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STRUCTURAL AND FUNCTIONAL ANALYSIS OF COMPLEMENT FACTOR H: A CRUCIAL PROTEIN IN SEVERAL DISORDERS

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Sommario

Il Fattore H del complemento (FH) è un importante regolatore della via alternativa del complemento: protegge infatti le cellule dell'ospite dall'attacco del sistema del complemento e carenze di FH sia qualitative e quantitative dovute a mutazioni nel gene CFH sono spesso associate ad una serie di malattie umane, come la glomerulonefrite membranoproliferativa (MPGN), la sindrome emolitico-uremica atipica (aHUS) e la degenerazione maculare della retina legata all'età (AMD). Mentre esiste una caratterizzazione genetica per tutte queste malattie, i dati funzionali a livello di proteine sono spesso carenti.

Inoltre, il FH gioca un ruolo significativo nelle malattie infettive: molti agenti patogeni sono infatti in grado di reclutare il FH sulla loro superficie sfruttandolo per proteggersi dagli attacchi del complemento. Mentre per alcuni agenti patogeni l'interazione con il FH è stata ben descritta, per gli altri gli "interattori" diretti sono ancora sconosciuti.

Tuttavia, lo studio del FH è complicato dalla presenza di proteine FH-related (FHRs) che posseggono un elevato grado di somiglianza con il FH e ne rendono quindi difficile la purificazione e la analisi diretta.

Il primo obiettivo di questo progetto è stato lo sviluppo di saggi quantitativi e funzionali FH-specifici, utilizzando un anticorpo monoclonale (Mab 5H5) prodotto nel nostro laboratorio, che si è dimostrato essere specifico per FH. Il secondo era la produzione del FH intero e di tre frammenti, contenenti diverse porzioni della proteina, sia in un sistema di espressione eucariotico che procariotico.

L'anticorpo 5H5 è stato prodotto in grandi quantità utilizzando un mini-fermentatore ed è stato utilizzato per purificare il FH mediante cromatografia di affinità. La purezza e l'attività funzionale del FH purificato sono state valutate e confrontate con gli altri metodi di purificazione (ad es. affinità su Eparina, HPLC, HIC).

È stato inoltre sviluppato un test ELISA per la corretta quantificazione del FH da fluidi biologici, utilizzando il 5H5 insieme ad un anticorpo policlonale anti-FH, anch'esso prodotto nel nostro laboratorio, e al FH purificato per affinità. Questo test di quantificazione è risultato affidabile ed è stato utilizzato per valutare diversi campioni di pazienti aHUS e AMD.

Inoltre, sono stati sviluppati anche due micro-metodi di purificazione del FH. Questi metodi consentono di purificare una buona quantità di FH altamente purificato partendo da una piccola quantità di campione e sono quindi uno strumento utile per studiare il FH specialmente in quelle patologie, quali aHUS, che colpiscono soprattutto i bambini.

Infine, sono stati sviluppati due saggi funzionali per valutare sia l'attività cofattoriale N-terminale che l'attività protettiva C-terminale del FH. Il FH è una grossa glicoproteina solubile che possiede funzioni diverse in differenti parti della molecola. Quindi diversi test sono necessari per valutare la completa funzionalità del FH.

Il primo test, il "cofactor assay", basato sul fatto che il FH possiede attività cofattoriale per il taglio del C3b mediato dal FI, è completamente funzionante ed è già stato utilizzato per lo screening di diversi campioni.
Il secondo, il test di emolisi, si basa sul fatto che il FH è in grado di proteggere gli eritrociti di pecora dalla emolisi mediata dal complemento. In questo test il FH purificato dai pazienti verrà utilizzato insieme ad un siero di controllo depleto di FH, al fine di avere un saggio in cui l'unica variabile sia il FH. Questo test è stato già messo a punto utilizzando un siero di controllo al fine di verificare tutte le condizioni, ed è ora nella fase finale di sviluppo, dopo di che sarà utilizzabile su materiale dei pazienti.
I quattro cloni contenenti la sequenza completa del FH e i frammenti parziali (SCRs 1-7, SCRs 8-14, SCRs 15-20) sono stati prodotti e saranno utilizzati per esprimere FH sia in forma glicosilata che non glicosilata. Sarà quindi analizzata la capacità di queste proteine ricombinanti di interagire con le proteine dei patogeni o di ripristinare la funzione di un FH difettoso.

Abstract

Complement Factor H (FH) is a major regulator of the Alternative Pathway of Complement: it protects host cells from complement attack and qualitative and quantitative deficiencies of FH due to mutations in the CFH gene are frequently associated with a number of human diseases, such as membranoproliferative glomerulonephritis (MPGN), atypical haemolytic uraemic syndrome (aHUS) and age-related macular degeneration (AMD). A genetic characterisation is available for all these diseases, whereas functional data on protein levels are often missing.

Furthermore, FH plays a significant role in infectious diseases, as many pathogens are able to recruit FH on their surface, thus protecting themselves from complement attack. Whereas interaction of FH with certain pathogens has been well described, for others the direct "interactors" still remain unknown.

However, the study of FH is complicated by the presence of FH-related proteins (FHRs) that share a high degree of similarity rendering its purification and direct analysis challenging.

The first focus of this project was the development of quantitative and functional FH-specific assays, using a monoclonal antibody (MAb 5H5) produced in our laboratory, which has been shown to be specific for FH. The second aim was the production of the whole recombinant FH and of three fragments, containing different parts of the protein, in both a eukaryotic and a prokaryotic expression system.

MAb 5H5 was produced in high amounts using a mini-fermentator and used to purify FH by affinity chromatography. Purity and functional activity of this affinity-purified FH were assessed and compared with FH obtained by other purification methods (eg. Heparin affinity, HPLC, HIC).

An home made ELISA for the correct quantification of FH from biological fluids, making use of 5H5 together with a polyclonal anti-FH antibody, also produced in our lab, and affinity purified FH was also developed. This reliable quantification assay was used to screen samples from aHUS and AMD patients.

Moreover, two micro-methods to purify FH were also developed. These methods enable to purify a good quantity of highly purified FH from a small amount of sample and are therefore a useful tool to study FH especially in those pathologies, such as aHUS, which mainly affect children.

Finally, two functional assays to assess both the N-terminal cofactorial activity and C-terminal protective activity of FH have been set up. FH is a big soluble glycoprotein that bears different functions in different parts of the molecule. Therefore different tests are needed to assess full functionality of FH.

The first test, the "cofactor assay", based on the fact that FH possesses cofactor activity for the FI-mediated cleavage of C3b, is now fully implemented and has already been used to screen different samples.

The second one, the "haemolysys test" is based on the fact that FH is able to protect sheep erythrocytes from haemolysis mediated by complement. In this test FH purified from patients will be used together with a control FH-depleted serum, in order to set up an assay where the only variable is FH. This test has already been set up with a control serum in order to test all the conditions, and is now in its fine tuning phase, after which it will be applicable to patient's material.
The four clones containing the complete FH sequence and partial fragments (SCRs 1-7, SCRs 8-14, SCRs 15-20) have been produced and will be used to express FH in a glycosylated or unglycosylated form. These recombinant proteins will be screened for their ability to interact with proteins from pathogens or to restore the function of a defective FH.

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ABBREVIATIONS

aHUS	atypical Hemolitic Uremic Syndrome
AMD	Age-related Macular Degeneration
AP	Alternative Pathway
AP	Alkaline Phosphatase
BCIP	5-bromo-4-chloro-3'-indolyphosphate p-toluidine
C1-INH	C1 inhibitor
C4bp	C4 binding protein
CA	Cofactor activity
CD	Cluster of differentiation
CFH	Factor H gene
CFHRs	Factor H related proteins genes
C	Complement
$C_{"n"}$	Complement component (from 1 to 9)
Cn	Clusterin(SP-40,40, apolipoprotein J)
CP	Classical Pathway
CR1	Complement receptor 1
CR2	Complement receptor 2
CRP	C-reactive protein
DAA	Decay accelerating activity
DAF	Decay-accelerating factor
DDD	Dense deposit disease
ELISA	Enzyme-linked immunosorbent assay
E_{Sh}	Sheep erythrocyte
FB	Factor B
FD	Factor D
FH	Factor H
FHL-1	Factor H-like protein 1
FHRs	Factor H related proteins
FI	Factor I
GAG	Glycosaminoglycan
GlcNAc	N-acetyl-glucosamine
GalNAc	N-acetyl-galactosamine

GBM	Glomerular basement membrane
GPI	Glycosylphosphatidylinositol
HRP	Horseradish Peroxidase
iC3b	Inactive C3b
iC4b	Inactive C4b
Ig	Immunoglobulin
IP	Immunoprecipitation
LP	Lectin Pathway
LPS	Lipopolysaccharide
MAb	Monoclonal antibody
MAC	Membrane attack complex
MASPs	MBL-associated serine proteases
MBL	Mannose-binding lectin
MCP	Membrane cofactor protein
NBT	nitro-blue tetrazolium chloride
P	Properdin
PAb	Polyclonal antibody
RCA	Regulators of complement activation
RPE	Retinal pigment epithelium
SCR	Short consensus repeat domain
SDS-PAGE	Sodium dodecyl sulfate – polyacrylamide gel electrophoresis
SPR	Surface Plasmon resonance
TM	Thrombomodulin
TMB	Tetramethylbenzidine
Vn	Vitronectin (S-protein)
WB	Western Blot

INTRODUCTION

1. The complement system

The complement system is a central component of innate immunity and is one of the principal mechanisms of defence against infections.

Complement was first identified in the 1890s by Jules Bordet as a heat-labile principle in serum that "complemented" antibodies in the killing of bacteria [1, 2]. Now we know that it consists of more than 30 soluble plasma and membrane-associated proteins interacting with each other to activate and regulate the system [3].

The complement system has three main physiologic activities: defence against bacterial infection, bridging of innate and adaptive immunity, and disposal of immune complexes and the products of inflammatory injury [4].

The activation of complement consists in an enzymatic cascade in which complement proteins, normally present in serum as inactive enzyme precursors (zymogens), are activated sequentially. Each component recognises and activates, generally by proteolysis, the following one.

Complement can be activated by three different pathways: the classical (CP), the lectin (LP) and the alternative (AP) pathways, according to the nature of recognition [5]. All these pathways converge after the activation of the central component C3 and are followed by a common non-enzymatic process consisting on the assembly of a cytolytic complex named the "membrane attack complex" (MAC), which forms a hole in the membrane and initiates cell lysis [6, 7].

Control of these enzymatic cascades is essential to prevent rapid consumption of complement in vivo, therefore a group of plasma and membrane-bound proteins act to inhibit the system at multiple stages.

Some of the proteolytic complement protein fragments, such as C3b, are also able to opsonize microbes thus stimulating phagocytosis and B-cell

responses, whereas other fragments, named “anaphylatoxins”, can attract and activate macrophages and neutrophils to the site of infection [8–10]. Two other important roles of the complement system are the clearance of apoptotic and necrotic cells by promoting their phagocytosis and the clearance of immune complexes from circulation by solubilizing and transporting them to the reticuloendothelial system [11, 12].

1.1. Complement activation

Depending on the stimuli, complement activation can follow three different pathways (Fig.1):

- ***Classical Pathway*** is activated by surface-bound antibodies (IgM and certain isotypes of IgG)
- ***Alternative Pathway*** is constitutively active but can be enhanced by certain molecules (carbohydrates, lipids or proteins) bound to foreign surfaces.
- ***Lectin Pathway*** is stimulated by the mannose-binding lectin (MBL) bound to mannose residues on the pathogen surface

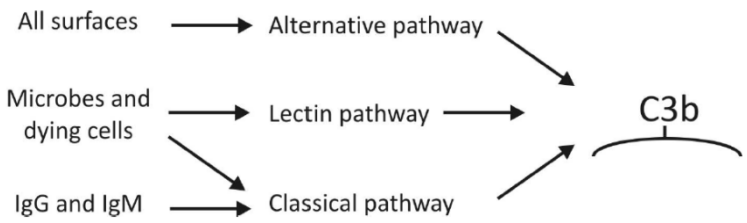


Fig. 1 Schematic representation of the activation of complement cascade.
After recognizing the target all the three pathways converge to covalent attachment of C3b on the surface.

The names *classical* and *alternative* arose because the classical pathway was discovered first but the alternative pathway is phylogenetically older.
All the pathways converge in the formation of an enzymatic complex called C3-convertase, composed of different parts in the various pathways but all with the same target, the molecule C3 which is hydrolysed into C3b and covalently attached to the target cell surface.

1.1.1. Classical Pathway

The Classical Pathway was the first one to be discovered, its components are designated from C1 through C9 in the order they were discovered but this does not quite correspond with the order of the reactions.

This pathway is initiated by the first component of complement system, **C1**, which is a macromolecular complex consisting of **C1q** and two molecules each of the proteases **C1r** and **C1s**, held together in a complex ($C1qr_2s_2$) stabilized by Ca^{2+}. C1q is composed of 18 polypeptide chains which form six monomers, composed of a collagen-like tail and a fibrinogen-like head domain, that associate in a "bouquet of tulips"-like configuration [13].

The pathway can be initiated in an Ab-dependent manner when C1q interacts with clusters of IgG_1 or IgG_3, or IgM on microbial surfaces or in immune complexes. The classical pathway can be initiated also in an Ab-independent manner occurring when C1q reacts directly with C-reactive protein (CRP) bound to apoptotic cells, or with polyanions (*eg*. heparin, protamine, DNA and RNA) displayed on their surface or when it interacts with lipopolysaccharide (LPS) on microbial surfaces [14–16].

Binding of C1q to its targets induces a conformational change in C1r that converts it into an active serine protease enzyme, which then cleaves C1s to a similar active enzyme [17]. Active C1s enzyme cleaves plasma C4 to C4a and C4b; the smaller fragment C4a has been shown to be an anaphylatoxin though considerably less active than are C3a and C5a [18, 19]. The bigger fragment C4b attaches to the target surface in the vicinity of C1, and acts as an acceptor for the next complement protein, C2. After the interaction with the cell surface–bound C4b, C2 is cleaved by C1s and generates two fragments: a smaller soluble fragment named C2b with unknown activity and a larger C2a fragment that remains physically associated with C4b on the cell surface, forming the **C4b2a** complex that is the **classical pathway C3 convertase**. (Note: the nomenclature of C2

fragments is different from the other complement proteins as for historical reasons the attached bigger fragment was called “a” and the released one “b”. An attempt to reverse these “a” and “b” designations was not universally accepted by experts in the complement field leading to confusion in textbooks and scientific literature) [20].

The **classical pathway C3 convertase** binds C3 thanks to the C4b component whereas the C2a component catalyses its proteolysis into C3a and C3b [21]. The small C3a fragment is an anaphylatoxin that stimulates inflammation [10]. The bigger fragment, C3b, can diffuse away and form covalent bonds with cell surfaces or with the antibody where complement activation was initiated, functioning as an opsonin, or it can remain bound to the complex to form a trimolecular complex **C4b2a3b**, called the **classical pathway C5 convertase**.

1.1.2. Alternative Pathway

The Alternative Pathway results in the proteolysis of C3 as the Classical Pathway does but without any need for antibody for initiation. This pathway of complement activation requires only four serum proteins: C3, factor B (FB), factor D (FD), and properdin (P) [22].

This pathway is normally constitutively active: C3 in plasma is in fact being continuously cleaved at a low rate to generate C3b in a process that is called “tick-over”. C3 is spontaneously converted to metastable C3* that undergoes hydrolysis to form $C3_{(H_2O)}$ which in turn interacts with factor B forming the initial convertase [23]. The complex $C3_{(H_2O)}B$ is a target for factor D which cleaves factor B releasing the Ba fragment and forming the enzyme $C3_{(H_2O)}Bb$. The enzyme $C3_{(H_2O)}Bb$ has a short half-life (90 sec.) but can be stabilized by the binding of properdin that acts as a positive regulator of complement [24]. $C3_{(H_2O)}Bb$ is a C3 convertase enzyme that cleaves C3 to C3a and to metastable C3b* that exposes a highly reactive thioester bond. The thioester domain can react with the amino or hydroxyl

groups of cell surface proteins or polysaccharides enabling C3b to become covalently attached to cell surfaces [25]. If these bonds are not formed, the C3b remains in the fluid phase, where is quickly hydrolysed and can form with FB the fluid phase $C3b_{(H_2O)}Bb$ that can cleave more C3. The C3b bound to the membrane can react with FB which is cut by FD with the release of Ba and the formation of the complex **C3bBb** that is the **alternative pathway C3 convertase** (Fig.2). **C3bBb** can cleave more C3 molecules, thus generating an amplification loop. Some of the C3b molecules originated can then bind to the convertase itself leading to the formation of the complex **C3bBbC3b**, which is the **alternative pathway C5 convertase**.

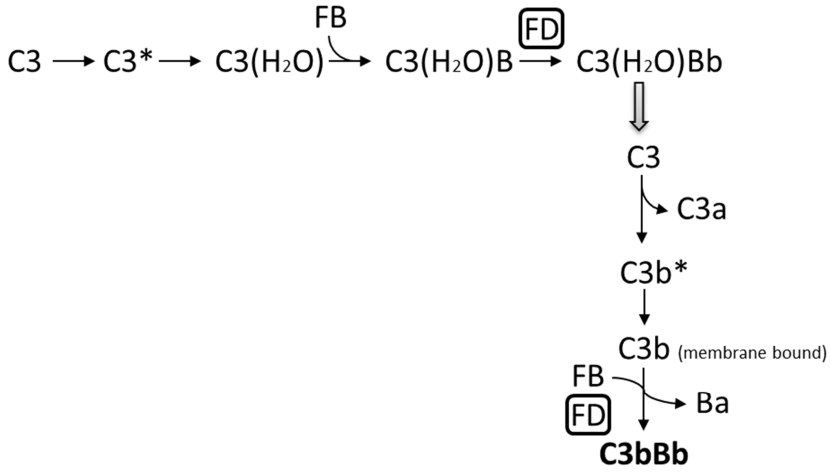


Fig. 2 Schematic representation of the AP activation. Details are discussed in the text.

1.1.3. Lectin Pathway

The lectin pathway is initiated in the absence of antibody by the binding of microbial polysaccharides to circulating lectins such as mannan-binding lectin (MBL) or ficolins (L, H or M). These soluble lectins are collagen-like proteins that structurally resemble C1q [26].

MBL, L-ficolin, and H-ficolin are plasma proteins whereas M-ficolin is mainly secreted by activated macrophages in tissues. These lectins are able

to recognize microbial sugar structures; MBL binds to mannose residues on glycoproteins or carbohydrates present on the surface of microorganisms whereas the ficolin fibrinogen-like domain can bind to different substances such as GlcNAc, GalNAc, fucose, sialic acid, lipoteichoic acid (LTA) [27, 28].
MBL and ficolins associate with MBL-associated serine proteases (MASPs) including MASP1, MASP2, and MASP3. The MASP proteins are structurally homologous to the C1r and C1s proteases and have a similar function but they are not able to bind C1q.
MASP-1 can cleave very efficiently C2 but not C4, a weak activity in cutting C3 has also been shown, and also has a role in activating MASP-2; MASP-2 can also cleave with a high efficiency C2 and to a lower extent C4; MASP-3 does not cleave C2 or C4 and its function is still uncertain but it seems to activate FD in vitro [26, 29]. MASP proteins lead to the formation of the same C3 convertase of the classical pathway (**C4b2a**) and the rest of the pathways are identical.

