outcome of the patients is completely different, the oldest patient being suffered severe and recurrent infections during his live that have left permanent sequelae. This study highlights the clinical relevance of an early detection of deficiencies on the complement system.

MATERIAL AND METHODS

Patients

The index case of family A attended and was diagnosed at the Internal Medicine Department of Hospital de Bellvitge (Barcelona, Spain) and was later referred to Hospital Universitari Vall d'Hebron (HUVH, Barcelona, Spain) to perform the genetic study. Family B attended the Pediatric Infectious Diseases and Immunodeficiencies Unit (UPIIP) of HUVH. 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 HUVH .

Complement function evaluation

The serum protein profile was assessed by Capillary Protein Electrophoresis (Capillarys2. Sebia, Evry Cedex, France). Complement C3 and C4 levels were measured by nephelometry (BNII. Siemens, Erlangen, Germany) and classical activation pathway, CH50, was measured using a liposome based assay (Wako. Neuss, Germany). Radial immune diffusion (The Binding Site. Birmingham, UK) was used to determine the activation of alternative pathway as well as circulating levels of complement Factors B, I and H.

Molecular studies

CFI gene (all exons and flanking regions) was sequenced in both index patients. In the rest of the family members only the exon containing the mutation was sequenced. Genomic DNA was extracted from

EDTA-containing whole blood samples using a QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Polymerase chain reaction (PCR) to amplify the 13 exons of CFI and their flanking regions was carried out (primers and PCR conditions are available upon request) and purified PCR products were sequenced on an ABI 3100 DNA Sequencer using the BigDye Terminator sequencing kit 3.1 (Applied Biosystems, Foster, VA, USA).

RESULTS

Complement function evaluation uncover a secondary C3 deficiency in two unrelated families

Family A

Patient 1 (P1) is a 62 year-old man born in Spain that was admitted for the first time to Internal Medicine department due to a native valve endocarditis caused by *Streptococcus bovis*. There is neither consanguinity nor reported history of infections or autoimmunity in the family. Since childhood the patient had several severe infections (pneumonia, meningitis, sepsis) of unknown aetiology, which caused important neurological impairment on the patient, affecting also to his relationship with the rest of family members. As a consequence, no P1 relatives were available for familiar studies. At the evaluation of the streptococcal endocarditis, we observed a normal immunoglobulin profile but we found altered values in the complement system. A total absence of Factor I was detected and, consequently, C3 and Factor B levels were also diminished, with the consequent dysfunction of classical and alternative activation pathways *in vitro* (**Table 1**).

Family B

Patient 2 (P2) is a 4 year-old girl, born in Spain from first-grade consanguineous Pakistani parents. She only presented a single episode of otitis media and recurrent abdominal pain. The detection of a flat beta2 zone at the serum protein electrophoresis performed as a screening led to complement system evaluation, which revealed low levels of C3. The patient has two younger siblings (a 21 month-old girl and a 2 month-old boy) without reported history of infections or autoimmunity in who complement was also assessed due to family history. The concentration of Factor I was clearly below the reference values (1.2 mg/dL; ref. 3-5). The patient also presented low levels of factor B, as a consequence of the uncontrolled amplification of alternative pathway. All the family members from P2 except the youngest brother

presented normal levels of complement proteins, and were also capable of activating both classical and alternative pathways normally. The 2-month old brother presented impaired activation of classical, alternative and lectine's pathway. Factor I and Factor B levels were very low, whereas C3 level was only slightly decreased (**Table 1**).

Genetic study of the *CFI* gene reveals a complete FI deficiency in the two families

Family A

CFI gene sequencing of the P1 revealed a homozygous nucleotide change in exon 4 (c.485G>A), affecting the second position of codon 162 and leading to an amino acid change (p.Gly162Asp) (**Figure 1**). To date, this mutation has not been reported in homozygosity but it had been previously described in heterozygosis associated to thrombotic microangiopathy [18] and as part of a compound heterozygous genotype in a patient with complete factor I deficiency [14]. As mentioned above, no familiars were available for genetic studies.

Family B

CFI gene sequencing of the P2 revealed a homozygous nucleotide change in exon 10 (c.1139A>G), affecting the second position of codon 380 and leading to an amino acid change (p.His380Arg) (**Figure 1**). This mutation had not been reported in the literature or databases and computational prediction using PolyPhen2 and SIFT tagged this mutation as clearly pathogenic (PolyPhen2 score=1, SIFT score=0). Familiar study showed that both parents and sister carried the mutation in heterozygosis, confirming the autosomal recessive pattern of complete FI deficiency, while the 2-month old brother was homozygous for the p.His380Arg mutation (**Figure 1**).

Molecular impact of the Gly162Asp and His380Arg mutations on FI protein

To assess the molecular impact of both mutations, we combined three sources of information: location of the mutations in the multiple sequence alignment (MSA) of the FI protein family and in the known experimental structure of this protein, and protein stability computations. Positions 162 and 380 are highly conserved in the MSA (**Supplementary Figure 1**), indicating that replacement of the corresponding native residues may be highly disruptive, either functionally and/or structurally. In the case of His380, this is consistent with the fact that this residue belongs to the catalytic triad of the serine

protease domain of FI. Mapping of the two mutations to the experimental structure of FI (PDB code: 2XRC) (**Figure 2A**), sheds further light on their molecular impact. We see that Gly162 is located in a highly packed region of the SRCR-LDL1 interface (**Figure 2B**); replacement of this residue by the relatively bulky, and less flexible, aspartic acid will disrupt this interface. In addition, amino acid volume differences will also affect SRCR domain stability by changing the pattern of intradomain contacts. The previous observations were consistent with the result of stability computations, done with I-Mutant2.0 [19], which show that Gly162Asp causes a large stability decrease (ΔΔG=-2.4 Kcal/mol). For mutant H380R, in addition to its expected functional impact resulting from the role of the native histidine in the catalytic triad (**Figure 2C**), stability computations show that the mutation may also have a destabilizing effect (ΔΔG=-1.16 Kcal/mol).

Clinical consequences of early versus late diagnosis of complete FI deficiency

The importance of an early diagnosis of FI deficiency is exemplified by the comparison of patient's clinical outcome. In P1, FI deficiency is diagnosed during the late adulthood, at the age of 60 years. Patient anamnesis revealed that he suffered from several respiratory infections since childhood. The most striking affectation was the meningitis episode at the age of 20 years, which lead important neurological sequelae in the patient including memory difficulties. After that episode, the patient had been suffering from many episodes of pneumonia and other infections by encapsulated bacteria. By contrast, P2 was diagnosed at the age of 4 after a single infectious episode and failure to thrive. This early diagnosis of FI deficiency permitted a prompt therapeutic intervention through vaccination of encapsulated bacteria (*Haemophilus influenzae*, *Streptococcus pneumoniae* and *Neisseria meningitidis*) and prophylactic antibiotics. At the time of the diagnosis, the patient never had any of the typical complications related to this disease. Additionally, the diagnosis of P2 allowed a very early detection of FI deficiency on his youngest brother, who at the age of 2 months was diagnosed, raising awareness on the clinicians of his disease and the consequently increased risk of infections.

A systematic review on the previously published cases of complete FI deficiency has been done and is summarized in **Table 2**. The average age at diagnosis is 19.4 years (n=25). 10 out of 25 patients were diagnosed within the first decade of life, six of them without having suffered any previous episode of meningococcal infection. Among the 25 patients, only one case presented an episode of meningococcal infection after the diagnosis [12]. Remarkably, this case had been diagnosed as part of a familiar study, but

the patient was considered as asymptomatic, and presumably no prophylactic therapeutic approach was followed until the first episode of meningitis appeared at the age of 18 [12]. Four out of five patients in whom Factor I deficiency was diagnosed at ages older than 35 years old referred not only a severe episode of meningococcal infection at a younger age, but also recurrent respiratory infections throughout the following years, which could have been avoided if their condition had been diagnosed earlier in their lives.

DISCUSSION

Complete FI deficiency is a rare autosomal recessive complement disorder, which leads to the uncontrolled amplification of C3 cleavage, resulting in a severe secondary C3 deficiency. In this study, we described three new cases of complete FI deficiency (from two different families) with novel homozygous *CFI* mutations. With the data presented here, the molecular basis of complete FI deficiency has been elucidated in 20 families with 23 different mutations described (**Table 2**).

The mutation identified in patient (c.485G>A / p.Gly162Asp) 1 had been previously found in heterozygosis associated to thrombotic microangiopathy after a renal transplant, and as part of a compound heterozygosis genotype in a case of complete FI deficiency [14,18]. However, it is the first time that this mutation is found in homozygosis and responsible of a complete FI deficiency. We have provided some information about the structural consequences of this mutation: The mutated amino acid (Gly162) is located at the SRCR (scavenger receptor cysteine-rich), a protein domain found in several extracellular receptors and associated with pattern recognition [20]. We observe that the substitution of a Glycine by an Aspartic Acid has, in our case, a damaging impact on the protein structure/function. This is, first, supported by the high conservation of Gly162 across species (**Supplementary Figure 1**), which is indicative of the important nature of this residue. Second, replacement of a small, uncharged amino acid such as Glycine by a big acidic residue disrupts both the SRCR domain network of interactions and affects the SRCR-LDL1 interface. As a net result, the Gly162Asp causes a substantial decrease in FI stability, -2.4 Kcal/mol, making it prone to degradation by the proteasome.

We have identified a novel mutation in family B affecting, for the first time, one of the FI conserved catalytic residues. The amino acid change H380R affects directly to the functionality of the peptidase activity in FI, since H380 is part of the active site of the peptidase domain, which is formed by the catalytic triad His380, Asp429 and Ser525 [21]. In our case, the function loss is further aggravated by the

effect of the mutation on protein structure stability, which drops -1.16 Kcal/mol. Overall, this could explain why, despite detecting some residual levels of FI in patient's serum, there is still a consumption of C3 that leads to a total absence of pathways activation. Both for His380Arg and Gly162Asp, the results of the structural analyses are consistent with those of the pathogenicity predictors SIFT and PolyPhen2.

Due to the dysregulation of the alternative pathway, complete FI deficiency leads to C3 consumption and to the absence of the opsonin C3b. Therefore clinical symptoms overlaps with those of a secondary C3 deficiency. These patients normally present with recurrent pyogenic infections of the respiratory tract, including otitis, sinusitis, bronchitis and pneumonias. Moreover, like in terminal complement components deficiency, these patients also have a higher risk of infections by *Neisseria meningitides*, which can be fatal if they lead to sepsis or can carry severe neurological consequences. Our study and previously published data illustrate the importance of an early diagnosis of complete FI deficiency for the establishment of prompt prophylactic proceedings, including vaccination of encapsulated bacteria and antibiotic prescription. Otherwise, the risk of severe infections (with possible permanent sequelae) dramatically increases. One example of this latter case is patient 1, who was not diagnosed until the age of 60, during an episode of streptococcal endocarditis. As mentioned, the patient had suffered several severe infections during his life, one of them (a meningitis episode at the age of 20 years) caused permanent neurological sequelae, including memory difficulties and affecting their ability to socializing. It is relevant to remark that despite the highest rates of invasive meningococcal disease are registered in children younger than 1 year, there is a second peak in adolescents and young adults (15-25 years of age) [22]. If the complement system had been studied before in this patient, the diagnosis of secondary C3 deficiency would have been established earlier and probably avoiding certain infections and its consequences. After the final diagnosis of complete FI deficiency, vaccination and antibiotic profilaxis were indicated, markedly improving his life-quality.

Interestingly, in the patient 2 the complete FI deficiency was diagnosed very early and using a simple and routinely used method in most clinical laboratories: the capillary protein electrophoresis (CPE). We recently reported the utility of CPE for the diagnosis of both primary and secondary C3 defects [17], since the detection of a flat beta2 zone usually reveals low levels of C3. The present study reinforces this idea.

The two cases presented here highlight the importance of keeping in mind, during the clinical practice, the warning signs that can alert of complement deficiencies [23]. Early diagnosis shortens the diagnostic delay that is distressing to the family, damaging to the patient and wasteful for the health-care resources.

CONFLICT OF INTEREST

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

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Table 1. Complement studies in patients and available family members.

	Family A	**Family B**					**Ref. Values**
	II.3	I.1	I.2	**II.1**	II.2	II.3	
C3 (mg/dL)	**29.6**	137	156	**35,2**	111	82	85 - 180
C4 (mg/dL)	**17.7**	17.8	37.9	**28,9**	18	31.6	10 - 40
CH50 (U/mL)	**<12.07**	60.27	95.16	**<12.07**	49.85	<12.07	34 - 71
AP50 (%)	**0**	n.d.	100	**0**	n.d.	0	40 - 100
Factor B (mg/dL)	**4.5**	40.76	59.05	**1.23**	30.22	4.3	20 - 40
Factor H (mg/dL)	**26.28**	71.21	78.76	**35.54**	53.77	34.9	12 - 56
Factor I (mg/dL)	**0**	3.38	3.09	**1.2**	2.53	2.1	3 - 5
CFI mutation	**p.G162D**	p.H380R	p.H380R	**p.H380R**	p.H380R	p.H380R	**-**
Genotype	**Hom**	Het	Het	**Hom**	Het	Hom	**-**

n.d.: not determined.

Table 2. Reported families/cases with complete Factor I deficiency.

Family	Exon	Mutation (cDNA)	Mutation (protein)	Genotype	Meningococcal infection (year of 1st infection)	Age at diagnosis (yr)	Year of publication	Ref.
1	11	c.1253A>T	p.H418L	Hom	Yes, meningitis (13)	18	1996	[10]
2	5, 11	c.772G>A / c.1253A>T	p.D220-K257del / p.H418L	Comp Het	Yes (7)	11	1996	[10]
3	11	c.1176insAT	p.W393Yfs*5	Hom	No	20	2003	[11]
	11	c.1176insAT	p.W393Yfs*5	Hom	No	3	2003	[11]
4	5	c.739T>G	p.C247G	Hom	Yes, recurrent meningitis and sepsis (16)	23	2008	[12]
	5	c.739T>G	p.C247G	Hom	Yes (18)	13	2008	[12]
5	5	c.772G>A	p.D220-K257del	Hom	Yes (17)	40	2008	[12]
	5	c.772G>A	p.D220-K257del	Hom	Yes (31)	37	2008	[12]
	5	c.772G>A	p.D220-K257del	Hom	No	34	2008	[12]
6	5	c.764G>A	p.C255Y	Hom	Yes, meningitis (10)	11	2009	[13]
7	5, 6	c.748C>A / c.803C>T	p.Q250K / p.S268L	Comp Het	No	23	2009	[13]
8	10	c.1060C>T	p.Q354X	Hom	No	3 months	2009	[13]
9	5	c.772G>A / c.1100T>G	p.D220-K257del / p.I357M	Comp Het	No	37	2009	[13]
10	4, 11	c.563G>T / c.1253A>T	p.G188V / p.H418L	Comp Het	Yes, sepsis (2 months)	2 months	2009	[13]
11	4, 11	c.485G>A / c.1176insAT	p.G162D / p.W393Yfs*5	Comp Het	Yes, sepsis (16)	37	2012	[14]
12	5	c.772G>A	p.D220-K257del	Hom	Yes, sepsis (1)	8	2012	[14]
13	2, 11	5-6Kb gene deletion / c.1420C>T	- / p.R474X	Comp Het	Yes, sepsis and meningitis (6)	6	2012	[14]
14	4, 13	c.559C>T / c.1610_1611insAT	p.R187X / p.V537Vfs*2	Comp Het	No	5	2012	[14]
15	2, 4	c.80_81delAT / c.559C>T	p.D27Afs*18 / p.R187X	Comp Het	No	6	2012	[14]
16	9, 13	c.1019 T>C / c.1571 A>C	p.I340T / p.D524V	Comp Het	No	16	2013	[15]
17	2	c.162C>G	p.C54W	Hom	No	28	2015	[16]
18	12	c.1450_1454delCTTCA	p.L484Vfs*3	Hom	Yes, sepsis (20)	53	2016	[17]
19	10	c.1139A>G	p.H380R	Hom	No	4	2017	this study
19	10	c.1139A>G	p.H380R	Hom	No	2 months	2017	this study
20	4	c.485G>A	p.G162D	Hom	Yes, meningitis (20)	60	2017	this study

FIGURE LEGENDS

Figure 1. *CFI* mutations causing complete Factor I deficiency in families A and B. (A) Familial trees showing the segregation of the CFI mutations. Index patients are indicated with a black arrow. n.d.: not determined. (B) The molecular basis of Factor I deficiency. In family A the antisense sequence is shown. (C) Schematic model of the CFI gene and the main protein domains. The mutations found in our families are located in exons 4 (family A) and 10 (family B).

Figure 2. Structural impact of the *CFI* mutations. (A) Mapping of Gly162Asp and His380Arg mutations to the structure of FI. The distance between Gly162 and His380 is a first hint indicating that the impact of the corresponding mutations will be different. (B) Gly162Asp is located at the SRCR (yellow)-LDL1 (green) interface where the introduction of an acidic residue such as Asp will have a disruptive effect. (C) His380Arg mutation implies the loss of the catalytic triad (red) of the protease domain of FI and also disrupts the network of residue-residue interactions between His380 and its neighbours. The remainder of the structure is shown in lilac. In both (B) and (C) residues having interatomic contacts (atom-atom distance below 5Å) with the mutated residue are labelled in white.

FIGURE 1

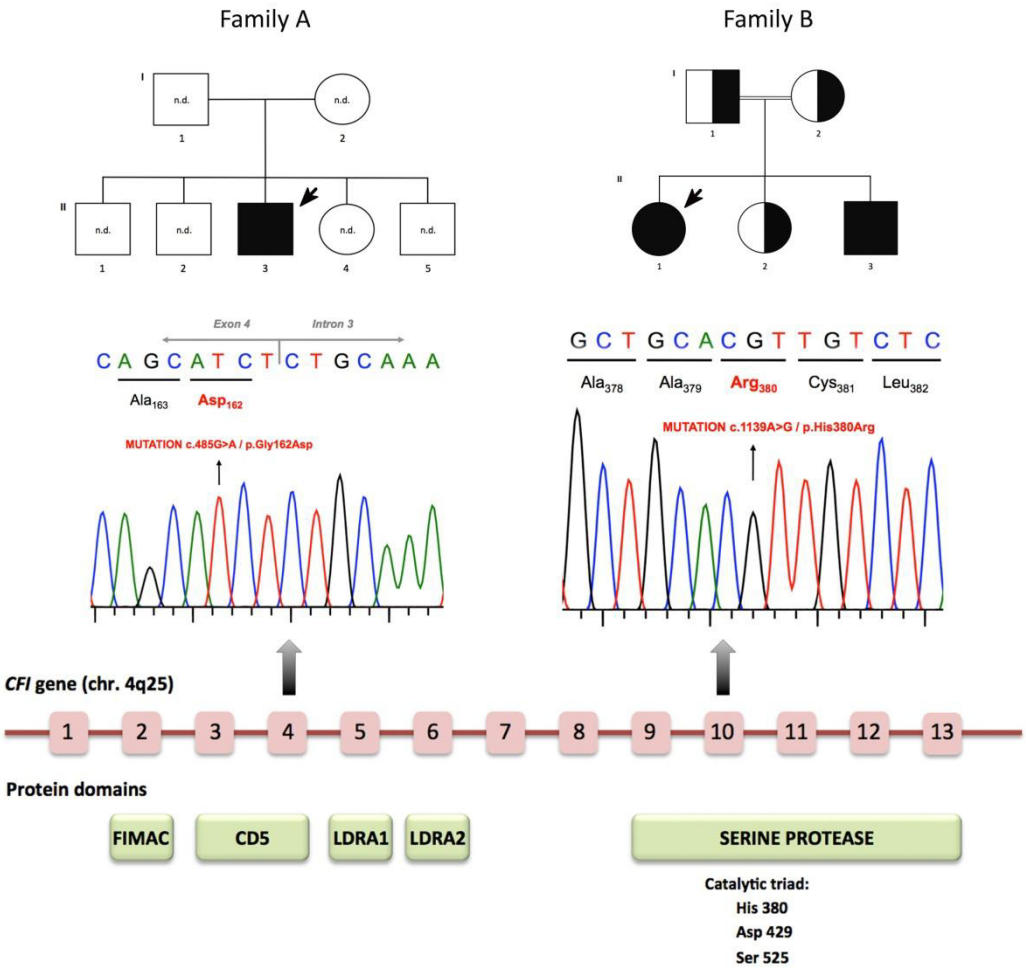
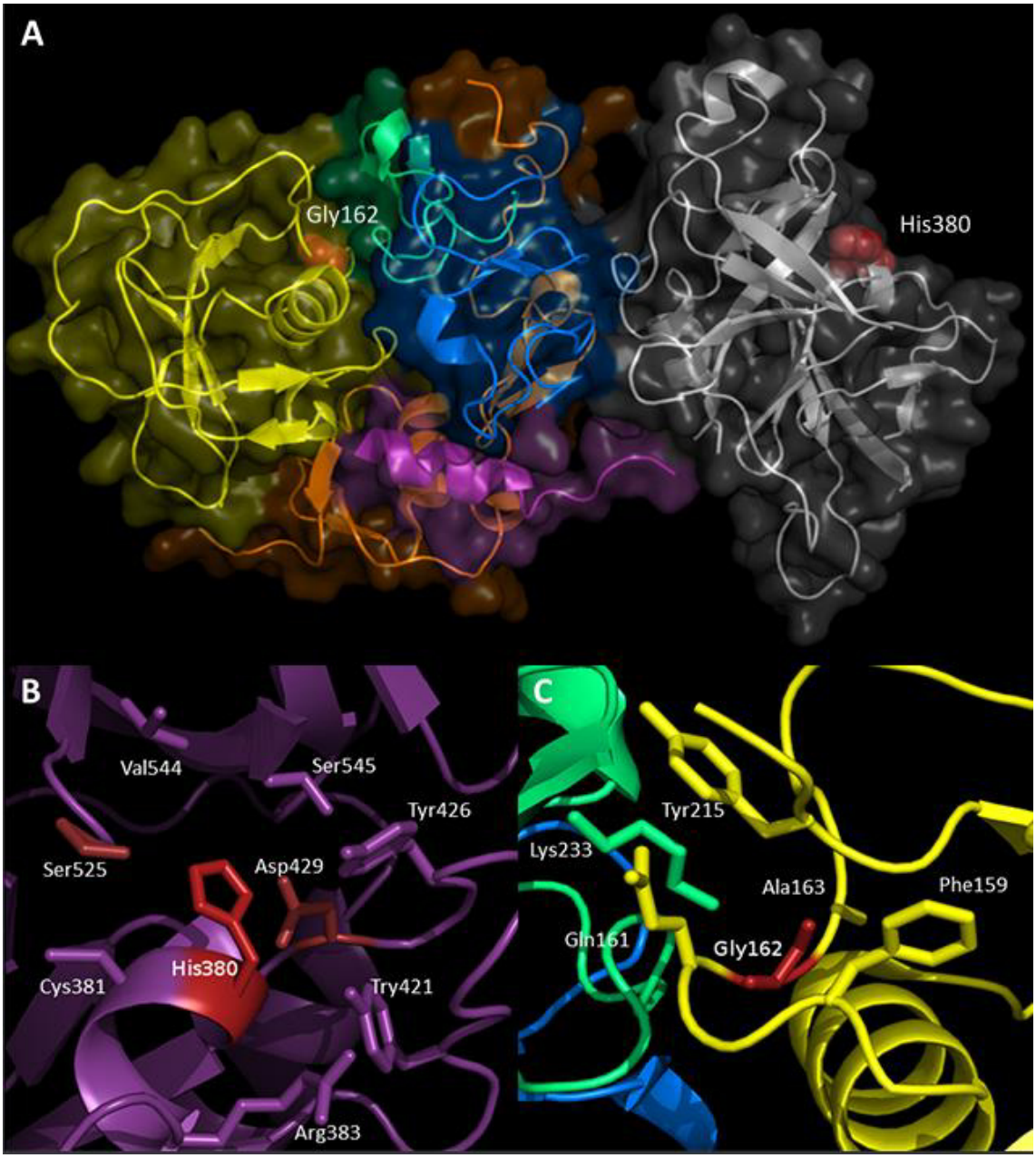