1.1.4. Properdin Pathway

Properdin role in stabilizing components of the alternative pathway convertases has been shown since the 1970's. More recently it has been discovered that properdin is also able to activate an "additional" pathway of complement that utilizes the same components as the alternative pathway, but that is initiated directly by properdin [30]. Properdin is able to bind directly to target cells and microbes, thus providing a platform for the assembly of convertase and leading to phagocytosis. Properdin bound on a surface has been shown to bind C3, the resulting C3bP complex can then bind factor B forming C3bBP that can in turn be cleaved by factor D, releasing Ba and generating surface-bound **C3bBbP**. This convertase is similar to the one of the alternative pathway, but its formation is initiated by properdin rather than nascent C3b [30]. The significance of this properdin-driven pathway is still elusive, but it has raised a lot of attention as a novel activation pathway.

1.2. The terminal pathway

The **C3 convertase** of the CP and LP (C4b2a) or that of the AP (C3bBb) perform a proteolytic cleavage of C3, releasing a small fragment, C3a and producing C3b. The C3 convertase is able to activate more C3 molecules leading to an amplification step that is necessary for proceeding to the terminal pathway. The terminal pathway of complement starts when large amounts of C3b have been produced and some bind directly to the C3 convertase forming the **C5 convertase** of the CP and LP (C4b2a3b) or that of the AP (C3bBbC3b) [31–33]. The addition of this C3b molecule on C3 convertases changes the substrate specificity of the active sites, located in C2a and Bb, from C3 to C5 component. The C5 is then hydrolysed by the C5 convertases forming the C5a and C5b fragments. C5a that is released to plasma is a powerful anaphylatoxin and chemotaxin, even more potent than C3a [34].

The generation of C5b activates a non-enzymatic process of assembling of the terminal complement components leading to the formation of the membrane attack complex (MAC) [6, 35]. C5b, which is extremely labile, serves as a platform for the sequential binding of C6 and C7 forming the C5b-7 complex. After the binding of C7, the C5b-7 complex becomes amphipathic and exposes hydrophobic regions which enable its insertion into the membrane [36]. C5b-7 anchored in the lipid bilayer is a receptor for plasma C8, the formed C5b-8 complex functions as receiver of multiple molecules of C9 (from 16 to 18) that create a cylindrical pore of about 30 nm in the membrane [37, 38].The C5b-9 complex, also called **membrane attack complex** (**MAC**), allows the diffusion of small molecules across the membrane to occur, and leads to rapid osmotic lysis of the target cell [39, 40].

2. Regulation of the complement system

The complement system is the first line of defence against infections, it is rapidly activated and ends in the lysis of foreign cells. Activation of complement could also be potentially harmful to the individual so, in order to prevent unwanted damage to host cells, the complement system needs to be tightly regulated. Complement is regulated by a set of proteins, both circulating or membrane-associated, that act at different steps of the cascade and enable fine tuning of complement activation in different physiological circumstances. Regulators of the complement system ensure that the activation is both effective and localized on foreign surfaces.

2.1. Regulatory proteins

Most complement regulators are encoded by a gene cluster called **RCA** (regulators of complement activation), located on chromosome 1 in the region 1q32 [41] (Fig.3).

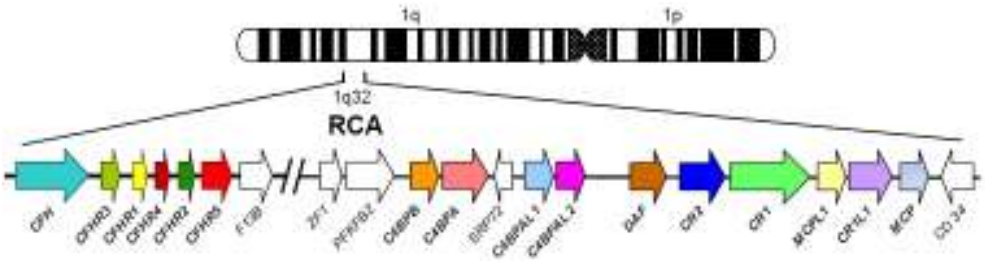


Fig. 3 Structure of the RCA gene cluster on human chromosome 1. The RCA cluster is located on the long arm of chromosome 1, 1q32 region. Colored genes encode for proteins related to the regulation of the complement system.

Genes for other complement regulators are spread throughout the genome, these include C1 inhibitor (C1-INH), CD59 (protectin), thrombomodulin (TM), vitronectin and clusterin.

2.2. Non-RCA regulators

C1-INH is a 105 KDa single-chian glycoprotein that belongs to the superfamily of serine proteinase inhibitor (serpin). It's composed of two domains: a C-terminal serpin domain that contains the protease recognition region and provides the inhibitory activity and an unique, heavily glycosylated N-terminal domain (~100 aa), not present in other serpins, that has no role in protease inhibition but is important in maintaining functional integrity [42].

C1-INH is the main regulator of both the CP and LP: it irreversibly binds and inactivates the serine proteases C1r, C1s [43] and MASP-1, MASP-2 [44]. C1-INH is the only plasma protease inhibitor of the CP, it disassembles C1 dissociating C1r and C1s and it can also suppress spontaneous activation of C1 via a reversible interaction with zymogen C1r and C1s. It can also regulate the LP via inactivation of MASP2, although α2 macroglobulin also is able to inhibit MASP2 [45].

C1-INH is a suicide protease inhibitor that irreversibly binds to its targets, its reactive loop behaves as a pseudo-substrate for the protease that cleaves the peptide bond and covalently binds to the inhibitor. After binding, C1-INH acts as a mousetrap swinging the inactivated protease from the upper to the lower pole and inserting the reactive loop of the protease inside its structure [46].

C1INH is also the major inhibitor of factor XIIa and kallikrein of the contact system, lack of inhibition of these enzymes results in inappropriate bradykinin generation and increase of vascular permeability. Inherited deficiency of C1-INH is the basis of hereditary angioedema [47].

CD59 also known as MAC-inhibitory protein (MAC-IP), membrane inhibitor of reactive lysis (MIRL), or protectin is the key regulator of the terminal pathway. CD59 is a cell surface GPI-anchored glycoprotein of 18-25 KDa that is heavily glycosylated.

CD59 can be found on all circulating blood cells (erythrocytes and leukocytes), endothelial cells, fibroblasts, and in most epithelial cells (in pancreas, epidermis, bronchi, kidney, and salivary glands, moreover it has also been found on sperm.
CD59 functions by binding to the α-chain of C8 in the C5b-8 complex and preventing subsequent binding of C9, and/or by binding to C9 in the C5b-9 complex and preventing recruitment of additional C9 molecules, it therefore blocks the polymerization of C9 and the formation of MAC [48]. Inhibition of MAC assembly by CD59 is species-specific, it only occurs if the MAC components are from the same species as the target cell.
This protein also plays a role in signal transduction pathways in the activation of T cells.
Deficiency of CD59 results in paroxysmal nocturnal hemoglobinuria, a rare disease characterized by destruction of red blood cells by the complement system [49].

Thrombomodulin (TM) is a transmembrane glycoprotein abundantly expressed on the surface of vascular endothelial cells and is mainly involved in anticoagulant pathway. TM binds thrombin forming a 1:1 stoichiometric complex thus sequestering it from its substrates, platelets and fibrinogen. Moreover, after the binding, thrombin changes its specificity from fibrinogen to protein C activating it. Activated protein C makes a complex with protein S and cleaves the activated factors V and VIII, inactivating them, thus acting as an antithrombotic factor.
TM can also act as a complement regulator in two ways: it enhances FI-mediated inactivation of C3b on the cell surface in the presence of the cofactor FH [50], and activates TAFI-mediated inactivation of the anaphylatoxins C3a and C5a [51].
Protein S has also been shown to participate in complement regulation by binding C4BP. Protein S in complex with C4BP loses its cofactor activity for protein C but enables C4BP to bind to negatively charged glycolipids [52].

This interaction thus promotes coagulation while controlling complement CP and LP activation.

Vitronectin (Vn, S-protein) and **clusterin** (Cn, SP-40,40, apolipoprotein J) are two soluble regulators of the terminal pathway that block the incorporation of MAC into membranes. Vitronectin binds preferentially to C5b-7 and interacts with C9 inhibiting its polymerization and preventing membrane insertion [53]. Clusterin binds not only to C7 in the complex of C5b-7 preventing its insertion into the cell membrane, but it can also bind to C8 and to C9, inhibiting the polymerization of C9 [54].
Soluble complexes of C5b-9 with vitronectin or clusterin (SC5b-9 complex) are normally present in serum during complement activation, thus a specific enzyme-linked immunosorbent assay (ELISA) for this complex can be used to monitor complement consumption.

Properdin as discussed earlier is a stabilizer of the AP.

Factor I (FI) is an 88 KDa serum glycoprotein predominantly synthesised by the liver and is a key regulator of complement activation. FI is a serine protease that cleaves C3b and C4b to their inactive forms iC3b and iC4b by releasing C3f or C4c fragments to the fluid phase. To exert its proteolityc functions FI requires cofactor proteins such as Factor H (FH), C4b-Binding Protein (C4BP), Complement Receptor 1 (CR1/CD35) and Membrane Cofactor Protein (MCP/CD46) [55]. Cleavage of C3b is mediated by FH, MCP or CR1, whereas in the proteolysis of C4b cofactor activity (CA) is performed by C4BP, MCP or CR1. These cofactor proteins will be discussed in more detail later.
Inactivating C3b and C4b, FI prevents the formation of the C3 and C5 convertases of both the AP and the CP, thus acting as regulator for both pathways. Moreover, since the AP comprises a feedback loop mechanism

triggered by C3b deposition on a target, inactivation of C3b by FI regulates the pathway also at a different level.

FI is synthesized as a single chain precursor (pro-I) that undergoes post-translational glycosylation and proteolytic processing before secretion [56]. The mature protein is composed of two polypeptide chains linked by a single disulfide bond [57]. The heavy chain (50KDa) comprises an N-terminal region, an FI membrane attack complex (FIMAC) domain, a CD5 domain (also known as the scavenger receptor cysteine-rich domain), two low-density lipoprotein receptor (LDLr) domains and a C-terminal region of unknown function. The light chain (38KDa) contains the serine protease (SP) domain with the conserved catalytic residues.

Inactivation of C3b

FI is able to hydrolyse both membrane bound and soluble C3b in the presence of its cofactors.

In a first step of hydrolysis, FI produces two cuts in the alpha' chain of C3b generating inactive iC3b and releasing a small peptide of 17 amino acids (C3f). This cut is performed using as cofactors FH, MCP or CR1. In the case of membrane bound C3b, the inactivated iC3b remains bound to the surface. The iC3b is then susceptible to another cleavage by FI that generates C3c, which is soluble in plasma, and C3dg, that remains bound to the surface if the starting point is membrane bound C3b. This second cut occurs only when CR1 is a cofactor [58]. (Fig.4).

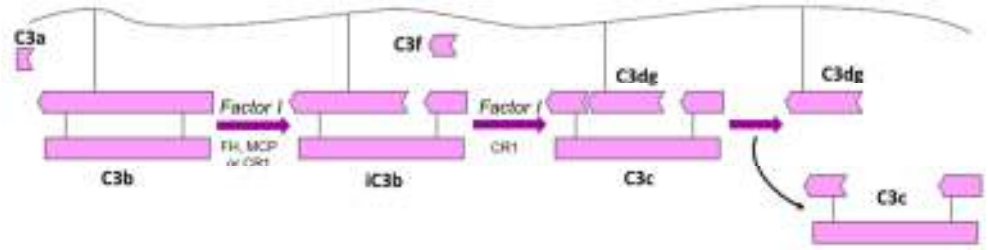


Fig. 4 Inactivation of C3b by factor I

C3dg can be further cleaved by plasma proteases to C3d that is thought to have similar biological activity than C3dg.

None of these fragments can activate the complement cascade, but the hydrolysis products iC3b, C3c and C3dg can bind to specific complement receptors and mediate functions such as the clearance of immune complexes, opsonization, B cell stimulation and immunological memory.

Inactivation of C4b

In the presence of the cofactors C4BP, CR1, or MCP, FI cleaves the membrane bound C4b inactivating it to iC4b. After a second cleavage the soluble fragment C4c is released and the residual C4d, with unknown biologic activity, remains bound to the membrane. (Fig. 5)

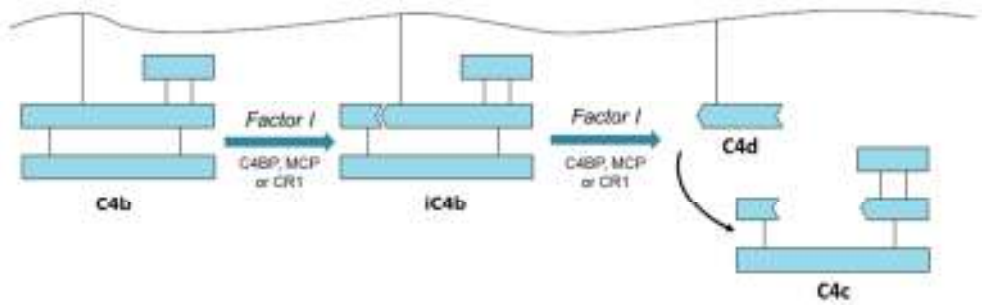


Fig. 5 Inactivation of C4b by factor I

2.2.1. RCA regulators

RCA regulators are a family of genetically, structurally and functionally related proteins.

They are composed of 4 to 30 homologous short consensus repeat (**SCR**) domains, also known as complement control protein (CCP), that have probably arisen by gene duplication events [59]. SCRs are small domains, composed of approximately 60 amino acids each, that are characterized by a high degree of conservation. They all contain four invariant cysteines that form two disulfide bridges essential to maintain a globular bead-like structure of the domain [60, 61]. (Fig. 6). The SCR domains are usually

arranged in proteins in a head-to-tail fashion making RCA proteins resemble beads-in-string [62]. The binding sites in SCR- bearing proteins may span over several domains and therefore the orientation between the SCRs is critical for the function of the protein.

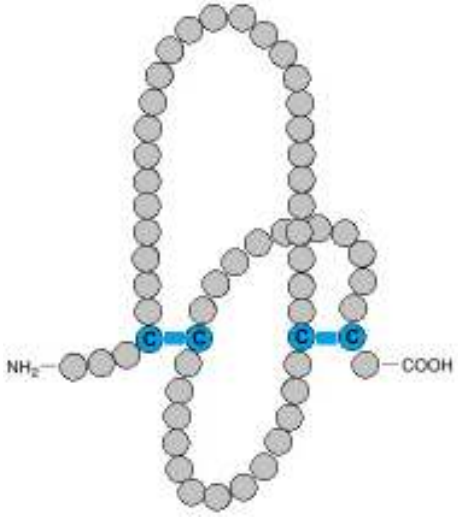


Fig. 6 Structure of a SCR domain. The SCR domain is composed of around 60aa and contains 4 cysteines that form 2 disulfide bridges essential to maintain its globular bead-like structure.

Another common feature of these components of the RCA is that their regulatory function are closely related. They act mainly on convertases by accelerating the dissociation of the C3 and C5 convertases or attending FI as cofactors in the proteolytic degradation of C3b and C4b.

Among RCA regulators we can find both cell membrane-associated and fluid phase proteins (Fig.7). Membrane-bound complement regulators include membrane cofactor protein (**MCP/CD46**), decay-accelerating factor (**DAF/CD55**), and complement receptor 1 (**CR1/CD35**), whereas complement receptor 2 (**CR2/CD21**), is encoded by the same locus, but has not been shown to regulate complement. Human cells are protected from complement-mediated lysis because they expose these regulators on their surface. On the other hand, soluble regulators limit the activation and the

consumption of complement in the fluid phase, those include C4 binding protein (**C4BP**), factor H (**FH**), factor H-like protein 1 (**FHL-1**), and factor H related (**FHR**) proteins.

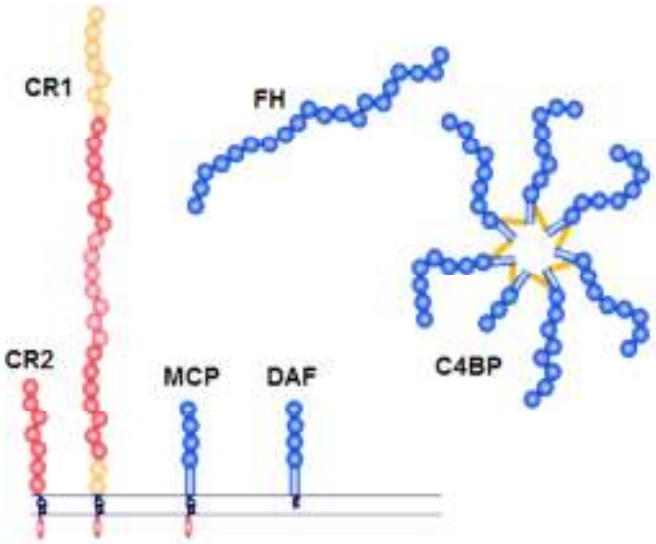


Fig. 7 RCA regulatory proteins. Membrane-bound and fluid phase RCA proteins are represented. They all are composed by SCR domains indicates as beads.

MCP (CD46) is a transmembrane glycoprotein widely expressed on cells that are in contact with the blood stream such as leukocytes, platelets, endothelial cells, epithelial cells and fibroblasts, with the exception that it is not present on erythrocytes [63]. It consists of an extracellular part formed by 4 SCRs followed by 1-3 serine/threonine-rich (ST) domains, a hydrophobic transmembrane domain and a cytoplasmic tail. More than 8 isoforms can be produced by alternative splicing.

MCP inhibits complement activation on host cells functioning as a cofactor for factor I mediated cleavage of C3b to iC3b and C4b to C4c and C4d, thus abolishing the ability of C3b and C4b to form C3/C5 convertases [64]. MCP is an “intrinsic regulatory protein” as it can bind to C3b/C4b deposited on the same cell membrane but not on other cell membranes, thus protecting from complement attack only those cells on which it is expressed.

DAF (CD55) is a 70 KDa glycoprotein bound to the cell membrane by a GPI-anchor followed by a serine/threonine-rich (ST) region and 4 consecutive extracellular SCRs. It's expressed on the surface of all blood cells and in various tissues and organs [65]. A soluble isoform generated by alternative splicing is present in body fluids [66].

DAF can control complement activation at different levels accelerating the dissociation of both C3 and C5 convertases. DAF binds to the AP C3 convertase, C3bBb, accelerating its dissociation and also decreases the stability of the CP/LP C3 convertase, C4bC2a, by accelerating its dissociation to C4b and C2a [67]. In this way C3b peptides can no longer be produced to bind to the surface of the cells. DAF can also bind and dissociate C5 convertases on the surface of cells, thus protecting cells from lysis mediated by the membrane-attack complex [67].