Family A
Family B
n.d.
Exon 4
Intron 3
C A G C A T C T C T G C A A A
Ala163 Asp162
MUTATION c.485G>A / p.Gly162Asp
G C T G C A C G T T G T C T C
Ala378 Ala379 Arg380 Cys381 Leu382
MUTATION c.1139A>G / p.His380Arg
CFI gene (chr. 4q25)
1 2 3 4 5 6 7 8 9 10 11 12 13
Protein domains
FIMAC
CD5
LDRA1
LDRA2
SERINE PROTEASE
Catalytic triad:
His 380
Asp 429
Ser 525

FIGURE 2


A
Gly162
His380
B
Val544
Ser545
Tyr426
Ser525
Asp429
His380
Cys381
Try421
Arg383
C
Tyr215
Lys233
Ala163
Phe159
Gln161
Gly162

Chapter 2.2: Clinical, functional and molecular characterization of patients with complement deficiencies affecting C5

There is some controversy regarding the need of performing screening tests for complement deficiencies on those patients presenting one single episode of meningococcal disease during childhood. For that purpose, in collaboration with the Infectious and Immunodeficiency Paediatric Unit from our hospital, we started a descriptive project that would include all patients aged under 18 years old diagnosed from a single invasive meningococcal disease during years 1997-2013. From the initial 160 patients who accomplished inclusion criteria, we could finally obtain samples from only 80 of them. Average age from the included patients was 10 years old (range: 11 months to 29 years old), and the average age for the meningococcal disease episode was 2 years old. Among the 80 patients, 64 (80%) presented infections due to serogroup B, 9 (11.25%) due to serogroup C and only two cases presented infections due to uncommon serogroups Y and E19. Meningococcal serogroup on 5 patients could not be determined. Complement initial screening tests were performed to all of them (CH50, AP50, C3 and C4). Results were normal for all the patients except for the two presenting the disease due to the uncommon serotypes, who lacked of classical and alternative pathways activity. Further studies (described in detail on next paper) finally allowed a diagnosis for C5 deficiency. Prior to our study, 50 families had been reported with C5 deficiency but only 15 mutations had been characterized. We performed molecular studies to determine the genetic defect underlying the C5 deficiency and identified two non-previously described mutations. Shortly after the characterization of these two families, a third one was referred to our hospital presenting also with C5 deficiency. The fact that this family shared the same mutation with one of the previous families drew our attention. These two families were non-consanguineous and the fact that they were both from the same region of Morocco (Alnif), prompt us to study the prevalence of this mutation in healthy donors' samples from the Middle East and North-Africa continental region (which are known to share the same genetic background). We stablished a collaboration with Evolutionary Biology Research Group from Pompeu Fabra University (Barcelona) and obtained 768 samples

from Morocco (254), Algeria (96), Tunis (247) and Lybia (171). We found one heterozygous sample for the mutation among the samples from Algeria (allelic frequency: 1/192). Although the mutation should be studied in larger population cohorts in order to stablish its real prevalence, the positive result in Algeria suggest that this mutation is found at low frequency within North-African countries.

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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

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.

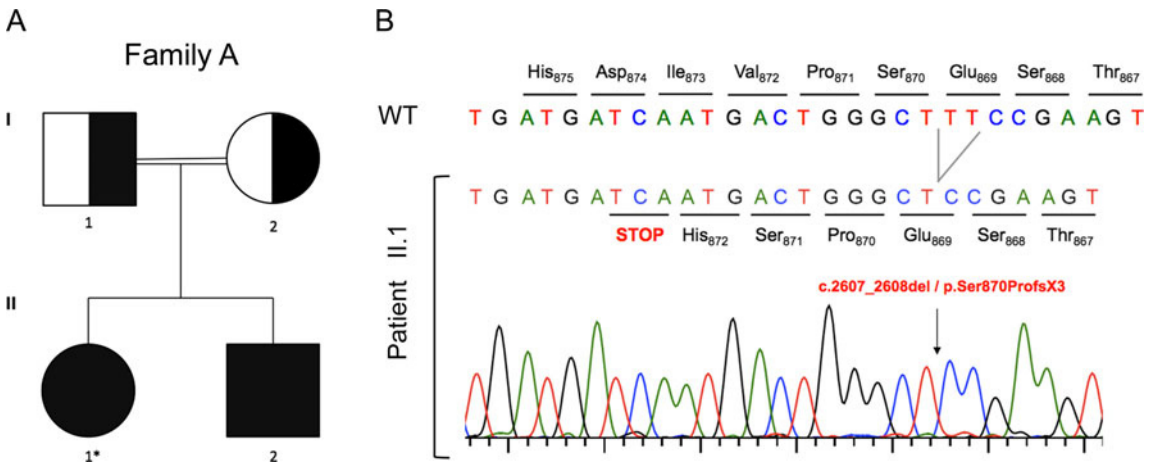

Fig. 1 C5 deficiency in family A. **a** Familial tree showing the segregation of the c.2606_2608del mutation. Both parents (consanguineous) carry the mutation in heterozygous state whereas the index patient (marked with an *asterisk*) and her brother are homozygous for this mutation. **b** The molecular 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

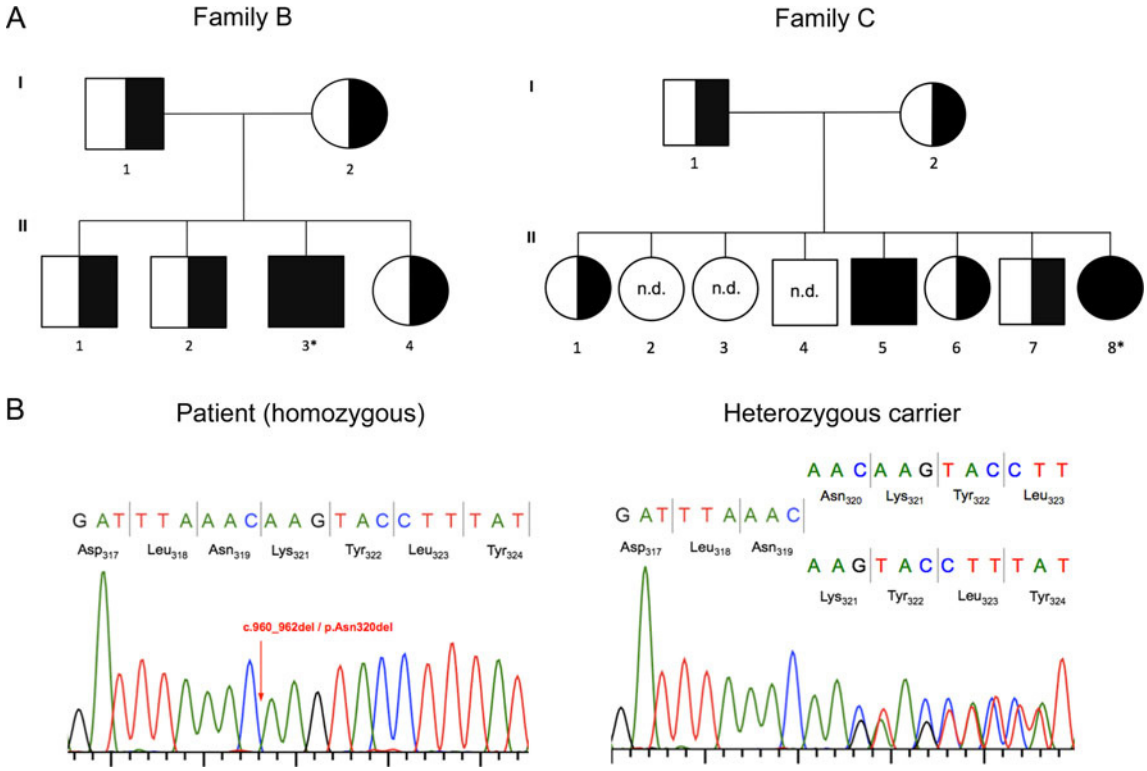

Fig. 2 C5 deficiency in families B and C. **a** Familial trees showing the segregation of c.960_962del mutation in the two families. In both families parents (non-consanguineous) are heterozygous and index patients (marked with an *asterisk*) are homozygous for this mutation. A sibling of family C index 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].

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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

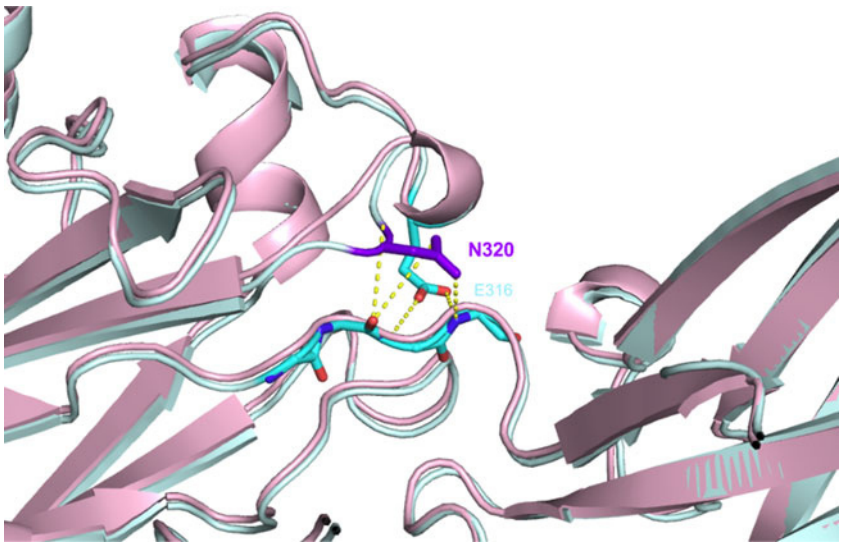

Fig. 3 Structure model of p.Asn320del. To emphasize the impact of this mutation we show the native structure (*light blue*) with the model of the mutant (*pink*). Asn320 (*shown in lilac*) is located in a structurally irregular region, where it interacts with neighboring residues (*dashed yellow lines*). 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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OBJECTIVE 3. TO INVESTIGATE RECURRENT MUTATIONS CAUSING C5 DEFICIENCY AT THE POPULATION LEVEL.

Materials, methods and results to this objective have been published and can be read in the following article:

Complement factor 5 (C5) p.A252T mutation is prevalent in, but not restricted to, Sub-Saharan Africa: Implications for the susceptibility to meningococcal disease.

Clara Franco-Jarava, David Comas, Ann Orren, Manuel Hernández-González, Roger Colobran.

Clin Exp Immunol. 2017 Apr 1. doi: 10.1111/cei.12967 *In press*

As has been previously introduced, prevalence of defects on complement components of the terminal pathway presents some geographical variations. One study from 2015 indicated that 18.5% of Black Africans with a history of meningococcal disease presented a complete defect of C6 molecule. With an incidence of 1/1000, C9 deficiency is one of the most common genetic defects in Japan. Moreover, it has been described that the prevalence of heterozygous carriers of C9 R95X (the most common mutation among C9 deficient patients in Japan) is of 6,7%.

During our studies on complement C5 deficiency, we observed that despite almost all the mutations were reported in one single family, there was a mutation that had been found in 12 families with C5 deficiency from South Africa. This mutation had also been described in three individuals from central African countries studied at the 1000 Genomes Project. Considering the high incidence of meningococcal disease at the region of central Africa, we thought that, if prevalent on the area, this complement deficiency could be one of the susceptibility factors for these epidemics. Additionally, we also found one data base (from NHLBI Exome Sequencing Project) describing the same mutation in 15/2090 Afro-Americans, but in none of the 4044 Americans from European origin. Therefore, we finally decided to expand our studies and look for the presence of this mutation in samples from as many different countries as possible. The presence of an elevated number of heterozygous carriers for a pathogenic C5 mutation would increase the possibility of homozygous C5 deficient individuals. Considering that South-Mediterranean countries are a common destiny for African immigrants, it is important to contemplate this possibility for the elaboration of a correct diagnostic algorithm.

We looked for the mutation in samples from the Human Genome Diversity Panel, which comprise samples from 51 different populations from all the continental regions. In order to

increase the size of the African samples, we also looked for the mutation in a collection of samples from Gabon and in the ones from North Africa used at the previous study. To carry out the screening of the p.A252T C5 mutation in this large number of samples (n=2710), we designed a qPCR SNP genotyping assay that would permit a rapid identification of individuals carrying the mutation. Since we did not have any positive samples to use as control for our technique, we established a collaboration with Ann Orren, an outstanding investigator in the complement field and the corresponding author in the paper describing the frequency of C5 mutation in South-Africa. She sent us samples from all three possible genotypes that were used as controls for each qPCR. We found 11 heterozygous carriers and, as expected, no homozygous samples. 9 out of 11 samples were form sub-Saharan African countries. However, the mutation was not restricted to that region. We also identified two carriers from Israel and Pakistan, thus highlighting the importance of migratory fluxes.

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Complement factor 5 (C5) p.A252T mutation is prevalent in, but not restricted to, sub-Saharan Africa: implications for the susceptibility to meningococcal disease

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Summary

Complement C5 deficiency (C5D) is a rare primary immunodeficiency associated with recurrent infections, particularly meningitis, by *Neisseria* species. To date, studies to elucidate the molecular basis of hereditary C5D have included fewer than 40 families, and most C5 mutations (13 of 17) have been found in single families. However, the recently described C5 p.A252T mutation is reported to be associated with approximately 7% of meningococcal disease cases in South Africa. This finding raises the question of whether the mutation may be prevalent in other parts of Africa or other continental regions. The aim of this study was to investigate the prevalence of C5 p.A252T in Africa and other regions and discuss the implications for prophylaxis against meningococcal disease. In total, 2710 samples from healthy donors within various populations worldwide were analysed by quantitative polymerase chain reaction (qPCR) assay to detect the C5 p.A252T mutation. Eleven samples were found to be heterozygous for p.A252T, and nine of these samples were from sub-Saharan African populations (allele frequency 0·94%). Interestingly, two other heterozygous samples were from individuals in populations outside Africa (Israel and Pakistan). These findings, together with data from genomic variation databases, indicate a 0·5–2% prevalence of the C5 p.A252T mutation in heterozygosity in sub-Saharan Africa. Therefore, this mutation may have a relevant role in meningococcal disease susceptibility in this geographical area.

Keywords: Africa, C5 deficiency, complement, infection, meningococcal disease, primary immunodeficiency

Introduction

The complement system has a major role in the innate immune system's defence against pathogens. Complement activation upon recognition of certain molecular patterns by any of the three pathways leads to activation of a common terminal complement pathway [1,2]. C5 is the initial component of this pathway, and activation through the C5 convertases releases the potent anaphylatoxin peptide C5a and generates C5b. After C5 cleavage, the terminal components C6, C7, C8 and C9 bind to produce the membrane attack complex (MAC), which forms a hydrophilic channel through the membrane of target cells, causing cell death [3]. The pores that the MAC generates on a pathogen's surface are key to host defence against Gram-negative bacteria, particularly species from the *Neisseria* genus [4].

During recent decades, deficiencies of the terminal complement components that form the MAC (C5-C9) have been described extensively [5]. The risk of meningococcal disease in patients with these deficiencies is 7000–10 000-fold higher than that of healthy individuals, and they also have a higher risk of recurrent invasive meningococcal infection [6,7]. In general, terminal complement deficiencies are rare, but the incidence varies considerably between different populations. A well-recognized example is C9 deficiency, caused mainly by the non-sense mutation, R95X. In Japan, it is one of the most common genetic disorders, with a population prevalence of one in 1000, whereas only a few patients with C9 deficiency have been identified in European countries [8]. C7 deficiency caused by the G357R mutation is reported as having a high

prevalence (1·1%) in the Israeli Moroccan Jewish population [9], and C6 deficiency has a high prevalence in Western Cape South Africans and in African Americans [10–13].

Focusing upon C5 deficiency, approximately 50 cases have been published from around the world [14–22] and most of the causal mutations have been described in only one family. A striking exception is the p.A252T mutation, reported recently to be responsible for C5 deficiency and found in approximately 7% of meningococcal disease cases in black Africans from the Western Cape (South Africa) [21]. This mutation has an allele frequency of 3% in the black African population and 0·66% in the Cape Coloured population of the Western Cape area [21].

Meningococcal meningitis cases occur throughout the world. However, large, recurrent epidemics affect an extensive region of sub-Saharan Africa known as the 'meningitis belt', which covers 26 countries from Senegal in the West to Ethiopia in the East [23]. Considering the previously observed allelic distribution of p.A252T in South Africa, the question arises as to whether the mutation may be prevalent in other parts of Africa or other continental regions. The aim of this study was to evaluate the presence of the C5 p.A252T mutation in Africa and other continental regions and discuss the implications for prophylaxis against meningococcal disease.

Materials and methods

Samples

In total, 2710 gDNA samples were tested. Almost half these samples ($n = 1064$) were a part of the Human Genome Diversity Panel (HGDP), a widely used resource for studies of human genetic variation. Genomic DNA samples from these fully consenting individuals were collected by the Human Genome Diversity Project in collaboration with the Centre Etude Polymorphism Humain (CEPH) in Paris. Fifty-one different populations from Africa, Europe, the Middle East, South and Central Asia, East Asia, Oceania and the Americas are represented in the HGDP [24,25].

To increase the number of samples from Africa, a collection of 352 samples from Gabon and 768 samples from North African countries (Morocco, Algeria, Tunisia and Libya) were also included. The presence of the mutated allele was also evaluated in 526 local samples (Barcelona, Spain) obtained from blood donors.