Complement receptor type 1 (**CR1**) is a large, multifunctional, transmembrane glycoprotein. It is composed by a cytoplasmic domain, a transmembrane region and a large extracellular domain composed of 30 SCRs in the most frequent allele. Three other alleles are possible containing respectively 27, 37 or 44 SCRs. On the basis of the degree of homology the SCRs are further grouped into larger units, called long homologous repeats (LHRs) and designated LHR-A, -B, -C, and -D. The number of LHRs in the different allelic forms varies from three to six [58].

CR1 is expressed on all peripheral blood cells with the exception of platelets, natural killer cells and most T cells [68] and is also found in glomerular podocytes, astrocytes and hyalocytes (vitreal macrophages).

CR1 binds C3b and C4b, and, with a lower affinity, iC3b and C3dg [58]. Different LHRs bind to different substrates and bear different activities.

CR1 on circulating cells acts mainly as an immune adherence receptor to facilitate the removal of C3b/C4b-opsonized immune complexes and pathogens from the circulation, which after binding are transported to liver and spleen where they are processed by macrophages [12].

CR1 also exerts complement inhibitory activities: it possesses decay-accelerating activity (DAA) for both CP and AP C3 and C5 convertases and it irreversibly inactivates all four convertases, due to its co-factor activity (CA) for factor I-mediated cleavage of C3b and C4b. CR1 is not particularly efficient in generating iC3b, but is the only co-factor protein able to carry out the third cleavage of C3b to generate C3c which is released to fluid phase and C3dg [58].
CR1 has also been reported to bind to the collagenous portions of C1q [69] and to mannan-binding lectin (MBL) [70].

Complement receptor 2 (**CR2**) which is expressed on B-lymphocytes binds to iC3b, C3dg, or C3d and has a role in B-cell activation, bridging innate and acquired immunity [71], but a specific role in complement regulation has not yet been discovered.

C4 binding protein (**C4BP**) is a large soluble glycoprotein, present in plasma at a concentration of approximately 200 μg/ml. C4BP exists in several isoforms, the major form (α7/β1) is composed of seven identical α-chains (70 KDa) and a single β-chain (45 KDa) all linked together at the C-termini by disulfide bridges in a unique "octopus-like" structure (Fig. 8) [72]. Other less abundant forms are composed of six α-chains and one β-chain (α6/β1) or exclusively of seven α-chains (α7/β0) or of six α-chains (α6/β0). Both α- and β-chains are composed of multiple SCRs domains, the α-chain contains eight and the β-chain three. The SCRs domains in both chains are followed by a C-terminal extension (~60 amino acid residues long) which contain an amphiphatic α-helix region and two cysteines that are required for intracellular polymerization and interchain disulfide bridging [73].
The genes encoding for both the α-chain (C4BPA) and the β-chain (C4BPB) are located in the RCA gene cluster in close proximity.

Each of the α-chains contains a binding site for the activated complement protein C4b, whereas the β-chain binds to coagulation system protein S [74, 75].

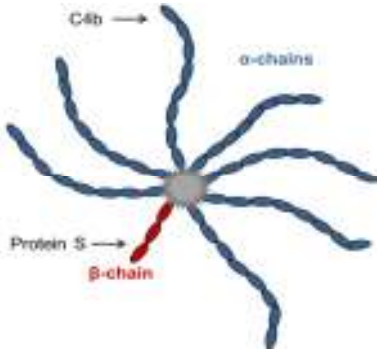


Fig. 8 Structure of C4BP. The major isoform (α7/β1) composed of 7 α-chains and 1 β-chain is indicated. This protein has a unique "octopus-like" structure.

C4BP is the main inhibitor of the CP and LP of complement, where it controls C4b-mediated reactions in several ways.

C4BP functions as a cofactor to factor I in the regulation of the complement system, with C4b as the primary target. C4b bound to C4BP α-chain is a substrate for FI, that cleaves it into inactive C4b (iC4b) [72]. However, C4BP can also serve as cofactor to FI in the regulation of the complement protein C3b, even though other proteins such as FH, MCP, and CR1 are more efficient in supporting C3b degradation.

Moreover, C4BP prevents the assembly of the classical C3-convertase by binding nascent C4b and it also accelerates the natural decay of the complex [76]. On the other hand, C4BP does not seem to be an inhibitor of the assembled alternative C3-convertase [77].

The binding of C4BP β-chain with protein S enables the S-C4BP complex to bind to negatively charged phospholipid membranes, thus providing C4BP with the ability to interact with certain cell surfaces, where it can express its complement regulatory activities.

During the apoptotic process, negatively charged phospholipids, that are normally located in the inner part of the cell membranes, are exposed on

the surface of the apoptotic cell. The protein S-C4BP complex can bind and inhibit the activation of complement on the apoptotic cell, which may be important for the controlling the inflammatory process. However, the protein S-C4BP complex does not stimulate phagocytosis of the apoptotic cells [78].

Factor H (FH), factor H-like protein 1 (FHL-1), and factor H related (FHR) proteins will be discussed in more detail.

3. Complement Factor H

Complement Factor H (FH), the main regulator of AP, was first identified in 1965 by Nilsson and Müeller-Eberhard as β1H globulin [79]. Factor H is a single polypeptide chain plasma glycoprotein of 155 KDa composed of 20 SCR domains [80].

FH is synthesized mainly by the liver [81], but extra-hepatic synthesis has been demonstrated in a variety of tissues including endothelial and epithelial membranes, glomerular mesangial cells, platelets, mesenchymal stem cells and retinal pigment epithelium [82–86].

FH plasma levels in humans is very variable (116-562 μg/ml) and it has been shown to be influenced by environmental factors such as age and smoke, although the genetic component accounts for the major part (~62%) [87].

3.1. FH functions

The main function of FH is down-regulation of AP which is achieved in three different ways: FH competes with FB for binding to C3b, thus preventing formation of C3 and C5 convertases of the AP [88]; FH also possesses decay acceleration activity (DAA) and promotes the dissociation of these convertases once they have formed [89]; finally it acts as an essential cofactor for FI in the proteolytic conversion of C3b to iC3b [90]. In

the absence of FH, spontaneous activation of AP occurs in plasma and leads to consumption of complement components C3 and FB [22].

The regulatory function of FH, which is mainly due to its interaction with C3b, is performed both in the fluid phase and on self-surfaces where C3b is bound. However, while FH binds and inactivates C3b promptly in fluid phase, the inactivation of surface-bound C3b by factor H is dependent on the chemical composition of the surface.

FH is able to distinguish between self and foreign surfaces and this is mainly due to its capacity to bind polyanions such as sialic acid and glycosaminoglycans (GAGs) such as heparin. The presence of these markers on self surfaces increases the affinity of FH for surface-bound C3b as a consequence of the simultaneous recognition of both types of molecules by the same FH molecule [91–93]. Thus, FH acts by selectively limiting the progression of the complement cascade on the surfaces of self-tissues and allowing amplification of the AP only on foreign surfaces.

3.2. FH structure and functional domains

FH mature protein is composed of 1213 amino acid residues that are organized in 20 SCRs of ~60 residues each joined in a “string-of-beads” arrangement by 19 linkers of 3-8 residues.

A multiple alignment of the 20 SCRs highlights four invariant Cys residues and a near-invariant Trp residue between Cys(III) and (IV). This consensus sequence also occurs multiple times in other members of the RCA family.

Each of the 20 SCRs is presumed to fold into a distinct three-dimensional (3D) structure termed the complement control protein module (CCP) stabilized by two disulphide bounds between Cys(I)-(III) and Cys(II)–(IV) [94].

Different parts of the protein contain binding sites for different ligands. (Fig. 9). Functional and interaction assays using the whole molecule or recombinant fragments enabled to establish a protein map characterizing

the binding sites for C3b and polyanions [95–102]. FH contains three regions important for binding C3b; two main binding sites located at each end of the molecule and a third one in the central part of FH. The binding site present in the N-terminal part (SCRs 1-4) is the only one necessary and sufficient to perform the cofactor function for FI. It also accelerates the dissociation of the convertase, although other regions of FH must contribute, as the dissociation capacity of the fragment FH-SCRs 1-4 is 100 times lower than that of the whole FH [100]. The binding site for C3b localised in the C-terminal region (SCRs 19-20) has the highest affinity to C3b, however, both sites at opposite ends must cooperate in binding to C3b. SPR interaction assays demonstrate that affinity of the entire FH is much higher than that of the N- and C-terminal fragments taken separately [103]. Moreover, SPR interaction assays also demonstrated that the three different binding sites on FH interact with distinct sites on C3b [99], and they can also bind C3b degradation fragments. The C3d fragment of C3b binds to SCRs 19-20 whereas the central binding site is specific for C3c.

The interaction of FH with GAGs and polyanions has been located mainly to two regions: SCR 7 with the contribution of SCRs 6 and 8, and SCRs 19-20, in which SCR20 is the main heparin-binding site [103]. Another central binding site (SCRs 12-15) has been proposed but never been confirmed. These sites enable the binding of FH to host cell surfaces, but not to the surfaces of pathogens, thus leading to the complement regulatory protection of host cells.

FH also binds C-reactive protein (CRP) at SCR 6-8 and SCR 16-20 [104]. CRP bound to damaged host cells can bind FH leading to C3b regulation.

Weak zinc binding sites are primarily located within SCR 6-8; these sites lead to the precipitation of FH–C3b complexes in the pathophysiological concentrations of zinc found in the retina [105]. In addition to these ligand interactions, FH self-associates with itself to form dimers and higher oligomers [106].

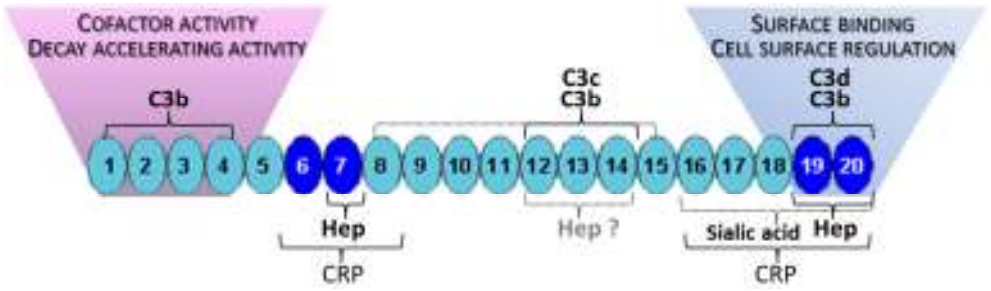


Fig. 9 Structure of FH. FH is composed of 60 SCR domains. Binding sites for different ligands are indicated.

The structure of FH, composed by 20 SCRs linked by short sequences, unlike it was previously believed, is not completely flexible, however N and C-termini are brought close to each other (Fig. 10). This architecture must be functionally relevant to allow both ends of FH to interact with the same molecule of C3b. This would explain how C3b binding sites, SCRs 1-4 and SCRs 19-20, cooperate and confer avidity. Several models of FH-C3b-Heparin interaction have been proposed. Morgan HP *et al* hypothesised a model of FH-C3b in which a single molecule of FH interacts with one of C3b in non-overlapping regions through binding sites located at opposite ends of FH (FH_{SCR1-4}-C3b and $FH_{SCR19-20}$-C3d/TED), and further enables the C-terminal region of FH (SCRs 19-20) to bind to polyanions on the surface [107].

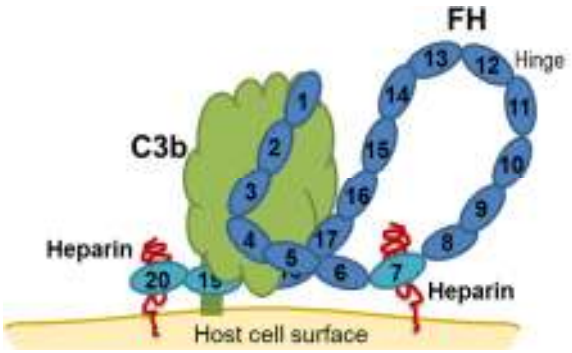


Fig. 10 FH-C3b-heparin complex. FH is a rigid structure but can fold in on itself, bringing the two ends close to each other and enabling interaction with C3b and polyanions.

The highly complex FH structure can be essentially divided into two functional regions:

- The N-terminal region, which binds to C3b and bears the regulatory activities, such as cofactor activity (CA) and decay acceleration activity (DAA)
- The C-terminal region, which binds to C3b and polyanions and provides the selective ability to interact with surfaces

The correct activity of FH depends on cooperation between these two regions and with the other interaction sites.

3.3. The CFH gene

FH is encoded by a single gene (CFH), located within the RCA in the region 1q32. The CFH gene contains 23 exons, the first exon codes for the 5'UTR and signal peptide. Each of the other 22 exons code for one of the 20 SCR domains except SCR 2 which is encoded by exons 3 and 4 (Fig. 11).[5]

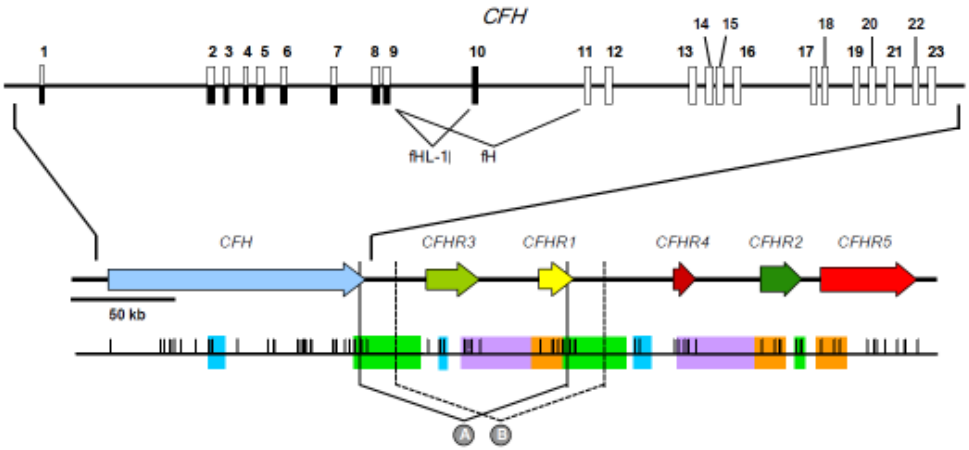


Fig. 11 Structure of the CFH gene. Structure of CFH gene and different exons coding for the two isoforms FH (white boxes) and FHL-1 (black boxes) are indicated. Location of the CFH gene in the RCA cluster, in close proximity to CFHRs genes, is also indicated.

From the same gene an alternative transcript is produced that codes for a 43 KDa protein named FH-like 1 (FHL-1). FHL-1 shares with FH the first 9

exons, which code for the first seven SCRs, plus exon 10, which is absent in the mature messenger coding for FH. Exon 10 possesses a premature stop codon and codes for a short tail of four amino acids (Ser Phe-Thr-Leu) [108]. Both proteins are mainly expressed by the liver, but the plasma concentration of FHL-1 is much lower (10-50 μg/ml). FHL-1 has complement regulatory functions, such as cofactor activity for FI and convertase C3bBb dissociation activity. FHL-1 is also capable of binding to heparin, various microbial proteins, and cell surfaces, being involved in cell motility processes [100, 109].

Despite FH and FHL-1 share the promoter region and the transcription start site, the regulation of their expression is different depending on the cellular type. The reason for this difference is unknown but it probably depends on different transcriptional and post-transcriptional mechanisms [110]. This suggests that there are specific functions for FH and FHL-1, where both the tissue and the local concentration can be very important. However, the precise role of FHL-1 in vivo is not determined.

3.4. Factor H protein family

In the RCA cluster, just downstream of the CFH gene, we can find five genes (*CFHR1, CFHR2, CFHR3, CFHR4, CFHR5*) coding for the FH-related proteins (FHRs) (Fig. 10). These proteins, together with FH and FHL-1 form a family of glycoproteins ("Factor H protein family") which are structurally and functionally related [111].

The chromosomal segment containing CFH and CFHR genes is characterized by several large genomic repeat regions, including different exons of CFH and CFHRs 1-5, which have a high degree of sequence identity. This situation favours non-homologous recombination processes that result in chromosomal rearrangements and can lead to both deletions and duplications. Chromosomal rearrangements can result in the loss of a

single or several CFHR genes, but can also give rise to new gene-compositions and hybrid genes including in some cases also the CFH gene. Rearrangements within the FH gene family may lead to defects associated with pathology [5, 112].

The FHRs 1-5, as FH, are composed exclusively of SCRs and contain from 4 to 9 domains. They possess two conserved regions: the N-terminal one, corresponding to the central region of FH (SCRs 6-9) with which it shows a high degree of sequence identity (36–94%) and the C-terminal one, which is very similar to the FH C-terminus (SCRs 19-20) (36–100% identity) (Fig. 12).

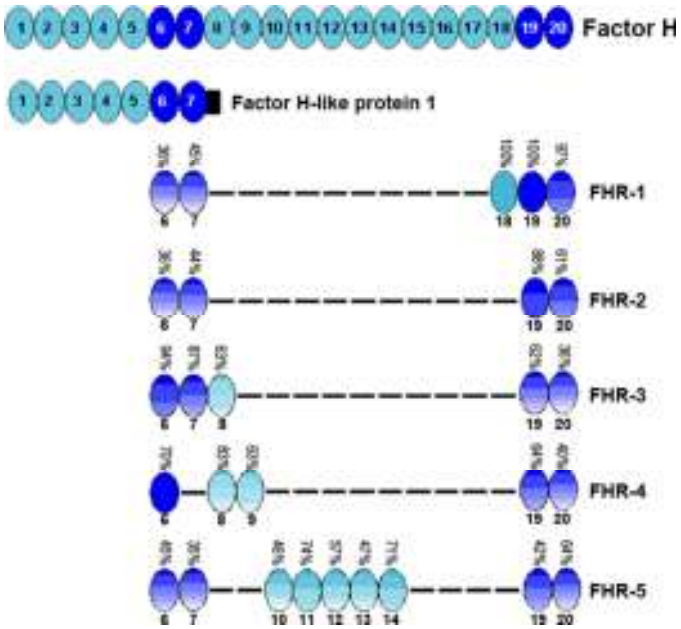


Fig. 12 Schematic representation of FH and FHRs. Similarity between SCRs of FH and FHRs is indicated and represented by different shades of blue.

The sequence similarity and the presence of conserved domains suggest that these proteins possess related and even shared functions. The conservation of the C-terminal region of the FHRs suggests that these proteins, similarly to FH, can bind to surface ligands and discriminate between self and non-self tissues. All five FHR proteins have been shown to

bind to C3b and to C3d. Of the two C-terminal SCRs in FHR proteins, the one that corresponds to SCR19 in FH is highly conserved whereas the one corresponding to SCR20 is more diverse. SCR20 of FH harbours an important heparin binding site, suggesting that the FHRs may recognize and bind to different cell surfaces.

On the other hand, the lack of a regulatory region such as the one present in SCRs 1-4 of FH, with cofactor and decay activity, implies that FHRs are not very potent in regulating complement [111].