Although all the samples included in the study were from healthy individuals, a history of previous meningococcal disease was not recorded specifically. All samples were collected with the informed consent of the volunteers participating in the study.

Screening for the C5 p.A252T mutation

Quantitative polymerase chain reaction (qPCR) assay design. The properly tagged consensus sequence containing the C5 c.754G > A (p.A252T) variant and all other single nucleotide polymorphisms (SNPs) in the vicinity was submitted to the design tool at the Applied Biosystems website for custom TaqMan assay design. The TaqMan genotyping assay mix contained a forward and reverse primer for the submitted sequence, one probe that matched perfectly the wild-type sequence variant (c.754G) labelled with VIC, and a second probe that matched the mutant (c.754A) variant, labelled with FAM.

Allele discrimination assay. To obtain a final PCR volume of 10 μl, a working master mix was prepared containing 0·25 μl of TaqMan genotyping assay mix (×40), 5 μl TaqMan UNG Master Mix ×2 (Applied Biosystems, Carlsbad, CA, USA) and 2·75 μl of water for each reaction. Samples were placed directly into each well of a 96-well plate. Two controls for each condition (wild-type, heterozygous for the mutation and homozygous for the mutation) were used in each assay. Plates were loaded onto an Applied Biosystems 7500 fast real-time PCR system, and the 7500 (version 2.0.5) software was used to run the assay, following the default standard allelic discrimination genotyping assay protocol. Briefly, this consisted of an end-point PCR in which fluorescence was read prior to PCR and after completion of the last PCR cycle. The software calculated the normalized dye fluorescence (ΔRn) as a function of cycle number for allele 1 (wild-type) and allele 2 (mutant). The results were displayed on a plot, where each axis represented the fluorescence level of each fluorochrome, thus grouping the samples on the three different genotypes (Fig. 1a).

Sanger sequencing. Samples identified as heterozygous for the c.754G > A/p.A252T mutation by the qPCR genotyping assay were then confirmed by Sanger sequencing of C5 exon 7 (Fig. 1b).

Results

The C5 p.A252T mutation is prevalent in, but not restricted to, sub-Saharan Africa

Screening for the C5 c.754G > A/p.A252T mutation was carried out in 2710 samples from different populations worldwide. Initially, to provide a general view of p.A252T distribution, we grouped the samples into seven continental regions and calculated the frequencies of the mutated allele in each one (Table 1). Results for individual populations are shown in the Supporting information, Table S1. In total, 11 samples heterozygous for the p.A252T mutation were found, resulting in an overall mutated allele frequency of 0·2% in our samples. However, the distribution of the mutation was not random: sub-Saharan Africa populations

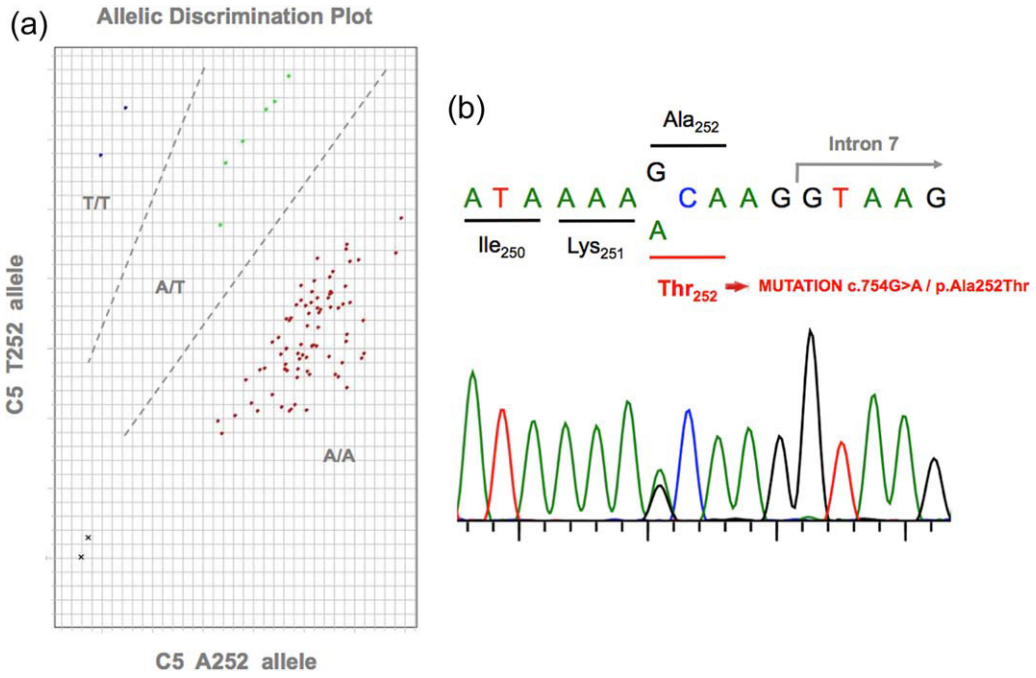

Fig. 1. C5 p.A252T mutation screening. (a) Cluster plot of 96 DNA samples that underwent C5 p.A252T mutation genotyping by TaqMan assay using the Applied Biosystems 7500 real-time polymerase chain reaction (RT–PCR) system. Red indicates wild-type homozygous samples (A252). Green indicates heterozygous samples. Blue indicates mutated homozygous samples (T252). Black indicates the negative controls. Two controls for each genotype were included in all plates. (b) Sanger sequencing of one heterozygous carrier of the C5 p.A252T mutation. All heterozygous samples detected by quantitative PCR were sequenced to confirm the results.

showed the highest number of mutated alleles, with nine heterozygous samples among the 958 tested (allele frequency 0·94%). These included seven heterozygous samples among 352 individuals tested in Gabon (allele frequency 1%), one of 25 in Nigeria (allele frequency 2%) and one of eight in South Africa (allele frequency 6·25%) (Table 2). In this last case, and to lesser extent in Nigeria, the allele frequency was probably over-estimated because of the small number of samples tested.

Interestingly, we found two heterozygous samples from other continental regions: one from Israel (Middle East and North Africa region) and one from Pakistan (Central and South Asia region). The mutation was absent in the remaining continental regions studied.

Table 1. Allelic frequencies of the C5 c.754G > A/p.A252T mutation

Continental region	Alleles tested	Mutated alleles	Mutated allele frequency (%)
Sub-Saharan Africa	958	9	0·94
America	216	0	0
East Asia	502	0	0
Central and South Asia	400	1*	0·25
Middle East and North Africa	1892	1†	0·05
Oceania	78	0	0
Europe	1374	0	0

All mutated alleles detected correspond to heterozygous individuals. *Individual from Pakistan; †individual from Israel (Negev).

Overall analysis of the C5 p.A252T mutation worldwide distribution

The C5 c.754G > A/p.A252T allelic variation is reported in the SNP database (dbSNP) (code rs112959008), and has an overall minor allele frequency (MAF) of 0·0012, as reported in the 1000 Genomes Project (http://www.1000genomes.org) [26]. However, as our results indicate, there are huge differences in this frequency depending on the continental region. Within the 1000 Genomes Project (1000G), the mutated allele was reported in individuals from Barbados (of African origin), Nigeria and Kenya (Table 3), with an overall estimated heterozygosity of 1–2% in these Central African countries. This data is concordant with our reported heterozygosity in Gabon (1%). The p.A252T mutation was not found in any of the other populations examined in 1000G, which unfortunately does not include samples from South Africa or countries in the Middle East/North Africa.

Further evidence of the higher prevalence of the p.A252T mutation in Africa relies upon a report from the National Heart, Lung and Blood Institute (NHLBI) Exome Sequencing Project (ESP) (http://evs.gs.washington.edu/

Table 2. Allelic frequencies of c.754G > A/p.A252T in sub-Saharan African countries

Country	Alleles tested	Mutated alleles	Mutated allele frequency (%)
South Africa	16	1	6·25
Kenya	24	0	0
Namibia	14	0	0
Central African Republic	72	0	0
Nigeria	50	1	2
Democratic Republic of Congo	30	0	0
Gabon	704	7	1
Senegal	48	0	0

EVS/). In the ESP, the mutated allele was identified in in 15 of 2090 samples from African American individuals (allele frequency, 0·36%) (Table 3). In contrast, it was not found in any of the 4044 samples tested from Americans of European ancestry.

Recently, Owen *et al.* investigated the incidence of the mutated allele in 1500 samples from South African neonates. The allele was present in both black Africans and Cape Coloured individuals, with allelic frequencies of 3 and 0.66%, respectively [21]. The populations in which the C5 p.A252T mutation has been detected, together with their heterozygosity frequencies, are summarized in Table 3.

Discussion

The MAC has a major role in defending the host from *Neisseria* infection, but it cannot form if any of the five final complement components are absent. Consequently, C5 deficiency (C5D, OMIM #609536) is associated with recurrent episodes of infection by *Neisseria* species, particularly meningitis and extragenital gonorrhoea. To date, studies to elucidate the molecular basis of hereditary C5D have included more than 30 families, with descriptions of 17 different mutations [22] distributed randomly along the C5 gene, and including missense, non-sense, insertion–deletion and splicing mutations. Most C5 mutations (13 of the 17) have been described in only one family [22]. The most relevant exception to this general observation is the p.A252T mutation, reported by Owen *et al.* [21].

The results of our study, together with data recorded in public databases, demonstrate the high prevalence of p.A252T in sub-Saharan Africa, and show for the first time the presence of this mutation in regions outside Africa (Middle East and South Asia).

Although it is difficult to establish the true frequency of the mutation, our results may be helpful for outlining an estimate. As shown in Table 3, various unrelated data have proved the presence of the A252T variant in heterozygosity in healthy individuals from several parts of Africa, and our results broaden the distribution of this mutation to other regions, specifically Israel and Pakistan.