According to their conserved domains, FHR proteins are divided into two major groups: group I includes FHR-1, FHR-2 and FHR-5 whereas group II is composed by FHR-3 and FHR-4 [113].

Proteins in group I are composed of a different number of SCR domains: FHR-1 has five, FHR-2 four SCRs and FHR5 nine. This group is characterized by their conserved N-termini (SCR1 and SCR2) which have more than 80% sequence identity. These proteins circulate in plasma exclusively as dimers (homodimers or heterodimers), and their dimerization is mediated by the two conserved N-terminal SCRs.

FHR-1 is composed of five short SCRs, it possesses a dimerization domain in the N-terminus and forms homodimers and likely also heterodimers with FHR-2 and FHR-5.

Two variants of FHR-1 have been identified, an acidic isoform (FHRA) that expresses the amino acids HLE in SCR3 and a basic isoform (FHRB) expressing YVQ in SCR3. FHR-1 circulates in human plasma in two glycosylated forms: FHR-1α with one and FHR-1β with two carbohydrate side chains. The plasma concentration of CFHR1 is about 70 to 100 µg/ml.

FHR-2 consists of four SCRs, it forms homodimers and likely also heterodimers with FHR-1 but not with FHR-5. The protein has two glycosylated forms, a single glycosylated (24 KDa) and a double glycosylated form (28 KDa). The plasma concentration is estimated to be about 1/10 of FH, around 50 µg/ml.

FHR-5 is composed of nine SCRs and is the longest FHR protein, it circulates in the plasma as homodimer and likely also as a heterodimer with FHR-1. FHR-5 is the only FHR protein that has five SCR domains with high sequence identities to SCRs 10–14 of FH. FHR-5 is a glycosylated protein of 62 KDa.

The group II FHR proteins, as well as factor H, lack the N-terminal dimerization domains. FHR-3 and FHR-4 show a high degree of amino acid identity to the SCRs 6–8 of FH (63 to 95%). SCRs 6–8 of factor H mediates binding to heparin and to monomeric C-reactive protein (CRP), which is conserved in FHR-4 [111].

FHR-3 consists of five SCR domains that show high amino acid identity to SCRs 6-7-8 and SCRs 19-20 of FH. It is detected in plasma in multiple variants (ranging from 35 to 56 KDa), reflecting the existence of four different glycosylated variants of FHR-3. The plasma concentration is estimated to be similar to FHR-1 (70–100 μg/ml).

FHR-4 is composed of nine SCRs and appears with an internal duplication similar to an internal dimer, as SCRs 1–4 show a high level of identity to SCRs 5–8 of the same protein. As FHR-3, it also has sequence identity to FH SCRs 6-7-8 and SCRs 19-20. The human *CFHR4* gene codes for two proteins, FHR-4A and FHR-4B. FHR-4B derives from an alternatively spliced transcript of the *CFHR4* gene and is composed of five SCRs that correspond to half of the SCRs of FHR-4A, in detail SCR1 and SCRs 5–9. FHR-4B has a mobility of 42 KDa and FHR-4A of 86 KDa. The plasma concentration of the two variants has not yet been determined.

The high amino acid identity among the family members is also reflected by the fact that antibodies raised against FH also detect different FHRs in plasma and that the antibodies generated against FHRs cross-react with other FHRs. Moreover, auto-antibodies against factor H in aHUS recognize not only factor H but also certain FHRs [114]. This cross-reactivity is a

challenge for FHRs purification from plasma, their concentration determination and in depth analysis.

The exact function of FHRs is still uncertain, but the functional relationship between them and FH is now clearer: they can cooperate with FH in the regulation of complement activation or compete for binding to ligands and surfaces, which would result in less regulation.

4. Factor H-Associated Diseases

Complement activation and its regulatory mechanisms are in strict balance, such that the effector activities of complement are normally directed towards foreign surfaces and are avoided against self ones. Any imbalance can lead to tissue damage, susceptibility to infection, as well as chronic pathology.

Complement regulatory defects due to FH mutations or anti-FH autoantibodies have been described in certain pathological conditions, and CFH polymorphisms have also been associated with disease.

Among FH-associated diseases, the best studied are atypical Hemolytic Uremic Syndrome (aHUS), Dense Deposit Disease (DDD) which affect primarily the kidney with predominant glomerular dysfunction and Age-related Macular Degeneration (AMD), a late onset eye dysfunction causing impairment of the central vision. aHUS and AMD well be discussed in more detail here.

4.1. Atypical Hemolytic Uremic Syndrome

The Hemolytic Uremic Syndrome (HUS) is a microvascular dysfunction mainly affecting the kidney, but also the gastrointestinal and central nervous systems can be involved. This condition is clinically defined as hemolytic angiopathic anemia, thrombocytopenia and acute renal failure.

The main events triggering the pathogenic process seem to be endothelial lesions in glomerular microvasculature due to either infection or other

exogenous damaging causes. These lesions show thickening of arterial and capillary walls, endothelial inflammation and detachment of the glomerular basal membrane (GBM), in addition to sub-endothelial accumulation of proteins and cellular debris as well as formation of microclumps of platelets and fibrin which may cause vascular occlusion. Generation of schistocysts (fragmented erythrocytes) due to the circulation through partially occluded renal microvasculature is another feature of HUS [115].

Two types of HUS are traditionally distinguished. The most frequent form (90% of the observed cases), called typical, mainly affects children and has an incidence of 2 cases per year every 100.000 inhabitants. Typical HUS is associated with infection by an entero-haemorrhagic strain of *Escherichia coli* (0157:H), which produces a highly toxic Shiga-like toxin. Although the mechanism of action of this toxin has not yet been totally clarified, its role in causing haemorrhagic diarrhoea and the evolution of this disease is not under dispute. After crossing the intestinal barrier and entering the systemic circulation, the Shiga toxins bind to receptors on the endothelial surface causing lesions and triggering an inflammatory status and pro-thrombotic episodes. Most cases of typical HUS have a positive outcome and kidney function is restored in at least 75% of the patients. The remaining cases can develop chronic kidney disease and in some cases might even die (3-5%). [115].

Approximately 10% of cases of the hemolytic–uremic syndrome are classified as atypical HUS (**aHUS**), since they are not caused by either Stx-producing bacteria or streptococci. Atypical HUS can affect both children and adults, its incidence corresponds to two cases per year per 1.000.000 inhabitants. Based on histological findings, the lesions observed in aHUS are indistinguishable from those of the typical form; however, aHUS has a much worst prognosis. Most patients show recurrent episodes and half of them develop chronic kidney disease (CKD). aHUS mortality is approximately 25% [116].

No special association has been found for aHUS, but a number of risk factors have been found which predispose to this disease. Among them, immune suppressing or anti-cancer therapies, oral contraceptives, pregnancy and post-partum.

On the other hand, several studies have found, in familiar cases of aHUS, and less frequently in sporadic cases, a clear genetic component. In some cases of aHUS, mutations were found in genes coding for pro-coagulant proteins, such as Thrombomodulin (TM), most patients were found carrying mutations on genes coding for the alternative pathway of complement (AP) [117].

The involvement of complement in HUS is known since 1974, but only in recent years a clear association between aHUS and specific complement proteins has been assessed. In many instances aHUS-associated mutations and polymorphisms have been identified in regulatory proteins such as FH, MCP, or FI and more recently to components FB and C3 [117]. In most of these mutations functional defects have been identified which affect up to half the aHUS cases. This implies that complement AP is somehow directly involved in the pathogenesis of this disease.

In spite of all this evidence, in most cases a specific genetic or environmental cause which might be considered the triggering event has not been found. This calls for the existence of additional factors that have not yet been identified. Among the mutations that have been described, most are located in the CFH gene, which deserves special attention in the pathogenesis of this disease [118].

4.1.1. FH in aHUS

Factor H mutations affect approximately 30% of aHUS patients, more than 100 mutations have already been described. An updated record of all mutations associated with aHUS can be found at the FH–HUS online database (http://www.fh-hus.org) [119].

Heterozygous mutations affecting various domains of FH have been associated with aHUS, however missense mutations in the C-terminal region (SCRs 19-20) are the most prevalent genetic alterations among aHUS patients. Furthermore, in a significant number of patients mutations affecting the functionality of the C-terminal region are the result of gene conversion events between exon 23 of *CFH* and the homologous exon 6 of *CFHR1* [120] or of genomic rearrangements creating *CFH::CFHR1* hybrid genes [121].

FH mutations associated with aHUS rarely result in decreased FH plasma levels, the majority of mutant proteins express normally and present normal co-factor activity for the FI-mediated proteolytic inactivation of C3b in plasma [122]. Functional analyses of several of these mutants showed an altered interaction with C3b, heparin and endothelial cells [122–124].

As mentioned above, aHUS-associated FH mutations cluster in the C-terminus of the protein, a region that is critical to control activation of complement on cell surfaces. Consistent with this location, carriers of these mutations express FH molecules that present normal regulatory activity in plasma but a limited capacity to protect self cells from complement attack [125].

Recent structural studies have provided new insights into how these mutations impair the function of factor H in host/non-host discrimination [107, 126]. Certain mutations in SCR20 were also found to reduce the binding of FH to CRP [127] and to PTX3 [114]. For many mutations, however, there is no functional effect known to date.

Moreover, anti-FH autoantibodies are detected in approximately 10% of aHUS patients, mostly children. These antibodies have functional consequences similar to those caused by the mutations in the C-terminal region of FH [128, 129].

Several studies using recombinant FH fragments have been performed to determine the binding sites of these autoantibodies and have shown that

they bind mainly to SCRs 19-20, although in some cases reactivity with other domains, such as SCRs 8-11, was also observed [130].

Functional studies indicated that the autoantibodies directed against SCRs 19-20 interfere with the FH recognition functions impairing its interaction with surface-bound C3b and thus inhibiting its complement regulatory activity on host surfaces [130]. This reduced protection from complement-mediated damage is likely to be involved in the endothelial injury associated with aHUS.

Interestingly, 90% of the affected individuals presenting anti-FH antibodies are also homozygous for the deletion of the *CFHR1* and *CFHR3* genes, it's still unclear whether the deletion of these genes predisposes to the development of FH autoantibodies or if they are independent risk factors for aHUS [129, 131, 132]. What is certain is that, due to the similar C-terminal SCRs of FH and FHR-1, most of the studied autoantibodies recognize both host complement regulators. FHR-1 in fact can hijack autoantibodies and rescue host cells when added to anti-factor H autoantibody-positive plasma [133].

A mouse model of aHUS is available, in which a truncated form of FH (FHΔ16-20) that lacks the terminal five SCR domains, equivalent to the location of the majority of aHUS-associated FH human mutations, is produced [134].

4.2. Age-Related Macular Degeneration

AMD is the main cause of elder blindness in the industrialized countries. It is an eye lesion affecting the central part of the retina, also known as macula. AMD diagnosis is based on the degeneration signs that are evident in the macula, independently from vision sharpness. Although substantial histological changes are associated with AMD in the retina retinal pigment epithelium (RPE), choriorcapillaris and choroid in middle age, AMD does not manifest clinically before 55 years of age [135].

The typical clinical sign of AMD are the drusen, the origin of which is still today totally unknown. As seen through the ophthalmoscope, they appear as white to yellow dots in the retina, sometimes with a crystalline and shiny aspect. They are extracellular deposits that accumulate between the RPE basal membrane and the inner collagen layer of the Bruch membrane [135].

The drusen are found in different eye diseases, and constitute a relevant risk factor for the development of AMD. This association is very strong, and as a consequence the presence of drusen is considered as a sign of an early-stage of AMD, even in the absence of vision loss. Both the size and the number of drusen are related to AMD severity. Drusen composition is variable and not well defined, and only some of its components are known. Their inside mainly contains glycoproteins and phospholipids, and in their outer layer several proteins have been identified: fibronectin, chaperones, apolipoprotein E, vitronectin, Amyloid P and complement proteins such as C1q, C4, C5, C3 (and its degradation fragments), as well as the C5b-9 complex. The have also been shown to contain RPE cellular debris [136].

There is an early, almost asymptomatic stage in AMD, which is characterised by the appearance of drusen as well as mild changes in RPE pigmentation. In the later stage there is a severe vision loss, and the disease may be classified as non-exsudative “dry”, or atrophic and exsudative “wet” or neovascular. In the former case, well-defined hypopigmented regions are produced, something known as “geographic atropohy”, and the typical symptoms are blind holes or spots of vision. The most advances stage is characterised by the presence of serum or hemorrhagic fluid in the extracellular space, which causes the detachment of the neuro-retina or of the RPE.

A typical sign is the choroidal neovascularisation, which can diffuse through the Bruch membrane until towards the RPE. These changes generate distorted images, which is often a sign of the wet form of AMD. Both types

of AMD can coexist in the same patient, and one type can change into the other. The general progression is from the dry to the wet form: from 10 to 15% of patients evolve from the dry to the wet form [135].

The RPE is the main component of the retina which is involved in the pathogenesis of AMD. The drusen structurally and functionally modify the RPE, induce thickening of the monolayer, removing it from the intimate vascular support of the choroidal region. This causes the interruption of nutrient and metabolite supply and disposal of waste between the retina and the choroid capillaries. Moreover, the modifications of the Bruch membrane cause the impairment of cell adhesion, a reduced regenerative capacity of the neuroretina by the RPE cells. All this, together with the effect of neovascularisation, has a deep impact on the photoreceptors with a direct negative effect on central vision. The extracellular deposits and some of the components present in the drusen, such as activated complement proteins and complement regulators, in addition unchain the local inflammatory reactions that are involved in the pathogenesis of AMD [135]. Changes in these eye structures, such as thickening of walls and loss of permeability are naturally occurring as an aging feature. However, the influence of environmental and genetic factors is very important.

AMD is a late onset complex disease caused, like many other multifactorial diseases, by the convergent action of multiple risk factors. Among these, we can quote aging, cigarette smoke, a high body mass index, and especially genetics [135]. Multiple genes appear to be associated with AMD, and particularly of note are Fib15, ABCA4, ApoE, HTRA1, ARMS2 and different complement genes: CFH, CFB, C2, C3 and CFI [137].

HTRA1 and ARMS2 are on locus 10q26 and are strongly associated with AMD. The former codes for a serine protease localised in the retina (as well as in other tissues) and the latter is a hypothetical gene with unknown function. An ARMS2 haplotype has been described with a sequence change (Ala69Ser) which increases the risk of AMD up to 6.3 times in homozygous

carriers. However, the existence of this theoretical protein has not been confirmed and its correlation with AMD pathogenesis is still elusive [137].
The most significative genetic association with AMD was found with the locus 1q32, where the CFH gene is found.

4.2.1. FH in AMD

The common FH polymorphism Tyr402His has been associated with AMD and the 402H variant represents the higher risk factor for developing the disease. This association has been confirmed in different human populations with an Odds Rate between 2.45 and 4.6 in heterozygous subjects and up to 7.4 in homozygotes [138–140]. The frequency of the 402H varies among different populations, which explains the differences in the incidence of this polymorphism for AMD.
Functional studies revealed different binding properties of the of the two FH variants (402Y and 402H). The 402H variant has a reduced binding affinity to CRP and a higher affinity for DNA and necrotic cells compared to the 402Y variant. Since residue 402 is situated in SCR7 which is involved in binding to the GAGs, there are also subtle differences between the variants in their interaction with heparin and GAGs [141, 142].
No difference in binding to retinal pigment epithelial cells was found, but the disease-associated variant binds less efficiently to both the extracellular matrix protein fibromodulin and the Bruch's membrane in the retina [143].
Additional SNPs in the CFH gene associated with AMD were identified.
A polymorphism in exon 2, resulting in a Isoleucin to Valine exchange at amino acid position 62 (I62V) of FH and FHL-1 has been reported. The 62I variant is associated with the protective form for AMD and most likely has a higher thermal stability [144].
The protective 62I variant has been shown to possess an increased affinity for C3b and enhanced cofactor activity for the FI-mediated cleavage of C3b

[145], even though, as shown by crystallographic studies, residue 62 is positioned outside the interaction interface of FH with C3b.

Recently, a new rare polymorphism (R1210C) was described in which the C allele confers a 20-fold higher risk of AMD and appears to be involved in a significantly earlier onset of the disease [146]. The same polymorphism was previously shown to be involved in atypical Hemolytic Uremic Syndrome [147].

In addition, a protective haplotype associated with the deletion of a 84-kbp fragment directly downstream of CFH gene, that includes CFHR1 and CFHR3 genes has been described [148].

4.3. Interaction with Pathogens

Since complement plays an important role in protection against infections, and is generally regarded as the first line of defence against pathogenic microorganisms it is not surprising that numerous pathogens (viruses, bacteria, fungi and parasites) have evolved strategies for attenuating or escaping complement attack.

Their mechanisms of action can be condensed to a few successful strategies: the recruitment or mimicking of complement regulators; the modulation or inhibition of complement proteins by direct interactions; and inactivation by enzymatic degradation.

By acquiring complement regulators onto their surfaces microorganisms gain the ability to regulate and control complement activation and can evade the destructive effects of this powerful defense system. Many pathogens are able to sequester FH on their surface in order to camouflage themselves as host-like cells and thus are protected from complement attack.

Binding of FH down regulates opsonization and lead to a rapid inactivation of the newly formed C3b at the microbial surface, thus preventing further amplification of the complement cascade and the formation of MAC. While

prevention of opsonization and subsequent phagocytosis is beneficial for practically all microbes, evasion of MAC formation is especially important for Gram-negative bacteria and spirochetes. Acquisition of FH is important or even essential for pathogens; increasing numbers of them have been shown to bind FH [149].

There are two main interaction sites on FH for microbial binding: one is within SCRs 6–7 and the other in the C-terminal SCRs 19–20.

Microbes such as group A streptococci [150] and *Neisseria* [151] have been shown to bind to the first site, for example the FH binding protein (FHbp) of *Neisseria meningitidis* binds to SCR6 [152].

Binding via SCRs 6–7 facilitates also utilization of FHL-1, which contains SCRs 1–7 of FH. Many microbes have been shown to bind both FH and FHL-1 [153].

The mechanisms of interaction with FH/FHL-1 and complement protection have been studied in more detail with the Gram-positive bacterium *Streptococcus pyogenes*. Both FH and FHL-1 bind to the streptococcal M5 and M6 proteins, and under physiological conditions a preferential binding of FHL-1 has been observed. In an M-protein bound conformation FHL-1 retains its cofactor activity and by promoting C3b degradation it can contribute to resistance against phagocytosis. The sites required for the interaction of the two proteins have been mapped: the surface-exposed hypervariable region of the streptococcal M protein interacts with the C-terminal SCR7 of the FHL-1 protein. Probably the M protein has an additional binding site for FH, but despite all the studies no other sites have been identified so far.

However, it seems that the majority of microbes utilize both sites to bind to FH: for instance, *B. burgdorferi* sensu stricto, which causes Lyme disease, binds to FH SCR7 using protein CRASP-1 [154] and to SCRs 19–20 using outer surface protein E (OspE) and its paralogs [155]. This ability for dual binding facilitates efficient protection against the AP attack.

Due to the high homology between the C-terminus of FH and C-termini of FH-related proteins (FHRs), some microbes bind also certain FHRs but the significance of this phenomenon for immune evasion is not yet clear.

The binding site in the C-terminal was analysed in more detail in a study comparing Gram-negative, Gram-positive, and eukaryote microbes known to bind FH, and using three microbial proteins, OspE (from B*. burgdorferi* sensu stricto), FhbA (from *B. hermsii*), and Tuf (from *P. aeruginosa*). All the pathogens and the proteins analysed share a common binding site in SCR 20 that overlaps but is not identical with the heparin and cellular binding sites [156].

Interestingly, recent studies indicate that the FH 402H polymorphism may relate to a better resistance from certain bacterial infections. Haapasalo et al. showed that the AMD-associated factor H 402H variant has a lower binding affinity to various streptococci compared to the 402Y variant, resulting in a more efficient opsonization and phagocytosis [157, 158]. These data point to a pathogen-driven establishment of this common polymorphism in the human population, with evolutionary advantage against bacterial infection at the expense of late-age adverse effects in developing AMD. This example raises the possibility that other factor H polymorphisms have similarly spread in the human population because of the evolutionary race between humans and their pathogens.

Moreover, interaction of FH with HIV has been previously described by our group [159–161]. Envelope glycoproteins gp41 and gp120 have been shown to bind to FH and target regions on these proteins have been characterized, a possible biding region in FH has been identified in SCR13 but a full characterisation is still needed.

AIM OF THE WORK

Quantification of FH serum levels and assessment of its functionality are crucial for patients characterisation in all FH-associated diseases but, due to the high degree of sequence similarity between FHR proteins and FH, tests specific only for FH are difficult to develop. Currently available methods for the quantification of serum FH are often not specific as they recognise both FH and FHR proteins, thus leading to an overestimation of real FH levels. Assays for the structural and functional characterisation of FH from individual subjects are available, but are usually cumbersome or time-consuming and require complex purification procedures [162].

Many methods for the purification of FH have been reported, these are mainly based on multiple chromatography steps needed to obtain a pure fraction with minor contamination from FHRs proteins, but they require long time and often lead to a partial degradation of FH.

FH has been shown to interact with several pathogens, but whereas for some of them interaction with FH has been well described, for others the direct "interactors" still remain unknown. Interaction of FH with HIV has been previously reported by our group [159, 160] but up to the present only target regions on HIV envelope glycoproteins have been characterized whereas an analysis of the binding domains on FH is still needed.

The present work had a dual purpose with the common aim to better understand the role of FH in different pathologies.

The first aim of my project was to develop FH-specific assays, using a monoclonal antibody (MAb 5H5) produced in our laboratory [160]. The antibody was first characterised and its ability to recognise specifically FH without any cross-reaction with FHR proteins was verified. The antibody was then used to purify large amounts of highly purified FH and to set up a group of FH-specific assays.

Two tests respectively for the specific quantification of FH in patients' sera and for the analysis of its structural integrity were first developed and used

to screen aHUS and AMD patients. Later two functional assays to assess both the N-terminal and C-terminal activity of FH have been set up and used to test different samples.

The second purpose was the cloning and expression of the entire FH or partial fragments in both an eukaryotic and a prokaryotic system, in order to produce glycosylated and unglycosylated FH.

The recombinant proteins will be useful for different purposes. First of all they can be used to screen which parts of FH are involved in interaction with proteins of known pathogens and to explore interaction with other pathogens, and also to test the importance of glycosylation in these interactions. Secondly, their ability to restore the function of a defective FH or to protect against FH autoantibodies can be analysed, with the final goal to use them for therapy in many diseases caused by FH defects for which the only possible treatment is currently plasma exchange.

MATERIALS AND METHODS

5. Production of 5H5 Monoclonal antibody

5.1. Suspension cell culture

A murine hybridoma (5H5) producing a monoclonal antibody (MAb) against human FH was thawed and cultivated in T25 flasks with RPMI1640 medium (Euroclone) containing 20% Fetal Bovine serum (FBS, Euroclone), 1x PenStrep (Euroclone) and 4 mM L-glutamine (Euroclone) in a humidified 37°C, 5% CO_2 incubator. Subsequently cells were subcloned and maintained in T75 flasks in RPMI1640 complete medium with FBS reduced to 10%. Aliquots were periodically harvested by centrifugation, frozen in 1 ml FBS aliquots containing 10% (v/v) DMSO (Sigma) and stored at -135°C. Production of MAb was periodically checked by a home-made ELISA on supernatants.

5.2. High density cell culture

In order to enhance antibody production the CELLine Bioreactor (CL1000) (INTEGRA Bioscience) has been used. This system permits to cultivate cells at high densities (up to 10^7-10^8 cells/ml) for a long periods allowing for a high production of antibodies, 50-100 times higher than in static culture. The bioreactor is a flask with two compartments, a cell compartment and a medium compartment, divided by a 10 KDa semipermeable membrane. This membrane allows small molecules, as nutrients and waste products, to diffuse from one compartment to the other, whereas higher molecular weight molecules secreted by the proliferating cells are retained within the cell compartment.

The medium compartment was filled with 1 L of RPMI1640 medium containing 1x PenStrep and 4 mM L-glutamine which was completely changed every 14 days. $2.5x10^7$ hybridoma cells were collected by centrifugation from static suspension cultures, resuspended in 15 ml

RPMI1640 medium containing 10% FBS, 1x PenStrep and 4 mM L-glutamine and seeded in the cell compartment. Every 7 days all liquid was collected from the cell compartment, cells were splitted 1:5 into fresh medium and reseeded into the same chamber. The supernatant was checked for the presence of antibody by ELISA test before storing it for subsequent antibody purification.

5.3. Purification of 5H5 antibody

A ProteinG-Agarose column was prepared in order to purify 5H5 monoclonal antibody from both static or high density culture supernatant.
1 g of Protein G-Agarose resin (Sigma P7700) was rehydrated in 25 ml of deionized water in agitation for 30 min. The resin was then thoroughly washed on a Büchner funnel with gentle vacuum using 50 ml of deionized water in order to remove the lactose stabilizer. The washed resin was then resuspended in PBS buffer and packed into the column.
For antibody purification the ProteinG-Agarose column was attached to the Econo-System (Bio-Rad). Column was washed at 2 ml/min rate first with PBS for 15 min, then with 10 ml of 0.1 M Glycine pH 2.7 and again with PBS until pH reached 7.4. 20 ml of cell culture supernatant were added to the column until they completely entered the column then the Ab-depleted fraction (Flow-through) was collected and column was washed again with PBS for 10min.
The antibody was then eluted in 0.1 M Glycine pH 2.7 monitoring OD in order to decide when to start collecting fractions. The eluates were immediately neutralised with Tris-HCl 1.5 M pH 8.8 (20 µl/ml). The collected fractions (Eluate) containing 5H5 antibody were stored for further analysis. Finally the column was washed with PBS for 15 min and stored in PBS-NaN_3 (2%) at 4°C until next purification.
Eluted fractions were analysed by SDS-PAGE on a 12% gel followed by Coomassie staining with BioSafe Coomassie (Bio-Rad).

Fractions from different runs containing the antibody were pooled together and concentrated using Centriprep-3 (Amicon) or Vivaspin-20 (30 KDa cut-off) (Sartorius) concentrators following manufacturer's instructions.

6. Purification of factor H

6.1. Preparation of 5H5-affinity column

Concentrated antibody preparations were desalted using EconoPac DG10 columns (Bio-Rad), according to the following protocol:

1. Remove the upper cap, and pour off the excess buffer above the top frit.
2. Add 20 ml of *coupling buffer* (0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.4) to the column (fill to the 30 ml mark), and snap off the bottom tip to start the column flowing.
3. Allow the buffer to drain to the top frit. The column will not run dry. Flow will stop when the buffer level reaches the top frit.
4. Add 3.0 ml of concentrated antibody to the column.
5. Allow entire sample to enter the column and discard the first 3.0 ml of effluent.
6. Add 4.0 ml of *coupling buffer* to elute the antibody, while collecting the 4.0 ml fraction from the column.

Protein amount from concentrated fractions before and after desalting was determined by Bradford Protein assay (Bio-Rad).

Column preparation:

1. Collect 500 mg of Cyanogen Bromide-Activated Sepharose 4B resin (Sigma) into a 50-ml tube
2. Washed four times with 50 ml of 1 mM HCl
3. Leave the last wash in incubation for 10 min
4. Filter the resin on a Büchner funnel
5. Wash with milliQ water
6. Wash with *coupling buffer* (0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.4)

7. Collect washed resin into a 15-ml tube and resuspend it in 10 ml of *coupling buffer* containing 5 mg of desalted 5H5 antibody
8. Incubate at RT in agitation for 2h
9. After incubation, collect the coupling buffer with unbound antibody
10. Wash the resin twice with coupling buffer
11. Incubate the resin in 0.2 M Glycine pH 8.0 overnight at 4°C
12. Wash the resin alternatively with *coupling buffer/acetate buffer* (0.1 M acetic acid, 0.5 M NaCl, pH 4.0) four times
13. Put the resin into a column and equilibrate with PBS for 15min.
14. Store the prepared 5H5-Sepharose column in PBS-NaN3 (2%) at 4°C until use.

6.2. Purification of factor H

Human factor H was purified from human serum by affinity chromatography on 5H5-Sepharose column. A set up of the protocol was necessary to achieve an efficient purification without any contaminant. It was necessary to test different elution protocols changing pH and salt concentration of solutions in order to obtain a perfectly purified protein. The final protocol is the following.

The 5H5-Sepharose column was attached to the Econo-System (Bio-Rad) and washed at 1 ml/min first with PBS for 10 min, then with 10 ml of 0.1 M Glycine pH 2.7 and finally with 0.5 M NaCl in PBS until pH reached 7.4. Before adding it to the column 2 ml of human serum were brought to a final concentration of 0.5 M NaCl and filtered through 0.22 µm filters. The filtered human serum was allowed to completely enter the column at 0.25 ml/min rate, then the column was washed with 0.5 M NaCl in PBS at 1 ml/min rate and the fraction of FH-depleted serum (about 15 ml) was collected until OD dropped. Finally FH was eluted from the column with 0.1 M Glycine pH 3.0 at 1 ml/min monitoring OD to decide when to start the collection, typically 6 ml of eluate (Purified Factor H) were collected. The eluate was immediately neutralised with Tris-HCl 1.5 M pH 8.8 (20 µl/ml).

The column was further washed with 0.1 M Glycine pH 2.7 in order to eliminate possible contaminants and finally washed with PBS and stored in PBS-NaN_3 (2%) at 4°C until next purification.
Eluted fractions were analysed by SDS-PAGE on a 8% gel in reducing and non-reducing conditions followed by Coomassie staining with BioSafe Coomassie (Bio-Rad).
Positive fractions containing purified FH were pooled together and concentrated using Vivaspin-20 (30 KDa cut-off) concentrators (Sartorius) following manufacturer's instructions.

7. Factor H ELISA

A home-made ELISA test to determine FH concentration in biological fluids was developed. This assay is a sandwich ELISA that uses MAb 5H5 as the catcher and a FH-specific chicken antibody, also produced in our lab, as the tracer together with 5H5-affinity purified FH as a standard.
Several adaptations have been made to the protocol to achieve the best results. Tris-Buffered Saline (TBS) pH 7.65 has been shown to be a better coating buffer than the traditional carbonate/bicarbonate buffer pH 9.6 for 5H5 antibody. The use of an HRP-conjugated secondary antibody instead of the previously used AP-conjugated, and the consequent change of chromogenic substrate from BluePhos® to TMB enhanced the sensitivity of the test.
Controls without coating and without FH were also included during the set up to assess test's specificity.
The final detailed protocol is described in the appendix.
When aqueous humour and vitreous humour samples were used in the test, working dilutions were as follows:

- aqueous humour: 1:5 and 1:10
- vitreous humour: 1:5, 1:10 and 1:50

8. IgG and Albumin Depletion

Albumin and IgG were depleted from normal human serum using Hi-Trap™ Albumin & IgG depletion columns (GE Healthcare) according to the following protocol.

Buffers:

- Binding buffer: 20 mM sodium phosphate, 0.15 M NaCl, pH 7.4
- Elution buffer: 0.1 M glycin-HCl, pH 2.7

Sample preparation:

Human serum was centrifuged at 14,000 g for 10 min. at 4°C to eliminate debris and 150 µl were diluted 1:10 in binding buffer.

Depletion procedure:

The column was connected to a peristaltic pump and a flow rate of 1 ml/min was used for the entire procedure.

A modified protocol from the manufacturer datasheet was used as follows:

1. Fill the pump tubing with *binding buffer*. Remove the stopper and the snap-off end from the column and connect it to the pump tubing 'drop-to-drop' to avoid introducing air into the system.
2. Wash the column with 5 ml *binding buffer* to remove the 20% ethanol storage solution.
3. Equilibrate with 10 ml of *binding buffer*.
4. Apply the diluted human serum to the column aspirating it directly from the tube and then wash with 7 ml of binding buffer. Collect the flow through (**depleted serum**) during sample application and wash (total 8.5 ml).
5. Elute albumin and IgG from the column with 5 ml *elution buffer* and immediately neutralise with 100 µl Tris-HCl 1.5 M pH 8.8.

9. Western Blot (WB)

Serum samples and samples deriving from different purification procedures were subjected to SDS-PAGE in both reducing and non-reducing conditions. For SDS-PAGE precast 4-12% or 12% NuPAGE® Novex®

Bis-Tris Protein Gel (LifeTechnologies) Mini or Midi were used and run in MOPS 1X buffer at 200 V for 40-60 min. depending on the different gels and formats.

Gels were blotted on a PVDF membrane using iBLOT (LifeTechnologies) with protocol P3 for 7-10 min. Membranes were blocked with 3% BSA in TBS/Tween 0.5% 2h RT or overnight at 4°C.

Different primary antibodies were used (Table 1) to reveal FH, FHRs, C3b and FI. Primary antibodies were typically diluted in 1% BSA in TBS/Tween 0.5% and applied to the membranes 1h RT in constant agitation. Membranes were then washed 3 times with TBS/Tween 0.1% and incubated 1h RT in constant agitation with the corresponding Alkaline phosphatase (AP) conjugated secondary antibody diluted in 1% BSA in TBS/Tween 0.5%. After 3 washes in TBS/Tween 0.1%, specific signal was revealed with SigmaFast™ BCIP/NBT (Sigma).

Table 1. Antibodies

Protein	***Primary Antibody***		***Company***	***Dilution***	***Notes***
FH	5H5	MAb	Our laboratory	1:15000	Specific for FH
	chicken anti-FH	PAb	Our laboratory	1:10000	Specific for FH
	sheep anti-FH	PAb	TheBindingSite	1:2000	Sees also two unspecified bands around 75 KDa
FHR-1	rabbit anti-CFHL1	PAb	Abcam	1:1000	Sees also FHR-2
	rabbit anti-CFHR1	PAb	Abnova	1:500	Sees also FHR-2
FHR-2	rabbit anti-CFHR2	PAb	Abnova	1:500	Sees also FHR-1
FHR-3	rabbit anti-CFHR3	PAb	ProteinTech	1:500	Sees FH, FHR-3 band is uncertain
FHR-4	rabbit anti-CFHR4	PAb	Abcam	1:500	Sees also FH
FHR-5	rabbit anti-CFHR5	PAb	Abnova	1:500	Specific for FHR-5
FI	mouse anti-FI (Supernatant)	MAb	Serotec	1:100	
	sheep anti-FI	PAb	Sigma	1:2000	
C3b	goat anti-C3	PAb	Sigma	1:2000	Sees different fragments of C3

10. Factor H Immunoprecipitation (IP)

To isolate FH from patients' sera an IP micro-method was developed using 5H5-Sepharose resin, in order to allow purification from small amounts of sample. The final standard IP protocol was as follows:

1. Incubate in a 2.0-ml eppendorf tube 50 µl of serum or plasma with 50 µl of 5H5-sepharose resin in 500 µl of PBS for 30 min on a rotator mixer
2. Centrifuge samples for 2 min at 13,000 rpm in a table-top microcentrifuge
3. Collect in a new tube supernatant containing FH-depleted serum
4. Wash resin once with PBS/Triton 0.1% and three more times with PBS, at each round centrifuge the tubes for 1 min at 13,000 rpm to pellet the resin and discard the wash buffer
5. Elute FH from the resin with 200 µl of 0.1 M Glycine pH 2.7, incubating it for 15 min on a rotator mixer and centrifuge the tubes for 1 min at 13,000 rpm to recover the eluate
6. Transfer the eluate into a new tube contain 4 µl of Tris-HCl 1.5 M pH 8.8 to neutralise it
7. Perform a second elution step in the same conditions to ensure that all the FH is eluted from the resin
8. Wash the resin several times with PBS

The method was further developed by the use of Micro Bio-Spin™ Columns (Bio-Rad) which allow for a faster procedure. The final protocol was as follows:

1. Incubate in the capped micro column 50 µl of serum or plasma with 50 µl of 5H5-sepharose resin in 500 µl of PBS for 30 min on a rotator mixer
2. Snap off the tip and place the column in a 1.5 ml eppendorf tube to collect the "FH-depleted serum". Remove the cap and push it back on the column to start the flow. Allow the sample to flow by gravity through the column and eventually centrifuge for 30-60 sec. at 1,000 g to allow all the sample to pass.

3. Wash resin once with PBS/Triton 0.1% and three more times with PBS allowing the buffer to flow by gravity through the column, after the final wash centrifuge for 30-60 sec. at 1,000 g to allow all the buffer to pass.
4. Place the column into a new tube contain 4 µl of Tris-HCl 1.5 M pH 8.8 and elute FH from the resin with 200 µl of 0.1 M Glycine pH 2.7, allowing the sample to flow by gravity through the column and eventually centrifuge for 30-60 sec. at 1,000 g to allow all the sample to pass.
5. Repeat the previous step once
6. Wash the resin several times with PBS

11. Heparin and hydrophobic interaction chromatography (HIC) purification

In order to compare our results, factor H was purified using two other methods (Heparin and HIC) which don't provide a complete separation of FH from FHRs proteins.

11.1. Heparin purification

Heparin purification was performed by the use of HiTrap Heparin HP Columns (GE Healthcare).

Method overview:

As FH and FHRs contain binding sites for Heparin with different binding affinity, they can be purified from serum by affinity chromatography on Heparin. Other serum proteins are also able to bind heparin are they can therefore be purified together with FH and FHRs. To separate proteins according to their binding capacity different salt concentrations can be used in the elution steps. The resin in the column is composed of Heparin covalently coupled to highly cross-linked agarose beads. Heparin used as a ligand is a naturally occurring sulphated glucosaminoglycan which is extracted from the native proteoglycan of porcine intestinal mucosa. It

consists of alternating units of uronic acid and D-glucosamine, most of which are substituted with one or two sulphate groups.

Protocol:

The protocol of the manufacturer was slightly modified as follow.