However, when interpreting the frequency of p.A252T, several factors must be considered, particularly the size and origin of the population studied. The specific populations included in the 1000G and HGDP used in this study are well defined, but relatively small in size (around 100 individuals or less). To increase the representation from Africa, we collected 352 samples from Gabon (Central Africa) and 768 from North African countries (Morocco, Algeria, Tunisia and Libya). Other relevant data were provided by the 2090 African American individuals within the NHLBI Exome Sequencing Project (although their specific origin is not reported) and the 1500 individuals from South Africa described by Owen *et al.* [21]. Based on the data from the present study and the above-mentioned available

Table 3. Review of the reported populations with presence of C5 p.A252T mutation

Population	Heterozygosity frequency (%)	Count (total)	Source
Africa			
Barbados (African Caribbean)	2·1	2 (96)	1000G
Nigeria (Esan)	2	2 (98)	1000G
Kenya (Luhya in Webuye)	1	1 (99)	1000G
Nigeria (Yoruba in Ibadan)	0·93	1 (108)	1000G
African American	0·72	15 (2090)	NHLBI Exome Sequencing Project
South Africa (black Africans)	6	45 (750)	Owen *et al.*
South Africa (Cape Coloured)	1·33	10 (750)	Owen *et al.*
South Africa (Bantu S.U. Zulu)	12·5	1 (8)	This study
Nigeria (Yoruba)	4	1 (25)	This study
Gabon	1·99	7 (352)	This study
South Asia			
Pakistan	0·5	1 (200)	This study
Middle East			
Israel (Negev)	2	1 (49)	This study

1000G = 1000 Genomes Project; NHLBI = National Heart, Lung and Blood Institute.

information, we estimated the heterozygosity frequency of the C5 p.A252T mutation in sub-Saharan Africa to be 0·5–2%. The true accuracy of this frequency and whether it substantially varies between the different populations in the region are questions that remain to be elucidated in studies with larger sample sizes. What seems clear, however, is that the high prevalence of this mutation in Africa is restricted to the sub-Saharan region, as no carriers were found among the 768 samples from North African countries. Nevertheless, we cannot exclude the possibility that the mutation is present at a low frequency in North Africa.

Based on these data, it seems likely that the C5 p.A252T mutation could have a relevant role in meningococcal disease susceptibility in sub-Saharan Africa. In all individuals homozygous for p.A252T, C5 protein levels are $< 4\%$ of the mean control level measured by enzyme-linked immunosorbent assay (ELISA) and are undetectable by Western blot [21], providing proof of the functional importance of this mutation. The amino acid A252 is highly conserved among the species (Supporting information, Fig. S1), and estimation of the putative pathogenicity of A252T using two widely used prediction software packages (SIFT and PolyPhen-2) indicated that pathogenicity was probable. Therefore, p.A252T homozygosity would lead to *de-facto* C5 deficiency (with extremely low serum C5 protein levels) and a high susceptibility to meningococcal disease.

Why this mutation has survived evolutionary pressure is a question still to be answered. It is reported that individuals with deficiencies of late complement components have a lower incidence of septic shock after *Neisseria meningitides* infection, which results in lower mortality from this disease [7]. Following this rationale, it is possible that carriers of the mutation in the meningitis belt countries of sub-Saharan Africa would have some protection from septic shock during infection by this pathogen, thus increasing their chances of survival. Nonetheless, meningococcal disease is often associated with serious sequelae, such as a loss of digits or limbs, or persistent neurological damage. Hence, emphasis should be placed upon prevention of the disease through vaccination.

Based on the lower range of the estimated frequency of C5 p.A252T heterozygosity in this study (0·5%) and considering a population of 1 billion individuals living in sub-Saharan Africa (source: http://www.worldbank.org), a few million heterozygous and thousands of homozygous individuals with C5 deficiency would be living in this area. These estimated values should be confirmed in further studies including a significantly larger sample size from countries within the meningitis belt. It would also be interesting to evaluate the prevalence of p.A252T in meningococcal disease patients in sub-Saharan African countries, which could be carried out with the simple, low-cost method used in the present study. Moreover, if the prevalence of the mutation is as high as suspected, a routine test could be used to detect it and facilitate a prompt diagnosis of complement deficiency [27].

Many countries outside Africa have been a common destination for African immigrants over the years; consequently, the mutation can be spread to other countries. An example of this occurred in the present study. We collected 527 local samples, and after rigorous selection to ensure their European origin, one of them was excluded from the study because it came from an individual from The Gambia. The sample was tested and was found to carry the mutated allele in heterozygosis.

In summary, the C5 p.A252T mutation is indeed prevalent in sub-Saharan Africa. Testing for this mutation in meningococcal disease patients would be of value to identify C5-deficient individuals. A further step would be newborn screening, which could enable more effective prevention of the primary disease through vaccination.

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Disclosure

None of the authors has any potential financial conflict of interest related to this paper.

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Supporting information

Additional Supporting information may be found in the online version of this article at the publisher's web-site:

Table S1. Allelic frequencies of C5 c.754G > A/p.A252T mutation

Fig. S1. Phylogenetic comparison of human C5 protein sequence. C5 multiple sequence alignment focusing on A252 is shown. Amino acid sequences from different organisms have been obtained from the UniProt database (http://www.uniprot.org).

OBJECTIVE 4. TO ASSESS THE PERFORMANCE OF AN IMPROVED DIAGNOSTIC ALGORITHM TO DETECT COMPLEMENT DEFICIENCIES IN PATIENTS WITH RECURRENT BACTERIAL INFECTIONS.

The role of the complement system in the protection against infections caused by encapsulated microorganisms is now better understood among clinicians due to the increasing numbers of complement deficiency cases reported. In the light of the results obtained from the different studies in this project, we present a diagram in order to highlight the warning signs that should alert of a complement primary immunodeficiency in cases of bacterial infections **(figure 6)**.

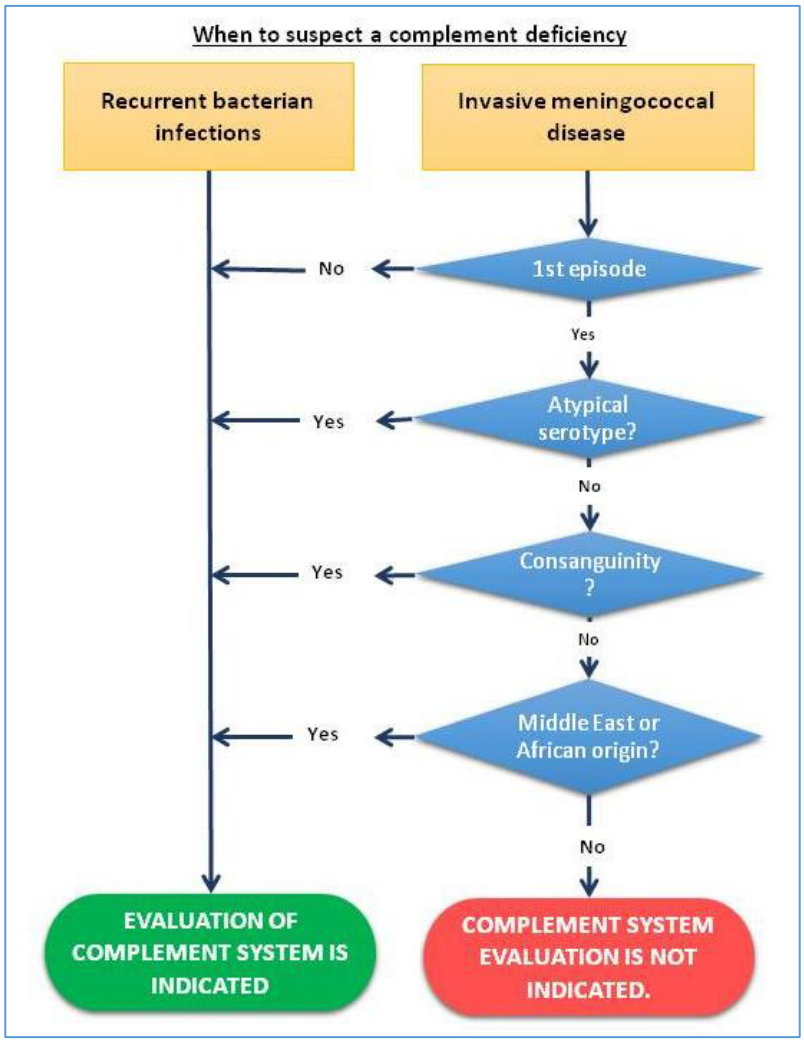

Figure 6 Evaluation of complement deficiency should be done if: i) first invasive meningococcal disease occurs in a patient who refers consanguinity; ii) an uncommon meningococcal serogroup is isolated; iii) in patients with family origin from Middle East or African countries

The increased number of new diagnostic tests added to the Immunology Department catalogue could lead to an indiscriminate demand immediately after the first suspicion of complement deficiency is raised. To avoid such shot-gun approaches we have designed an algorithm aiming to facilitate a step-wise complement analysis **(figure 7)**. The diagnostic approach starts by screening the function of each activation pathway and proceeds with the characterization of the defect at the protein and molecular levels). Functional tests, either by ELISA or by hemolytic/liposome-based assays, provide information about the integrity of the entire complement cascade, since their read-outs imply the formation of membrane attack complex (detection of C5b9 neo-antigen in ELISAs and pore formation in hemolytic/liposome-based assays). As we have previously mentioned, temperature is the most limiting pre-analytical factor that has to be cautiously observed for a correct analysis of complement system functions. Therefore, we strongly recommend to obtain serum samples immediately after blood extraction and store them in as many aliquots as possible at -80ºC (-20ºC can be sufficient if tests are performed within one month). In the case of complement deficiencies, good laboratory practices such as avoiding freeze/thaw cycles become of real importance. Although not depicted in figure 7, it is important to repeat functional tests with new samples to confirm that abnormal results are not a consequence of incorrect pre-analytical conditions or due to an excessive consumption secondary to the infectious process. This last observation can also be ruled out by studying the soluble form of C5b9 complex (sC5b9). Elevated levels will indicate an increased activation of the complement system, whereas defects on terminal pathway components will lead to an absence of sC5b9.