Buffers:

- Binding buffer: 10 mM sodium phosphate, pH 7 + 50 mM NaCl
- Elution buffer: 10 mM sodium phosphate pH7 + NaCl
    - **E1** 100 mM NaCl
    - **E2** 150 mM NaCl
    - **E3** 200 mM NaCl
    - **E4** 250 mM NaCl
    - **E5** 300 mM NaCl
    - **E6** 400 mM NaCl
    - **E7** 500 mM NaCl
    - **E8** 1 M NaCl

Sample preparation:

300 µl of human serum were diluted 1:10 in binding buffer and filtered through a 0.45 µm filter immediately before to be applied to the column.

15 ml IgG-Albumin depleted serum, which correspond to 300 µl of undiluted serum were brought to a final NaCl concentration of 50 mM before being applied to the column.

Purification procedure:

The column was connected to a peristaltic pump, a flow rate of 0.5 ml/min was used for sample loading whereas 1 ml/min was used for washing and elution.

1. Fill the pump tubing with binding buffer. Remove the stopper and connect the column to the pump tubing, “drop to drop” to avoid introducing air into the column.
2. Remove the snap-off end at the column outlet.
3. Wash out the preservative and equilibrate the column with 10 column volumes of binding buffer.

4. Apply the sample into the column aspirating it directly from the tube
5. Collect the flow through (F.T.) into a new tube
6. Wash with 10 ml of binding buffer
7. Elute different fractions using 5 ml of each elution buffer and wash after each step with 5 ml of the same buffer
8. Store all the samples (F.T. and Eluates) for further analysis

All the fractions were then concentrated and desalted in PBS Buffer using Vivaspin-500 (5 KDa cut-off) (Sartorius) concentrators following manufacturer's instructions. Fractions were subsequently analysed by SDS-PAGE followed by Coomassie staining with BioSafe Coomassie (Bio-Rad), and later by WB.

11.2. Hydrophobic interaction chromatography (HIC)

For HIC purification a column containing Macro-Prep Methyl HIC Support (Bio-Rad) was prepared, serum was loaded in the presence of ammonium sulphate and different fractions were eluted by gradually diminishing the salt concentration.

Method overview:

Hydrophobic interaction chromatography (HIC) separates proteins on the basis of their relative hydrophobicity. The principle for protein adsorption to HIC media is complementary to ion exchange and size exclusion chromatography. Sample molecules containing hydrophobic and hydrophilic regions are applied to an HIC column in a high-salt buffer. The salt in the buffer reduces the solvation of sample solutes. As solvation decreases, hydrophobic regions that become exposed are adsorbed by the media. The more hydrophobic the molecule, the less salt is needed to promote binding. Usually a decreasing salt gradient is used to elute samples from the column in order of increasing hydrophobicity.

FH has been shown to bind to hydrophobic resin and therefore can be isolated with this procedure [163].

The Macro-Prep Methyl HIC Support which is ideal for purifying proteins that have strong hydrophobic regions was chosen for this procedure.

Buffers:

- Buffer A: 10 mM Tris, pH 7.2 + 1.5M Ammonium Sulphate
- Buffer B: 10 mM Tris, pH 7.2

Column preparation:

The Macro-Prep Methyl HIC Resin was resuspended into storage buffer (50% slurry) and packed into a column. The column was extensively washed with Buffer B and later equilibrated with Buffer A.

Sample preparation:

1 ml of human serum was diluted 1:3 into Buffer A and brought to 1.5 M $(NH_4)_2SO_4$ adding 200 mg Ammonium Sulphate. The tube was left in the end-to-end platform until the salt was dissolved. The sample was centrifuged at 15,000 g for 15 min. to recover precipitates whereas the supernatant was used for chromatography.

Setting up the Econo Chromatography System:

The column was connected to Econo-System (Bio-Rad) and the sample supernatant (about 2.5 ml) was directly loaded on the Methyl HIC column. Flow through which doesn't bind to the column was collected (100% Fraction). A gradient, mixing Buffer A and Buffer B in order to gradually diminish Ammonium Sulphate content, was set on the instrument and three fractions (66%, 33% and 0%) were collected monitoring OD_{280}.

Collected fractions:

- **100%** 1.5 M Ammonium Sulphate
- **66**% 1 M Ammonium Sulphate
- **33%** 0.5 M Ammonium Sulphate
- **0%** no Ammonium Sulphate

All the fractions were concentrated and desalted in Buffer B using Vivaspin-500 (5 KDa cut-off) (Sartorius) concentrators following manufacturer's instructions. Fractions were subsequently analysed by SDS-PAGE followed by Coomassie staining with BioSafe Coomassie (Bio-Rad), and later by WB.

12. Factor H integrity assay

FH (150 KDa) can be cleaved by a serum protease between amino acids 341-342, thus generating two fragments (142 KDa and 38 KDa) that are held together by a disulphide bridge and are visible as separate molecular species only after reduction. This cleavage can be mimicked in vitro by trypsin. In order to develop a test capable of detecting the presence of the cleaved form of FH it was necessary to test the ability of 5H5 to bind it, using trypsin-cleaved FH.

12.1. Trypsin cleavage of FH

Reagents:

- Trypsin TPCK treated from bovine pancreas (Sigma - T1426) - 1mg/ml in 1mM HCl
- Trypsin inhibitor from Glycine max (soybean) (Sigma - T9003) - 1mg/ml in H_2O
- 5H5-affinity purified FH

Protocol:

Trypsinization of FH was performed with a FH:trypsin ratio of 100:1 (w/w) (1 µg of FH + 10 ng of trypsin) in TBS pH 7.5, incubating at 37°C for different times.

Digestion was stopped by adding twofold weight excess of soybean trypsin inhibitor.

12.2. IP of Trypsin-cleaved FH

A sample with 50% of trypsin-cleaved FH was immunoprecipitated with 5H5-coupled Sepharose resin, as described previously. Samples before IP, and different fractions of IP were analysed by SDS-PAGE followed by Coomassie staining and densitometric analysis with the program TotalLab.

12.3. Integrity assay

After demonstrating that 5H5 recognizes and immunoprecipitate with the same affinity both the uncleaved and cleaved form of FH, this important feature was used to set up a test for the integrity of FH in different samples.

The test consists in the 5H5-affinity immunoprecipitation of FH from serum samples or from FH preparation followed by SDS-PAGE and Coomassie staining. A 50% trypsin-cleaved FH is used as a control.

13. Factor H functional assays

Two tests to screen N-terminal cofactorial activity and C-terminal protection activity of FH have been set up, namely the “Cofactor assay” and the “Haemolysis test”.

13.1. Quantification of Purified Factor H

The exact quantification of purified FH samples was a crucial point in order to perform the functional assays. Different quantification methods were used and compared: Bradford method with Bio-Rad protein assay, our home-made FH ELISA and spectrophotometric determination at 280 nm.

Bio-Rad protein assay was performed following manufacturer instructions, the home-made ELISA developed for serum and plasma sample was used as described previously.

Spectrophotometric determination was done with a Nanodrop instrument. PBS was used as a blank and absorbance at 280 nm was measured. To establish FH concentration an extinction coefficient for a 1% solution ($E_{1\%}$) of 12.4 was used, as reported by others [164].

This formula was used to calculate the concentration expressed in µg/ml:

$c = (A_{280}/E_{1\%} * 10) * 1000$

13.2. Cofactor assay:

This test is based on the fact that FI can cut C3b in vitro only in the presence of FH; if FH is not functional C3b cannot be cleaved.

All the samples to be used in this test were brought into PBS buffer and their concentration was established as previously reported.

In the test we used purified C3b and FI (Merck Millipore) and 5H5-affinity purified FH.

The test was set up with different amounts of purified proteins and with different incubation times. FH concentration was gradually scaled down to assess test sensitivity.

The final protocol used to screen samples was as follows:

500 ng of purified C3b and 250 ng of purified FI were incubated in PBS for 30 min at 37°C alone or in the presence of different amounts of 5H5-affinity purified FH. After incubation samples were put on ice and SDS sample buffer was immediately added to stop the reaction. Samples were loaded on a SDS-PAGE gel and analysed by WB using a polyclonal anti-C3b antibody (Sigma) or by Coomassie staining.

13.3. Haemolysis test

This test to assess the activity of the C-terminus is based on the fact that FH is able to protect Sheep erythrocytes from complement attack. If complement is activated erythrocytes undergo lysis that can be measured by the release of haemoglobin using a microplate reader.

The test has been first set up using a control serum with known amount of functional FH. Protocol was adapted from "AH_{50} Assay for Total Alternative Pathway Hemolytic Activity" Giclas (2001) [165].

Due to the impossibility to purchase Veronal buffer that is normally used in this kind of test it was necessary to set up the test using two different buffers:

- A modified Veronal buffer adapted using a commercially available buffer similar to the one needed: Veronal Buffer 5X (Lonza)
- HEPES buffer adapted from Moreno-Indias et al. [166]

The final detailed protocol is described in the appendix.

The test was then performed adding different concentration of EDTA to serum. This was necessary to test the minimum concentration of EDTA that still allows lysis, since depleted serum needs to be prepared in the presence of EDTA in order to prevent complement activation during the procedure.

The final part of the test that makes use of FH-depleted serum alone or with different amounts of FH to be tested is still under development.

14. Cloning of factor H

The sequence of human factor H RNA (NM_000186.3) has been divided into three main fragments containing approximately 7 SCR each (SCR 1-7, SCR 8-14, SCR 15-20) and specific PCR primers were designed (Table 2). Quality and specificity of primers was checked with Primer Premier software (Primer Biosoft).

14.1. Retrotranscription

Human Liver total RNA (Life Thechnologies) was retrotranscribed with two methods: iScript cDNA Synthesis Kit (Bio-Rad) which contains a mix of Random Hexamers and Oligo(dT) or with ImProm-II™ Reverse Transcription System (Promega) which enables the use of Oligo(dT) or Specific primers in different reactions. A modified protocol was used.

14.1.1. RNA denaturation

Samples for different retrotranscription reactions were prepared as follows and denatured at 70°C for 5 min followed by a 10 min incubation on ice.

	cDNA-1 (ImProm)	*cDNA-2 (ImProm)*	*cDNA-3 (iScript)*
Oligo(dT)$_{15}$	1 µl	-	-
FH-specific primers	-	1 µl	-
RNA	1 µl	1 µl	1 µl
Nuclease free H_2O	3 µl	3 µl	4 µl

14.1.2. Retrotranscription reactions

Different reaction mixes were prepared and added to the samples:

RT mix ImProm		RT mix iScript	
5x ImProm reaction buffer	4 µl	5x reaction mix	4 µl
MgCl2	2.5 µl	iScript RT	1 µl
dNTPs	1 µl	Nuclease free H_2O	5 µl
RNAse Inhibitor	1 µl		
ImProm RT	1 µl		
Nuclease free H_2O	5.5 µl		

Retrotranscription was performed with the following thermic protocol:

25° C	5’
42° C	60’
85° C	5’

cDNA quality was checked by PCR using beta-actin primers when possible.

14.2. Amplification

The entire FH and the three fragments have been amplified using Phusion™ DNA Polymerase (Thermo). Regular HF buffer was substituted with CG Buffer, which is normally used for difficult templates, as FH cDNA tends to form secondary structures and amplification results challenging.

Mix			Thermic protocol		
5x CG buffer	4 µl		98° C	30”	
dNTPs (10mM)	0.4 µl		98° C	10”	
Primer F (10µM)	1 µl	*	64° C	20”	X 35
Primer R (10µM)	1 µl		72° C	50”*	
Phusion	0.2 µl		72° C	5’	
cDNA	2 µl				
dH_2O	up to 20 µl				

* for the amplification of the entire FH 0.8 µl of dNTPs and 2’ extension were used.

Amplification products were checked and quantified on a agarose gel.

14.3. Features of the pFastBac™/HBM-TOPO® vector

The pFastBac™/HBM-TOPO® vector (Life Technologies) was chosen for cloning and expression into insect cells. The vector contains the following elements:

- Strong **polyhedrin (PH) promoter** for high-level baculovirus-based protein expression in insect cells
- **TOPO® Cloning site** for rapid and efficient cloning of blunt-end PCR products amplified with proofreading polymerases
- N-terminal **Honey Bee Mellitin (HBM) secretion signal** coding sequence for secretion of the cloned gene product into the extracellular medium
- C-terminal **polyhistidine tag** for simple purification of recombinant proteins
- **TEV protease cleavage site** for removal of the polyhistidine tag following protein purification using AcTEV™ protease
- **SV40 polyadenylation signal** for efficient transcription termination and polyadenylation of the recombinant transcript
- **Mini-Tn7** elements for site-specific transposition of the gene of interest into the baculovirus shuttle vector (bacmid DNA) propagated in *E. coli*
- **Ampicillin (bla) resistance gene** (β-lactamase) for selection of transformants in *E. coli*

- **pUC origin** for high copy replication and maintenance of the plasmid in *E. coli*
- **Gentamicin resistance gene** for selection of transformants containing recombinant bacmid DNA

14.4. Blunt-End TOPO® Cloning Reaction

The amplified fragments were inserted into the pFastBac™/HBM-TOPO® vector (Life Technologies) using TOPO-cloning.

All the samples were brought to a concentration of 5 ng/µl and an insert:vector ratio of 2:1 was used in the in a TOPO® Cloning reaction. The 6-µl reactions were prepared as follows:

PCR Product (5 ng/µl)	4 µl
Salt Solution (200 mM NaCl, 10 mM $MgCl_2$)	1 µl
pFastBac™/HBM-TOPO® vector	1 µl

and incubated 5 min at RT.

14.5. Transformation and analysis of tranformants

The TOPO reactions were used to transform Mach1™ T1R Chemically Competent *E. coli* (Life Technologies) following the manufacturer protocol. Plates were incubated overnight at 37°C.

Some colonies were screened by Colony PCR to check the orientation of the insert, two parallel PCR reactions were performed:

- **PCR Forward**: which detects the fragment inserted in the correct orientation.
  In this PCR a forward primer on the insert together with SV40 reverse primer on the vector is used. (Table 2)
- **PCR Reverse**: which detects the fragment inserted in the correct orientation
  In this PCR a reverse primer on the insert together with SV40 reverse primer on the vector is used, if the fragment is in the wrong orientation the reverse primer on the insert functions as a forward primer giving amplification. (Table 2)

After this first screening, plasmid DNA from positive ones was isolated by Mini-prep using Eurogold Plasmid kit (Euroclone) according to the manufacturer protocol.

Plasmid DNA was then sequenced using primers located on the vector (Table 2) and sequences were analysed with CLC Sequence Viewer (CLC bio).

14.6. Cloning into a prokaryotic system

Primers to clone the same fragments into a prokaryotic system were also designed. (Table 2)

These primers will be used to amplify the fragments directly from the previously generated pFastBac™/HBM clones, fragments will be subsequently inserted into *E. coli* expression vector using the StarGate® IBA System (IBA BioTAGnology).

Table 2. Primers

Primer	***Sequence*** (5'-3')
PCR primers for cloning into the eukaryotic system	
FH-SCR1-F	GAAGATTGCAATGAACTTCCTC
FH-SCR7-R	GACACGGATGCATCTGG
FH-SCR8-F	GTCAAAACATGTTCCAAATCAAG
FH-SCR14-R	TTTTTCAACACAGAGTGGTATTG
FH-SCR15-F	AAAATTCCATGTTCACAACCAC
FH-SCR20-R	TCTTTTTGCACAAGTTGGATAC
Colony PCR primers	
SV40 polyA-rev	GGTATGGCTGATTATGATC
	Used together with PCR specific primers
Sequencing primers	
pFASTBAC-F	TCCGGATTATTCATACCGTCCC
pFASTBAC-R	CCTCTACAAATGTGGTATGGCTG
PCR primers for cloning into the prokaryotic system	
Star-FH-SCR-1F	AGCGGCTCTTC**AATG**GAAGATTGCAATGAACTTCCTC
Star-FH-SCR-7R	AGCGGCTCTTC**TCCC**GACACGGATGCATCTGGGA
Star-FH-SCR-8F	AGCGGCTCTTC**AATG**GTCAAAACATGTTCCAAATCAAG
Star-FH-SCR-14R	AGCGGCTCTTC**TCCC**TTTTTCAACACAGAGTGGTATTG
Star-FH-SCR-15F	AGCGGCTCTTC**AATG**AAAATTCCATGTTCACAACCAC
Star-FH-SCR-20R	AGCGGCTCTTC**TCCC**TCTTTTTGCACAAGTTGGATAC

15. Production and Purification of 5H5 antibody

High density hybridoma cultures with CELLine Bioreactor enabled to produce a consistent amount of MAb 5H5, mean antibody production was about 1.5-2 mg/day.

The quality of purified antibody checked by SDS-PAGE in reducing conditions followed by Coomassie staining was good, and purity was about 90-100%. Two major bands are visible on the gel, a 50 KDa band corresponding to antibody's heavy chains and a 25 KDa band corresponding to light chains, an upper band due to incomplete reduction can also be present. (Fig. 13)

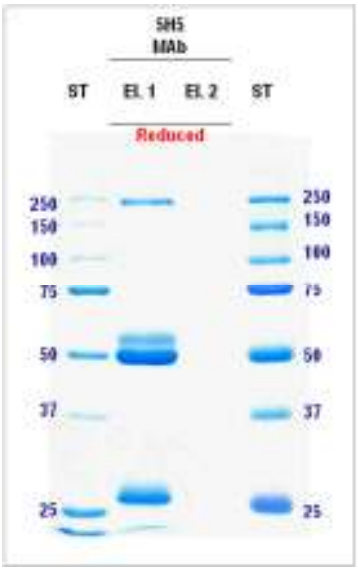


Fig. 13 Coomassie of purified MAb 5H5: two elution fractions are shown (El.1 and El.2), antibody elute in the first fraction and bands corresponding to heavy (50 KDa) and light (25 KDa) chains are clearly visible.

MAb 5H5 antibody has been tested in Western Blot and fractions of total human serum, albumin- and IgG-depleted serum and albumin and IgG purified from serum have been analysed. Under non-reducing conditions a clear band around 150 KDa corresponding to FH is visible in total serum

and depleted serum. Both in total serum and in the fraction containing albumin and IgG a non-specific signal is present, due to cross-reaction of secondary antibody (anti-mouse IgG) with human immunoglobulins as confirmed by specific controls (Fig. 14). WB with MAb 5H5 was performed only in non-reducing condition as the antibody recognises a conformation-dependent epitope.

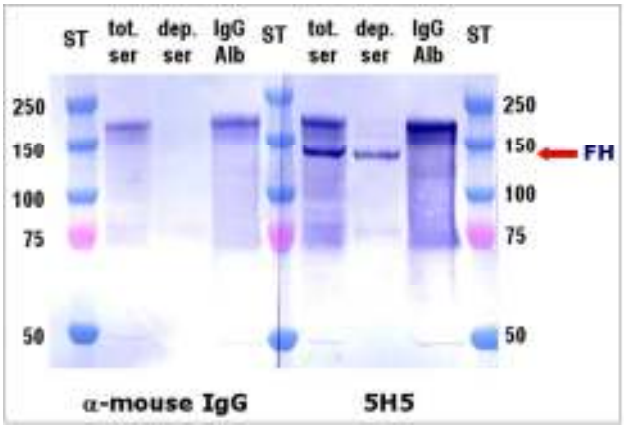


Fig. 14 WB of FH from human serum: fractions of total human serum (tot. ser), albumin- and IgG-depleted serum (dep. ser) and albumin and IgG purified from serum (IgG Alb) have been analysed with 5H5 antibody in non-reducing conditions. FH band around 150 KDa is visible in total serum and depleted serum, non-specific signal present in total serum and in IgG-albumin fraction is due to cross-reactivity of secondary antibody as shown by specific control on the left. (FH = complement factor H)

16. Purification of FH

Thanks to the high affinity of MAb 5H5 for FH, it was possible to setup a "one-step" purification which gives a highly pure protein, starting from total human serum.