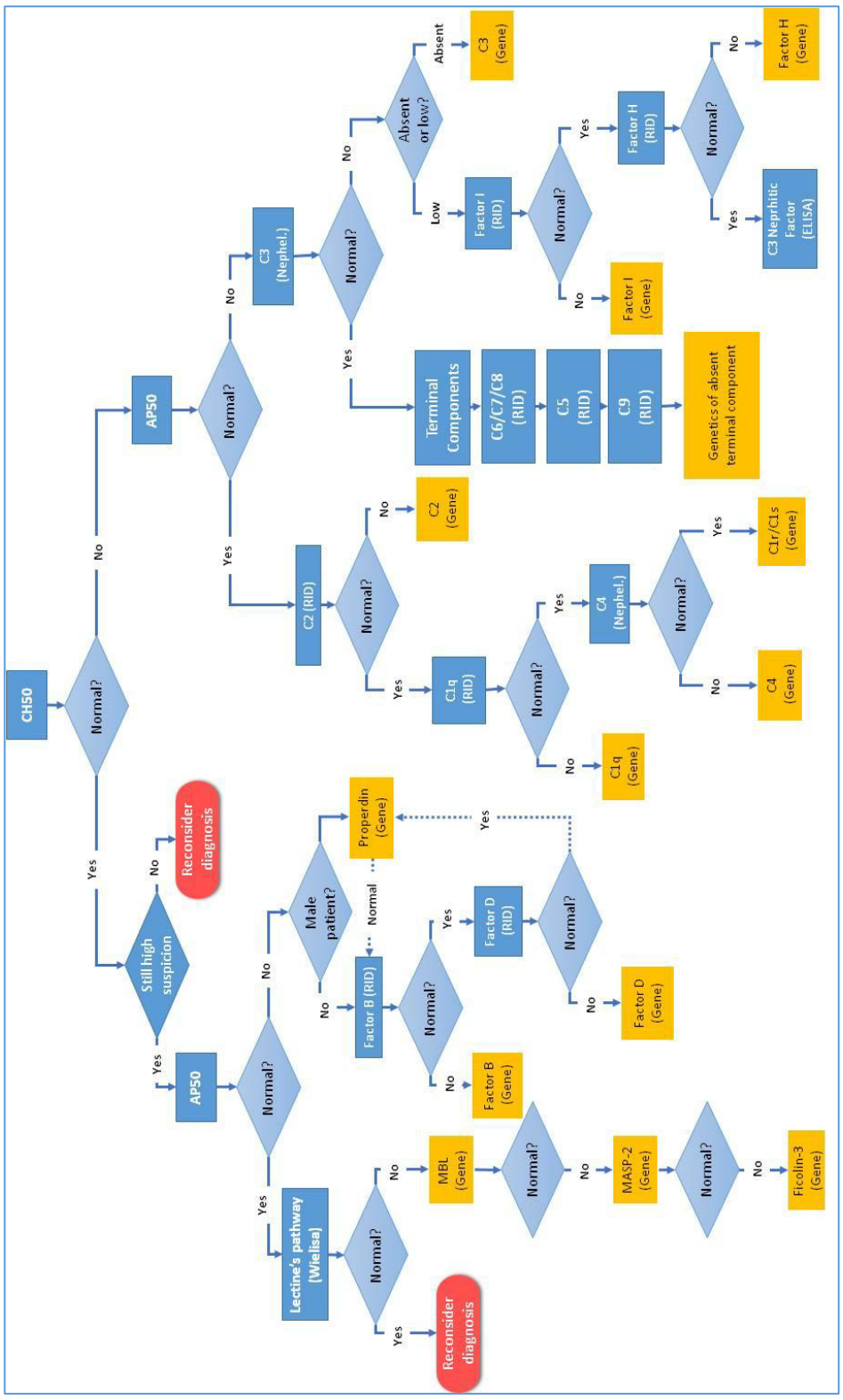

Figure 7 Algorithm for laboratory techniques on complement evaluation. Affected molecules have been ordered following available data on frequencies of registered cases. All functional tests with abnormal results must be repeated with a new sample, to avoid false negative results due to pre-analytical errors. Radial Immunodifusion (RID) should be performed –when available- before genetic testing. Genetic testing to rule out functional defects –not affecting protein structure- is also indicated under strong suspicion of complement deficiency due to recurrent infections.

4 DISCUSSION

The complement system has not been a popular research topic among immunologists, maybe because of its complicated nomenclature or because of the mere complementary activity originally assigned to it. Following the same rationale, few industries focused their commercial strategies on the design of commercial kits for complement system analysis. Therefore, clinical laboratories willing to perform advanced studies on complement had to design their own time-consuming and tedious in-house assays. Until now, in Catalonia there were no specialized clinical immunology laboratories where advanced complement studies –beyond CH50, C3 and C4- could be performed. Whenever a patient raised a suspicion of complement deficiency, samples had to be sent to a reference laboratory from a different region. As previously mentioned, pre-analytical conditions have to be very strict regarding time and temperature to avoid intrinsic complement consumption. Therefore, sample's shipment was always a source of variation and error. Increased awareness on complement system and its defects during the last decade prompted the industry to develop more commercial kits that could be easily implemented on the daily routine of clinical laboratories. However, in order to add new tests to the laboratory catalogue from a public health institution, their use must be demonstrated to be clinically relevant. The results derived from this project, together with an increased demand from clinicians, allowed the authorization and implementation of new techniques for the complete study of complement system at the Immunology department of the Hospital Universitari Vall d'Hebron. Therefore, clinicians from our hospital, but also from other related centres in our region, can now rule out complement deficiencies in a rapid and easy way.

It is important to remark that despite the availability of commercial kits, not every laboratory should perform such diagnosis. Specialized immunologists should apply their knowledge and experience for a correct interpretation of the results. Capillary electrophoresis is one of those techniques that generate controversy regarding which department should it belong to. Despite the fact that this technique is part of the clinical pathology or clinical chemistry departments in many laboratories, the interpretation and acceptance of results at our clinical laboratory is done by immunologists. In my opinion, a wider experience in immunology gives an extra value

to capillary electrophoresis results in immunology disorders, other than monoclonal gammopathies. It has been already more than 20 years since capillary electrophoretic techniques substituted previous protein separation techniques, like paper chromatography or agarose electrophoresis. Capillary electrophoresis resolution permits a better discrimination of the serum proteins' mobility zones. The best example is that it allows the differentiation of two segments at the beta zone (beta-1 and beta-2). The most abundant protein in beta-2 fraction is C3, and therefore a low percentage of this fraction can alert of low serum levels of C3. After our first identification of C3 deficiency through capillary electrophoresis, special attention was paid to all the electrophoresis performed. Two other samples from different cases also revealed an absence of C3; one of the patients presented C3 nephritic factor and her C3 deficiency is now under investigation. However, it is noteworthy that C3 is naturally degraded in serum within two or three days after blood drawing. Therefore, the beta-2 zone can only alert of C3 deficiencies when capillary electrophoresis is done with fresh serum.

Serum capillary electrophoresis also allowed us to diagnose of Factor I deficiency a 4-year old patient, before she had suffered any severe infection. A systematic review of the Factor I deficiency cases reported so far, indicated that 5 out of 7 patients diagnosed at ages older than 30 years referred an episode of severe invasive meningococcal disease. Median age of meningococcal disease in Factor I deficient patients is of 13.5 years. This result is consistent with the information regarding carriage rates in Europe and North America, which are low during the first years of life, but increase dramatically during the adolescent age. Although identifying index patients before any meningococcal disease has occurred is difficult, what is important is to vaccinate the patient directly after the diagnosis, in order to prevent further infections. Another relevant point to be discussed is the importance of performing familial studies after the diagnosis of index cases. Due to the autosomal recessive pattern of the disease, siblings from an index patient that has inherited the bi-allelic mutations from his parents have 25% probability of being homozygous for the mutated allele. But, should asymptomatic familial cases follow the same therapeutic strategies as the index patients? Data obtained from reported Factor I deficiency cases

show that the only individual presenting meningococcal disease after the diagnosis was derived from a familial study. Since this individual did not present any clinical symptoms, she was left unvaccinated until two years after the diagnosis, when the patient presented with meningococcal disease. Therefore, we strongly recommend familial asymptomatic cases always to be vaccinated against the most typical encapsulated bacteria and meningococcal B serotype (MenB). In three of the six families we studied, one more sibling was diagnosed of complement deficiency apart from the index patient. More importantly, the diagnosis in two of them was done within the first two months of age, and meningococcal serogroup B vaccine was then added to their vaccines calendar.

Deficiency in Factor I highlight the importance of maintaining the complement system tightly regulated. The fact that the 2-month old patient with a functional Factor I defect still presented normal C3 levels, suggests that the alternative pathway amplification loop is still not fully implemented at that age. In our opinion, effects derived from the absence of functional Factor I do not acquire some relevance until patient's exposure to pathogens increases. However, this is only a hypothesis. In order to shed some light onto this aspect, patient's complement components will be closely monitored during the following years. Some of the patients described with Factor I deficiency present the disease due to compound heterozygosity, and this fact increases the number of mutations reported in comparison with the number of cases described. From the patients with Factor I deficiency reported in this project, one carried a mutation previously described on a compound heterozygous case. However, the other two mutations were reported for the first time. This implies that the mutations have to be molecularly characterized in order to establish the reason of its pathogenicity. Despite prediction software provides very useful information, it is highly recommendable to collaborate with specialized bioinformaticians in order to provide a better description of the effect that the mutation exerts on protein structure and function. Through the study of Factor I deficiencies we observed that not all the mutations were named taking different sequences as reference sequence. Some of the authors did not include the signal peptide on the amino acid count. However, this is a mistake, because the first residue should always be the one codified by the first ATG

codon. The differences among both designations lead in case of Factor I to an incorrect designation of the serine-protease catalytic residues in some of the publications. For that reason, we unified the amino acid designation in all Factor I published cases, and will report them together in our publication. Hopefully, this will avoid mistakes on the designation of key structural and functional amino acids and misinterpretation of mutation effects. The use of different nomenclatures adds difficulty in identifying previously reported mutations.

Reporting newly described mutations is necessary and beneficial for future diagnosis. Sometimes, one same mutation can be highly prevalent in a specific community, thus revealing a founder effect on the area. When this happens, molecular diagnosis can be firstly orientated to detect the most frequent mutation if the patient is from the described area. As an example, in Japan, C9 deficiency has an incidence of 1/1000 individuals, and the prevalence of carriers with C9 R95X mutation is about 6.7% (1/15). Therefore, if an individual from Japan or with Japanese origins is diagnosed of C9 deficiency, we recommend starting genetic studies by looking for R95X mutation. Following this rationale, the identification of the same non-previously reported mutation in two families from the same geographical localization (Alnif, Morocco), prompted us to study whether this mutation was more frequent in that geographical area. As stated in the publication, we did not restrict our search to Berber population, since there are no clear genetic differences between Berbers and Arabs. Therefore, samples from all North-Africa and Middle-East continental regions were used to study the presence of Asn320del C5 mutation. From our results it can be suggested that this mutation may exist at low frequencies in the North-African population. However, a larger cohort of individuals should be studied in order to establish heterozygous frequencies and to estimate possible homozygosity cases.