It has been necessary to refine the procedure in order to obtain a 100% pure preparation. Several buffers with different salt concentration and pH have been tested.

The addition of NaCl to a final concentration of 0.5 M into the binding buffer was shown to prevent unspecific binding to the 5H5-affinity column. The best elution buffer was found to be 0.1 M Glycine pH 3.0. At pH 4.0 elution of FH is only partial whereas at pH 3.0 the maximum of elution is achieved, lowering the pH to 2.7 only traces of FH still elute (Fig. 15). If elution is performed directly at pH 2.7 also a small amount of serum albumin co-elutes with FH when purification is performed from total (non albumin-depleted) serum (data not shown).

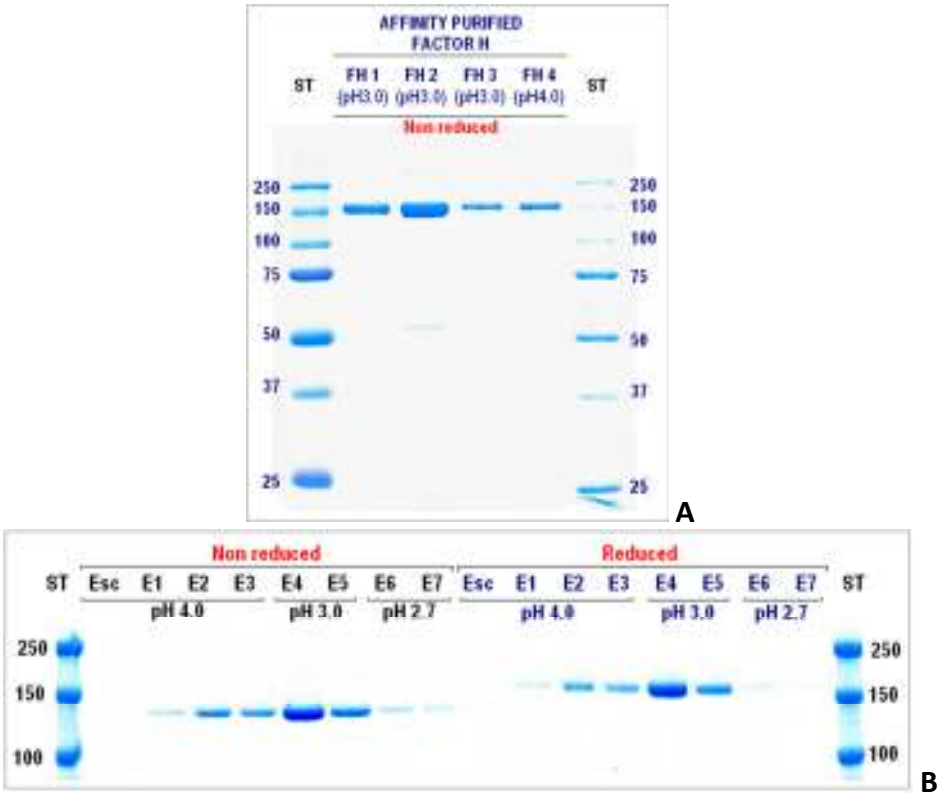
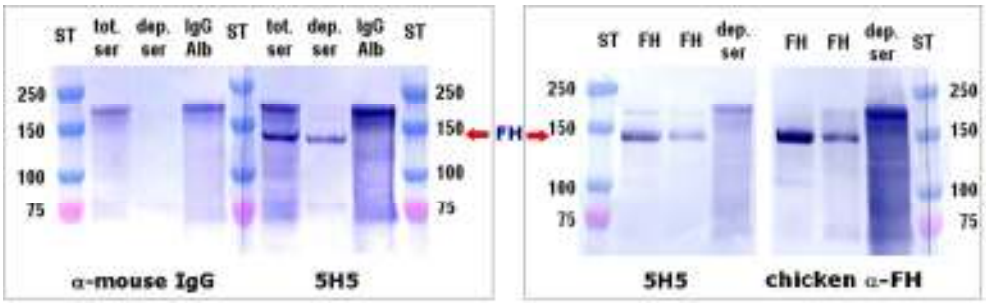


Fig. 15 Coomassie of purified FH (A): three fraction from different purifications at pH 3 (FH1, FH2 and FH3) and one residual fraction at pH 4 (FH4) are shown. Under non reducing conditions a single band corresponding to FH is visible at 150 KDa. **(B)**: Excluded fraction (Esc) and different elution fractions of factor H (E1-7) are shown both in non-reducing and reducing conditions. The maximum of elution is achieved at pH 3.

Efficiency of the method and purity of the affinity purified FH were checked by Western Blot.

Western blot on total serum, purified FH fractions and FH-depleted serum was performed in non-reducing conditions with both MAb 5H5 and polyclonal chicken anti-FH. With both antibodies, a 150 KDa band is present in purified FH fractions whereas it is missing in FH-depleted serum, confirming that depletion was complete (Fig. 16). Non-specific signal due to cross-reaction of the polyclonal or the secondary antibodies with human immunoglobulins is present in total serum and FH-depleted serum (Fig. 16).



A B

Fig. 16 (A) WB of FH from human serum: fractions of total human serum (tot. ser), albumin- and IgG-depleted serum (dep. ser) and albumin and IgG purified from serum (IgG Alb) have been analysed with 5H5 antibody in non-reducing conditions. FH band around 150 KDa is visible in total serum and depleted serum, non-specific signal present in total serum and in IgG-albumin fraction is due to cross-reactivity of secondary antibody as shown by specific control on the left. (FH = complement factor H) **(B) WB of purified FH**: fractions of purified FH (FH) and FH-depleted serum (dep. ser) have been analysed in non-reducing conditions with both MAb 5H5 and polyclonal chicken anti-FH antibodies. FH band (150 KDa) band is present in purified FH fractions whereas it is missing in FH-depleted serum.

Depletion of FH from serum and efficiency of purification was also checked in an ELISA test using purified FH as a standard for precise quantification (Fig. 17).

As shown in Figure 17, a single step of affinity chromatography was able to almost totally deplete the sample of FH, less than 1 µg/ml is still present in depleted serum.

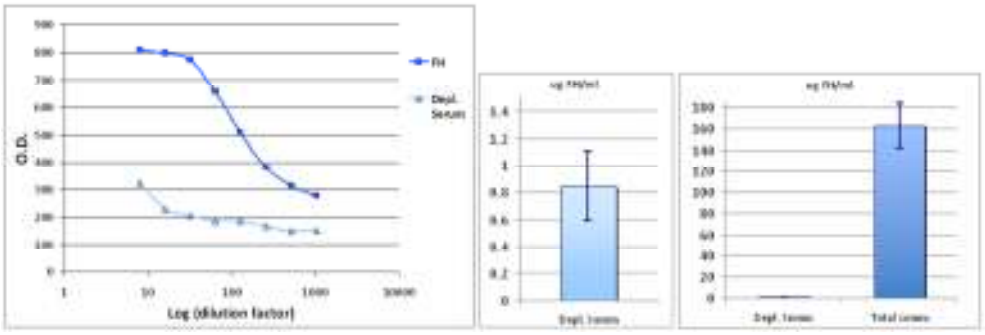


Fig. 17 FH ELISA from depleted serum: OD at different dilutions of FH-depleted serum compared to purified FH (A) and quantification of residual FH in FH-depleted serum alone or compared to total serum (B) are shown.

Moreover purity of the fractions and absence of "contaminating" FHRs proteins was demonstrated by WB using antibodies against different FHRs proteins. The FH purified by 5H5-affinity chromatography was compared with fractions obtained with other purification methods such as Heparin-affinity chromatography and HIC chromatography. In fractions obtained by 5H5-affinity, unlike the ones coming from other purification methods, no FHRs proteins are present (Fig. 18).

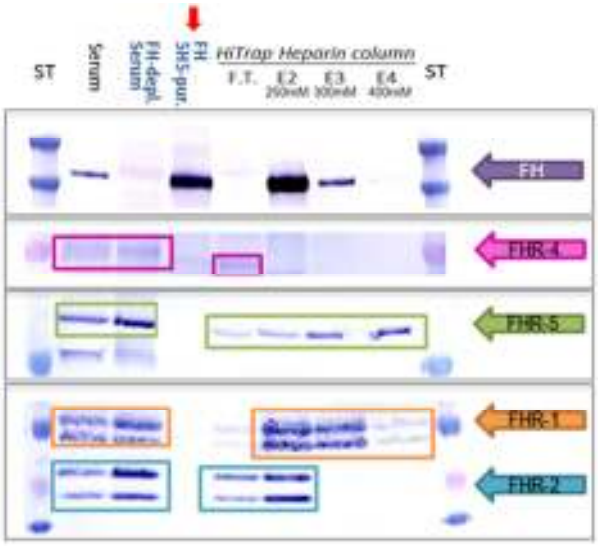


Fig.18 WB of total serum and different fractions of 5H5-affinity purification and Heparin purification developed with antibodies for FH and FHRs: FHRs are completely absent in the 5H5-affinity purified FH indicated with the red arrow.

17. FH quantitative ELISA

An ELISA assay for the specific quantification of serum FH was developed. This assay is a sandwich ELISA that uses MAb 5H5 as the catcher and a FH-specific chicken antibody, also produced in our lab, as the tracer. Serial dilutions of 5H5-affinity purified FH are used to generate a standard curve.
This high specificity of the test is based on the fact that the MAb 5H5, which was shown to recognise only FH, is used in the solid phase.
This test was fine tuned in order to achieve the maximum sensitivity. Efficiency of capture was higher when Microtiter plate coating was performed in TBS at pH 7.65 than it was carbonate/bicarbonate buffer pH 9.6, which is the standard coating buffer, thus coating conditions were accordingly modified. The shift from AP-conjugated to an HRP-conjugated secondary antibody and the consequent change of chromogenic substrate from BluePhos® to TMB also enhanced the sensitivity of the test. The test was validated using control sera sample from human donors and turned out to be reliable, reproducible and with a very good dynamic range (almost three orders of magnitude).
The assay was later used to quantify FH serum levels from aHUS patients and their relatives, compared to controls (Fig. 19). The analysis revealed a FH deficit in four patients, two of them with really low FH levels (<100µg/ml), in the other patients FH serum level was within the normal range.

Finally the method was also used to quantify FH levels in different samples from AMD patients such as plasma, aqueous and vitreous humour.

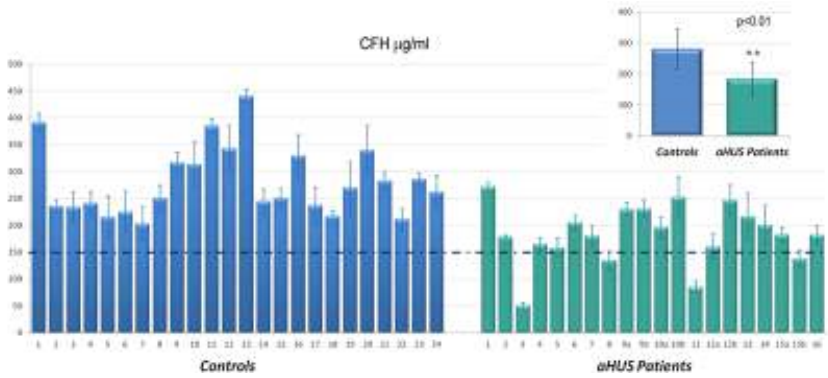


Fig. 19 FH ELISA: Bar Graph representing FH serum levels in controls and aHUS patients are shown. In 4 patients FH levels are below the normal range (indicated by dotted bar). Mean of FH levels is significantly reduced in patients compared to controls

18. Factor H Immunoprecipitation (IP)

A micromethod to purify FH from small amounts of sample was developed using 5H5-coupled Sepharose resin. The minimum amount of plasma needed is 20 µl but the best results are achieved with 50 µl. The method resulted to be really efficient as FH is completely depleted from plasma and a single band corresponding to FH is present in the purified fractions (Fig. 20).

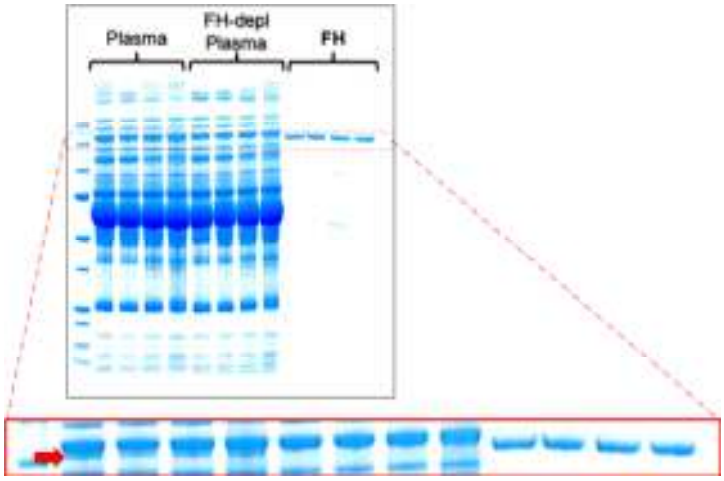


Fig. 20 FH IP: IP from 4 plasma samples; total plasma, FH-depleted plasma and immuoprecipitated FH are shown. Depletion of FH from plasma is complete as shown by the arrow

The method was further improved by the use of Micro Bio-Spin™ Columns (Bio-Rad) which allow for a faster procedure.

19. Trypsin cleavage and integrity assay

19.1. Cleavage of FH by trypsin

FH (150 KDa) can be cleaved by a plasmatic protease into two fragments (142 KDa and 38 KDa) that are held together by a disulphide bond. This cleavage can be mimicked *in vitro* by trypsin. In order to develop a test to detect the presence of cleaved FH it was necessary to obtain trypsin-cleaved FH. 5H5-affinity purified FH was subjected to digestion with trypsin using different incubation times and samples were analysed by SDS-PAGE under reducing conditions followed by Coomassie staining.

As the incubation time increases the band corresponding to intact FH (180 KDa, in reducing conditions) decreases, whereas the bands corresponding to the two cleaved fragments (142 KDa and 38 KDa) appear. The 50% of cleavage is achieved after only 5 min. Increasing again the time other bands due to other cuts performed by trypsin also appear fractions (Fig. 21).

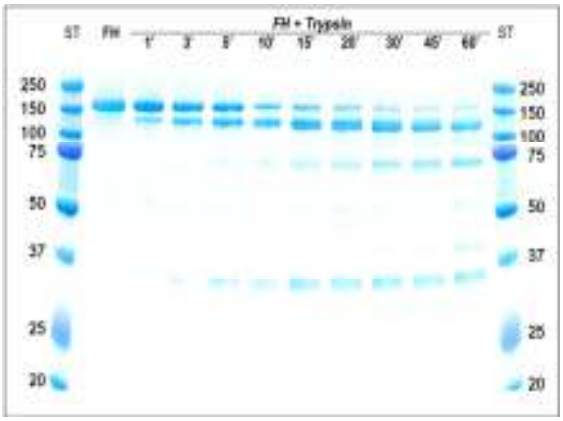


Fig. 21 Trypsin cleavage of FH: trypsin cuts FH into two fragments, as the incubation time increases the cleaved form increases compared to the uncleaved form.

19.2. Affinity of 5H5 for cleaved and uncleaved FH

To test the ability of 5H5 to bind both the cleaved and uncleaved form, affinity purified FH was cleaved in vitro with trypsin for 5 min. in order to obtain 50% of cleavage and subsequently immunoprecipitated with 5H5-coupled Sepharose resin. Analysis of immunoprecipitated samples on reducing SDS-PAGE followed by Coomassie staining revealed that the antibody is able to bind and immunoprecipitate both forms fractions (Fig. 22). Moreover, to assess the affinity of 5H5 for the two forms, gels were analysed by densitometry with the TotalLab program. This analysis revealed that 5H5 binds both forms with the same affinity, this is probably due to the fact that the antibody recognises only the non-reduced FH in which the two fragments of the cleaved form are held together by a disulphide bond.

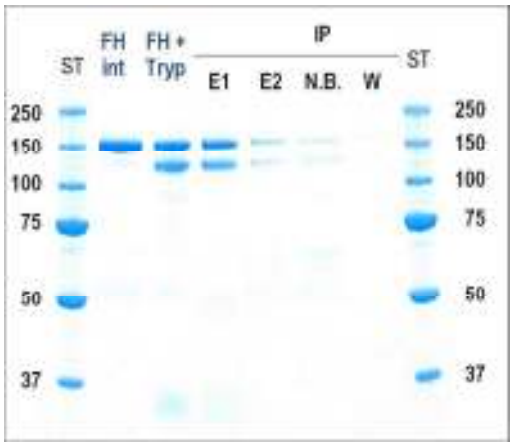


Fig. 22 IP of trypsin-cleaved FH: both bands bind with the same affinity to 5H5 Mab and are eluted from the beads (E1). A small amount is present in the second elution (E2) and in the not-bound (N.B.) fraction.

19.3. FH integrity assay

The ability of 5H5 to immunoprecipitate with the same affinity both forms was exploited to set up a test to check integrity of FH in different samples,

such as patient's sera or FH preparations obtained with other purification methods.

FH was immunoprecipitated from 16 aHUS patients and their relatives and protein integrity was verified on a SDS-PAGE followed by Coomassie staining, the analysis revealed that all the patients possess the intact form (Fig. 23).

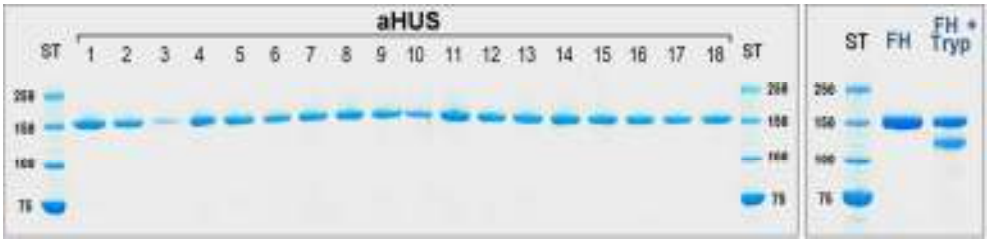


Fig. 23 aHUS FH-IP: FH immunoprecipitated from aHUS patients in comparison with entire FH and trypsin-cleaved FH. All the patients possess the intact form.

The same method was used to test some FH preparations obtained with a purification procedure consisting of two chromatography steps. The analysis revealed that these samples contain also the cleaved form of FH, the amount of which was ranging from 5 to 25% depending on different preparations (Fig. 24).