We have also observed that the mutation A252T in C5 gene is highly prevalent on Central African countries, but it is not restricted to that region. As has been mentioned in the introduction, patients with complement defects on the terminal pathway have a higher risk of suffering from meningococcal infections, but also have a better outcome regarding septic shock, due to the lower amount of endotoxin released and the

absence of anaphylatoxin C5a. Considering that the region where this mutation seems to be more prevalent is where the largest epidemics of meningococcal infections occur, one could understand that carrying the mutation in homozygosis, despite the higher risk of recurrence for the infection, conferred the patients with a protection against a fatal outcome, therefore allowing the mutation to be maintained through evolutionary pressure. However, a real incidence of the C5 mutation among patients with meningococcal disease in the area cannot be estimated from our results due to the small size of samples from each country. For that purpose, further studies should be done increasing the sample size and focusing on the population from countries at the Meningitis Belt area. Although the primer sequences we designed provide a tool that could help making this expanded study possible, lowering costs and complexity, the main obstacle would be obtaining the samples from these developing countries. If such expanded study confirmed the mutation's high prevalence, it would be a good idea to implement this genetic study as a routine test on these countries (whether on already affected patients or as a neonatal screening would depend on the results of the expanded study). Nowadays that important vaccination campaigns are being implemented in these countries, with the high economic cost that this implies, knowing if the A252T C5 mutation is one of the main causes of meningococcal disease on the area, could allow a more accurate vaccination approach.

Anyhow, all these studies also served to address the question of the importance of evaluating the complement system as a screening method on patients suffering from a single episode of invasive meningococcal disease. At a first glance one would say from the results of our retrospective study (unpublished data) that a single episode of invasive meningococcal disease is not indicative of a complement deficiency, since only 2.5% (n=80) of the cases studied resulted from a defect of the complement system. However, results from this study coincide with previous reports indicating that complement system should be studied when the infection is due to an uncommon serotype or if consanguinity is described. From the results obtained, we would add to these considerations that the ethnical background should be also considered as a key point for suspecting a complement deficiency following an episode of invasive meningococcal disease. The fact that two of the three Moroccan families diagnosed

with C5 deficiency carry the same mutation (Asn320del), together with the fact that the A252T C5 mutation is not confined to sub-Saharan African countries, reveal that individuals carrying the mutation have followed similar migratory fluxes. Taking into account that our country is an important migration destiny, a complement deficiency should be suspected in any case of meningococcal disease in patients with family origins from regions where the incidence of the mutation is higher.

The last objective of this project was to design an algorithm that would help both clinicians and immunologists in the diagnostic approach of this type of complement deficiencies. We think that laboratory tests should follow some rationale and that shotgun approaches should be avoided. Therefore, the designed algorithm is intended as a guide through the sequential selection of the most adequate laboratory technique in each case. Reduction in the cost of genetic panels may lead to a change in the way that genetic testing is done in clinical laboratories, and the use of radial immunodiffusion to study individual components is debatable. In my opinion, high-throughput genetic studies should complement but not substitute protein studies. Genetic results are sometimes difficult to interpret so information regarding serum levels of the suspected protein can be helpful to detect partial defects. Maybe the algorithm will be modified in the future, placing genetic studies after functional assays, and leaving specific components protein tests to be performed only once the candidate gene has been identified. Like all algorithms, this one will be subjected to constant revisions and modifications to keep it updated.

To give an answer to the global hypothesis of this project (i.e. complement deficiencies are more common than what is reported) we first had to know the exact number of cases registered around the world. This would be an easy task if we had demographic registries of complement deficiencies in each hospital, so that data could be related to total number of individuals attending that hospital. However, at the moment these types of registries are only applied for very few diseases, often with a higher incidence. We inquired the International Complement Society, regarding the existence of a registry for primary complement deficiencies but as we obtained no reply, we had to limit our observations to data obtained from European Society of Immunodeficiency Diseases (ESID) registry. It is important to notice that ESID registry is

not a demographic registry, and therefore it depends on clinicians' will of reporting their cases. The latest information published on the ESID registry was that of 2014 (with reference in the introduction of this thesis). However, thanks to the help of Dr. Kindle, from the ESID registry, we could obtain more updated information.

Table 7 Complement deficiency cases included in ESID registry (date: 16.03.2017)

Deficiency	***Registered cases***	***Deficiency***	***Registered cases***
C1 deficiency	4	C9 deficiency	1
C2 deficiency	64	FH deficiency	2
C3 deficiency	2	FD deficiency	2
C4 deficiency	8	FI deficiency	17
C5 deficiency	8	MBL deficiency	18
C6 deficiency	7	Properdin deficiency	6
C7 deficiency	10	Unclassified	92
C8 deficiency	17	C1inhibitor deficiency	543
		TOTAL	***801***

There are so far 801 properly registered cases of complement deficiencies. Excluding patients with hereditary angioedema due to mutations on *SERPING1*, which do not course with increased susceptibility to infections, data from the ESID registry reflect the frequencies reported in the literature. The most frequent deficiency of complement system components is C2 deficiency (n=64), followed by clinically-associated MBL deficiency (n=18), complement component 8 (n=17) and Factor I deficiencies (n=17). Unclassified complement deficiency cases are those in which, after functional defective results, a complement deficiency has been suspected despite the exact missing molecule has not been found yet. Surprisingly, the total number of complement deficiency cases in 2017 (801) is smaller than the number of cases reported on the registry summary in 2014, which was 946. The explanation for this is that a new registry was implemented after 2014, and the process of transferring all the data from the previous one is still on course. The data we were given reflect the number of *verified patients* as for 16th March, 2017. However, there are near 200 more cases that have not yet been transferred from the previous registry system. That would make a total number of around 1000 cases. Nevertheless, these data indicate that not all the reported cases are registered: from the work presented in this thesis we already know of at least 10 more cases that should be registered. From my point of

view, it is crucial to either create a common international registry or find an easy way to gather all the information from the different registries. I understand that creating and keeping updated these international networks are expensive and time-consuming tasks. However, only by collecting data from all the diagnosed cases will we have the sufficient numbers to know the real situation of complement deficiencies. Moreover, studies involving rare diseases require collaborations within different centres, in order to increase sample sizes and give sufficient relevance to the results. Despite publications can provide appropriate contacts, I think that registries can render better results because not all the immunodeficiency cases can be published, and because writing a paper requires more time than registering a patient in a database.

As an example, registering the new cases identified in this project on the ESID registry allowed Prof. Lennart Hämmarstrom, in Sweden, to ask for our collaboration. His new project is focused in developing new techniques for the neonatal screening of complement defects, and samples from our registered cases will be used as positive controls to validate these techniques.

The identification of three families with complement C5 deficiency has allowed us, in collaboration with Immunology Department at Complutense University in Madrid, to establish T-cell(HTLV-1) and B-cell(EBV) lines from the patients and their relatives. These cell lines are going to be used to study the recently described C5's role on the maintenance and regulation of Th1 response. This work will be done in collaboration with the group of Innate Immunity from the Universidad de Murcia, nowadays one of the most relevant groups in the field in our country. As was mentioned in the introduction, identifying the molecular defect underlying already defined clinical conditions, not only permits a more accurate diagnosis and familiar counselling; but also a better understanding on the complex immune system network.

5 CONCLUSIONS

Conclusions to this work have been grouped according to the objectives proposed.

Objective 1 *To expand and improve complement system tests in use in Vall d'Hebron clinical immunology laboratory.*

- This project has allowed the internalization of 10 new diagnostic tests for the evaluation of the complement system. Due to this internalization, the Immunology Department of Hospital Universitario Vall d'Hebron has been positioned as a reference centre for this type of studies in Catalonia.
- Capillary electrophoresis makes the detection of both primary and secondary C3 defects feasible when the beta-2 fraction is reduced, but only when fresh serum is used.

Objective 2 *To apply improved functional and molecular tests to patients with suspected deficiencies in Factor I and C5 complement components to reach a complete molecular and functional diagnosis.*

- We have identified two cases of complete Factor I deficiency in adult patients from Spanish origin (53 and 60 years) who had suffered invasive meningococcal disease at young adulthood, leaving one of them with severe neurological sequelae and memory loss. Capillary electrophoresis alerted of a new Factor I deficiency case in a 4-year old patient who had never suffered from meningococcal invasive disease. Vaccination and prophylactic antibiotic therapy have been indicated to avoid future infections.
- We have reported two new mutations (c.1450_1454delCTTCA/p.L484Vfs*3 and c.1139A>G/p.H830R) in *CFI* gene that cause a complete defect on Factor I when found in homozygosis. The deletion of 5 nucleotides in c.1450_1454delCTTCA/p.L484Vfs*3 causes a truncated Factor I protein of only 485 amino acids. The mutation c.1139A>G/p.H830R directly affects the functionality of the peptidase activity in factor I, since H380 is part of the catalytic triad.

- It is the first time that the mutation c.485G>A/p.Gly162Asp is found in homozygosis, although it had been found as part of a compound heterozygosis genotype in a case of complete Factor I deficiency and in heterozygosis associated to thrombotic microangiopathy. Molecular characterization of c.485G>A/p.Gly162Asp reveals that the substitution of a Glycine for an Aspartic Acid may prove very damaging for the protein structure.

- The high number of paediatric patients reported during the last decade with Factor I deficiency show that clinical awareness towards complement deficiencies is increasing.

- We have identified two new mutations (c.2607-2608del/p.Ser6870ProfsX3 and c.960-962del/p.Asn320del) in C5 gene which in homozygosis lead to complete C5 deficiency. The mutation c.2607-2608del/p.Ser6870ProfsX3 leads to a truncated protein of 872aa. The impact of c.960-962del/p.Asn320del mutation indicates that loss of Asn320 induces an unstable local unfolding in the vicinity of this residue.

- The studies on probands' relatives lead to the early detection of one Factor I and one C5 deficiency cases in two neonatal siblings, indicating the importance of familiar screening.

Objective 3 *To investigate recurrent mutations causing C5 deficiency at the population level.*

- Although C5 deficiency has been diagnosed in many populations, we have reported for the first time the molecular defect in three North-African families.
- We have screened *C5* c.960-962del/p.Asn230del mutation in 768 samples from Middle-East and North-Africa region. The fact that we found one heterozygous allele in a sample from Algeria indicates that this mutation exist at low frequencies in North-African population.

- The screening of *C5* p.A252T mutation in 479 samples from Sub-Saharan Africa region concludes that estimated frequency of heterozygosity in that geographical region is 0.5%-2%. The identification of two heterozygous carriers in Israel and Pakistan among 2710 samples from different populations worldwide indicates that *C5* p.A252T is not restricted to Sub-Saharan Africa.

Objective 4 *To assess the performance of an improved diagnostic algorithm to detect complement deficiencies in patients with recurrent bacterial infections*.

- We have proposed a diagnostic algorithm to evaluate the complement system in patients presenting invasive meningococcal disease. We propose to expand the situations where the complement system has to be evaluated to the following: i) presence of consanguinity ii) isolation of an uncommon serotype and iii) African familiar origins.

- We have reported nine complement deficiency cases in less than three years, indicating that complement deficiencies might be more common than what can be expected from the registered cases.

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