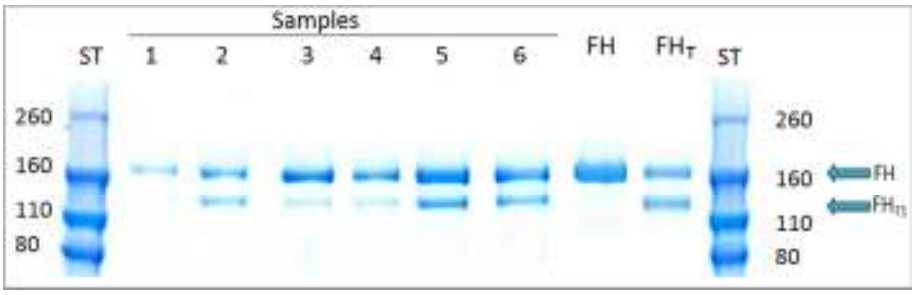


Fig. 24 FH-IP from FH preparations. FH immunoprecipitated from FH preparations obtained with biochemical methods. The samples have different amounts of cleaved FH. FH = purified FH; FH_T = trypsin-cleaved FH

20. Quantification of Purified Factor H

The exact quantification of purified FH samples was a crucial point in order to perform the functional assays and to compare different samples.
Different quantification methods were compared. Bradford assay turned out to be totally unreliable for this purpose, whereas ELISA and spectrophotometric determination at 280 nm were more reliable and gave comparable results. ELISA resulted to be the most sensitive method as it was possible to quantify low FH concentrations. However, OD_{280} was sensitive enough to quantify FH samples to be used for functional assays and was in general preferred as it is faster.

21. FUNCTIONAL ASSAYS

Two assays to test respectively the N-terminal cofactorial activity of FH and its C-terminal protective activity were developed.

21.1. Cofactor assay

A "cofactor assay" to assess N-terminal functionality of FH making use of purified C3b and FI was set up. FI is able to cut C3b converting it to the inactive form iC3b only in the presence of its cofactor FH. C3b is composed of two chains: α'-chain (104 KDa) and β-chain (70 KDa), the α' is cleaved by FI into two fragments, α-68 (68 KDa) and α-43 (43 KDa).
Purified C3b was incubated with FI alone or together with different amounts of FH and the presence of different bands was checked on a reducing SDS-PAGE followed by WB with a polyclonal antibody against C3b. In the absence of FH (C3b and C3b + FI) the α'-chain is not cleaved and a single band around 104 KDa (α') is observed whereas in the presence of FH (C3b + FI + FH) α' is cleaved and a faint band around 104 KDa is still visible but α-68 and α-43 are also visualized (Fig. 25). In all the samples a band of 70 KDa corresponding to β-chain is also present and remains constant.

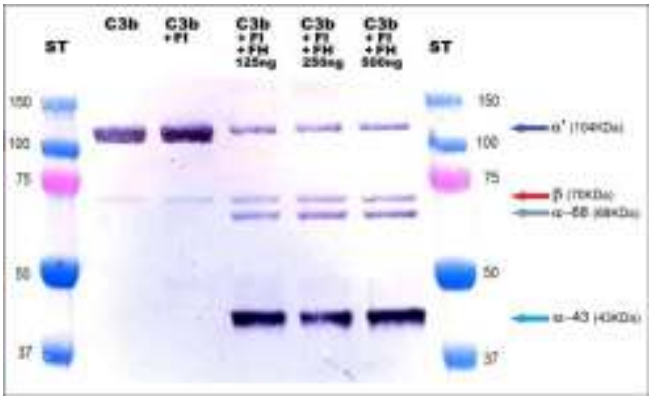


Fig. 25 Cofactor assay: Cofactor assay to assess functionality of FH, C3b is cleaved by FI only in the presence of FH. Without FH (C3b and C3b + FI) the α'-chain is not cleaved and a single band around 104KDa (α') is observed, with different amounts of FH (C3b + FI + FH) α' is cleaved and a small band around 104KDa is still present but α-68 and α-43 are also visualized. The β-chain band at 70KDa is also present in all the samples

Different amounts of FH were used in the test to find the minimum quantity of FH which is still able to act as cofactor for FI. Samples were analysed both in WB and by Coomassie staining and results were compared (Fig. 26). Test sensitivity was high also in Coomassie staining and presence of other bands corresponding to FI heavy (50 KDa) and light (38 KDa) chains, not visible in WB, didn't interfere as they migrate under the C3b bands used for the analysis. For these reasons and because the Coomassie method is much faster, this was used for the following tests.

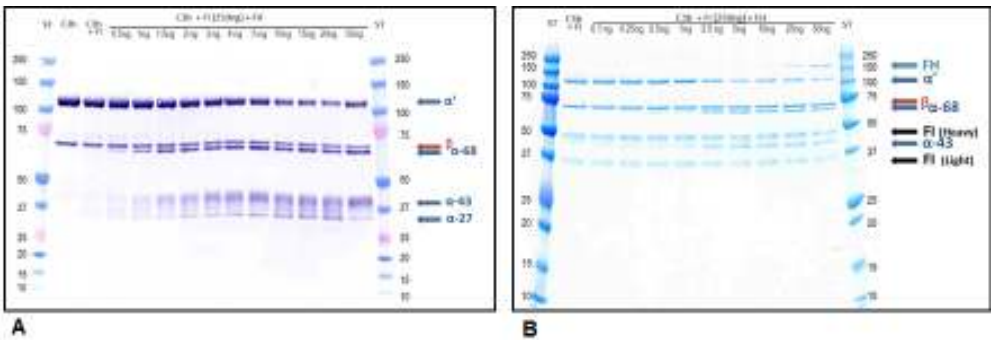


Fig. 26 Cofactor assay developed in WB (A) or Coomassie (B): the two methods give comparable results.

Gels were analysed by densitometry with the TotalLab program and the intensity of α'-chain or α-68 band was compared to that of β-chain (70 KDa) which remains constant. The cofactor activity was calculated as a decrease of α'-chain or an increase of α-68. The first one was preferred as the α'-chain is subject to a single cut by FI, whereas α-68 can be further cleaved into α-27 and C3dg.

To assess functionality of different FH samples, cofactor assay was performed in parallel on test samples and 5H5-affinity purified FH as a control. A sample in which FH was absent was always included for quantification of the results. At least three different concentrations of FH were used for each sample.

The percentage of α'/β was calculated as follows: the intensity of α' band was first normalised to the intensity of β band in each lane and then divided by the α'/β ratio obtained in the control where FH was absent as this corresponds to 100% of α'-chain.

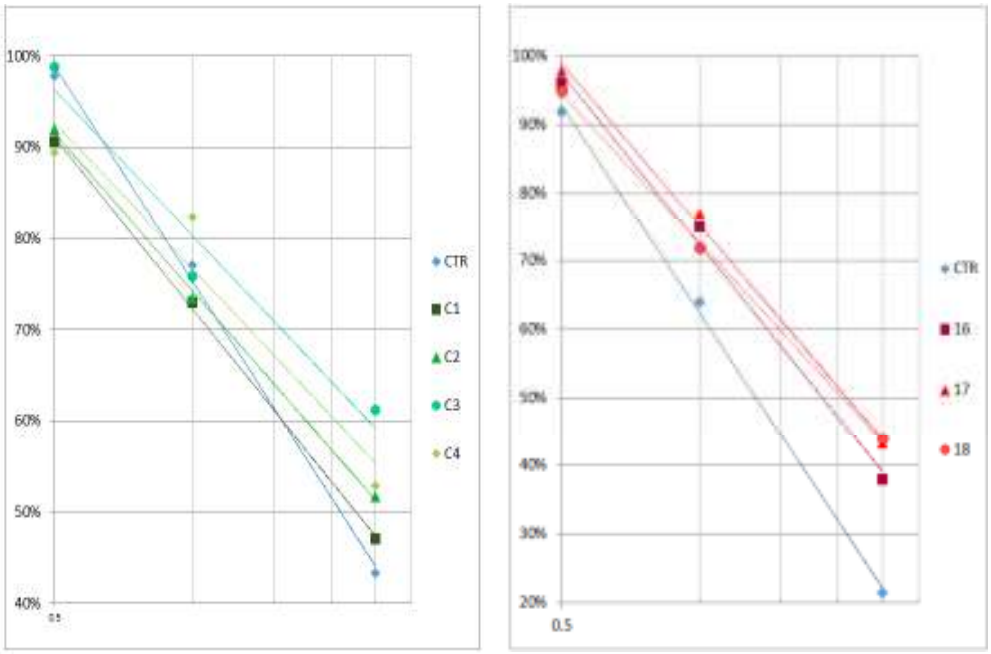
Data corresponding to the percentage of α'/β relative to the ng of FH used were put on a graph and a Logarithmic regression model was used to build a curve using the equation: $y = a + b \ln(x)$, where y = % α'/β and x = ng FH. The curve was then visualized in a graph in which x-axis is in logarithmic scale (Log10) in order to visualize a straight line. The curve obtained allows the direct comparison of different samples.

This test was used to compare different samples of FH purified by 5H5-affinity IP from sera of normal donors. The analysis revealed that cofactor activity in these samples was normal and comparable to that of the control (Fig. 27-A).

This test was also used to analyse some samples of FH, purified with a biochemical method consisting in a two-step chromatography, that were produced for therapeutic purpose.

Different samples and different fractions coming from different purification steps were analysed and compared with a 5H5-affinity purified FH as a

control. The analysis revealed a decrease in cofactor activity in some of these samples, probably due to the purification procedure (Fig. 27-B).



A **B**

Fig. 27 Cofactor assay: (A) on 5H5-affinity purified FH and **(B)** on FH purified with biochemical methods.

21.2. Haemolysis test

An "Haemolysis test" to test the protective activity of FH, which depends on the C-terminal part of the molecule, was set up for sera samples.

This test is based on the fact that FH is able to protect sheep erythrocytes from complement attack; in the absence of FH or in the presence of a defective FH erythrocytes undergo lysis which can be measured spectrophotometrically following the release of haemoglobin.

A minimum of haemolysis is still observed using high concentrations of sera and can be used to test alternative pathway (AP) activation.

The assay was first set up using a control serum to test all the conditions needed to develop the final test.

Due to the impossibility to purchase Sodium Barbital needed to prepare the right buffer for this assay, it was necessary to adapt the protocol using two different buffers (V-AP and H-AP).

The first buffer used (V-AP) was a prepared Veronal buffer commercially available already containing Sodium Barbital. The formulation of this buffer was not ideal for this test, especially for the presence of Calcium that needs to be chelated. Some adaptations were made to the buffer in order to obtain a formulation the most similar to the one needed. The second buffer (H-AP) was made substituting Sodium Barbital with HEPES.

Several dilutions of the control serum were tested in the two different buffers and two dilutions of heat-inactivated serum were used as controls for each buffer. Data were normalised subtracting the OD_{415} of the 0% lysis control (only buffer) and percentage of haemolysis was calculated relative to the 100% lysis control obtained by lysing erythrocytes with water.

The test was functioning with both buffers and almost no haemolysis was present in heat-inactivated serum. Using the H-AP the percentage of haemolysis was higher (Fig. 28).

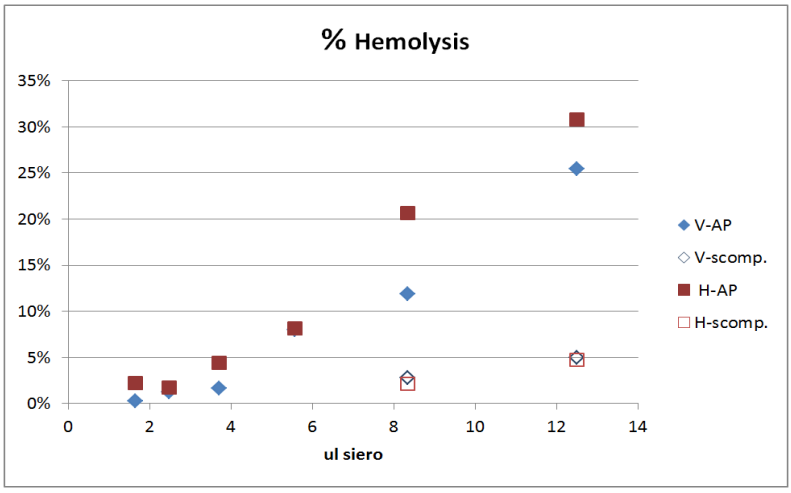


Fig. 28 Haemolysis assay: assay performed with two different buffers.

In the final test FH-depleted serum needs to be used. Since subtracting FH from serum leads to an indiscriminate activation of complement with the consequence of consuming all the complement proteins, activation needs to be inhibited by adding EDTA throughout the depletion procedure. On the other hand, EDTA will also chelate Magnesium ions needed to activate AP in the haemolysis test. For these reasons, different concentrations of EDTA were tested in this assay in order to establish the minimum concentration that prevents activation of complement in FH-depleted serum but still allows the test to function.

The test was performed diluting the serum 1:5 in H-AP buffer with the addition of different amounts of EDTA. As a negative control PBS was used instead of serum and prepared in the same way. Percentage of haemolysis was calculated as before and visualized on a graphic relative to final EDTA concentration (Fig. 29).

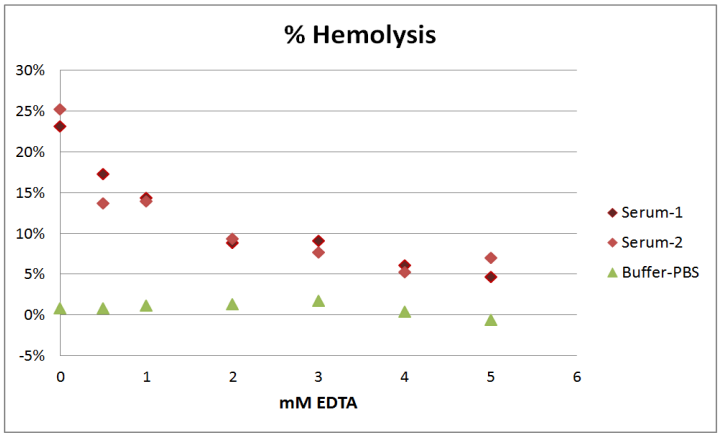


Fig. 29 Haemolysis assay: assay performed with different EDTA concentrations.

Haemolysis resulted to be inhibited by the presence of EDTA and decreases as the concentration of EDTA increases. A final concentration of 1 mM still allows the test to function. Considering that serum is used at a 1:5 dilution and is then diluted 1:1 with erythrocyte preparation, the final dilution is 1:10. That allows to prepare depleted serum with 10 mM of EDTA and to perform the test with a final concentration of 1 mM.

22. Other purification methods

22.1. Heparin affinity purification

Human serum was applied to a Heparin column in order to purify heparin binding proteins such as FH and FHRs. Fractions were analysed by WB with different antibodies for FH, FHRs and C3b (Fig. 30). All the proteins were present in serum before purification and some traces were still present in the flow through probably due to column overload. FH eluted at 250 mM NaCl with some residual at higher salt concentrations (300-400 mM). FHR-1 eluted both at 250 and 300 mM NaCl with some residual at 400 mM, whereas FHR-2 eluted at 250 mM together with FH and no residuals were visible at higher salt concentrations. FHR-5 had a different pattern as is eluted at higher salt concentrations with a maximum at 300 and 400 mM and only a small amount eluted earlier. Finally FHR-4 didn't seem to bind to the Heparin column as all the signal was present in the flow through.

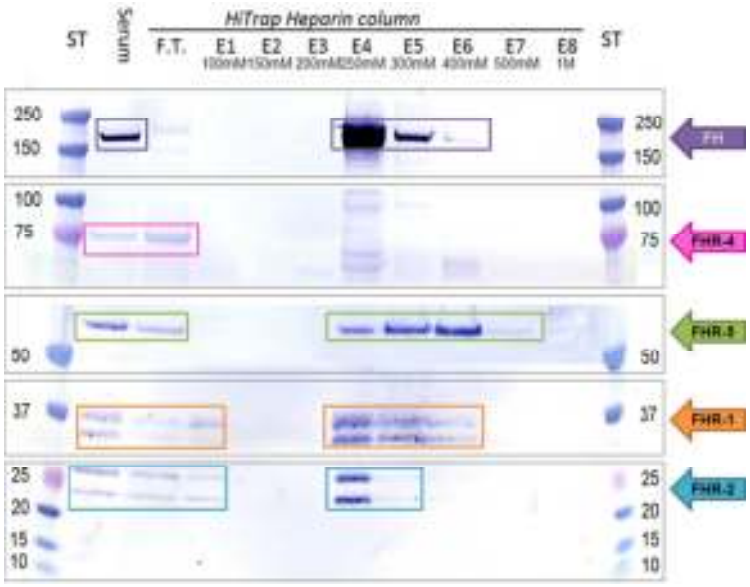


Fig. 30 WB of Heparin fractions developed with antibodies for FH and FHRs.

C3b eluted together with FH at 250 mM NaCl with some traces at higher salt concentrations. (data not shown)

22.2. Hydrophobic Interaction Chromatography (HIC)

Serum proteins were also isolated with a hydrophobic column (HIC) and fractions were analysed by Coomassie staining and by WB with different antibodies for FH, FHRs and C3b (Fig. 31).

Albumin did not bind to HIC column and is present in the 100% fraction containing 1.5 M ammonium sulphate. FH and FHRs eluted in the 33% fraction containing 0.5 M ammonium sulphate except FHR-5 which was present in the 0% fraction. Finally C3b was present both in the 66% and 33 % fractions (1 and 0.5 m sodium sulphate) with a residual of α-43 in the 0% fraction.

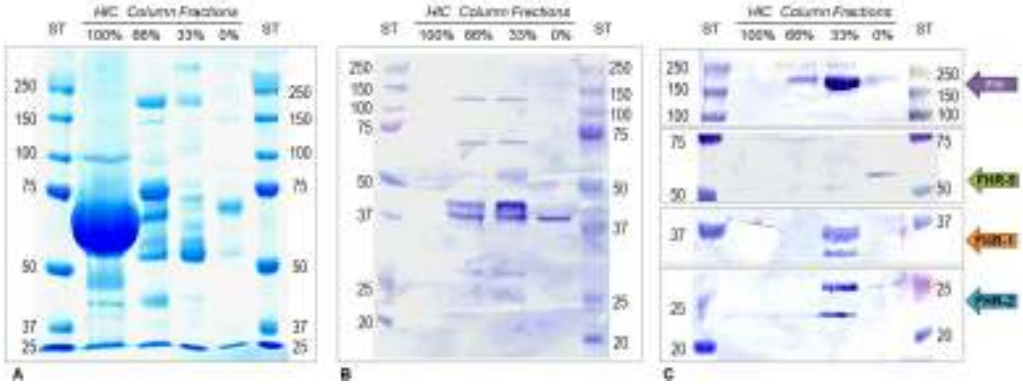


Fig. 31 Coomassie e WB of HIC fractions.(A) Coomassie, (B) WB for C3b and (C) WB for FH and FHRs.

23. CLONING OF FACTOR H

The entire FH and three fragments containing approximately seven SCRs each (SCR 1-7, SCR 8-14, SCR 15-20) have been amplified from human liver RNA using Phusion™ DNA Polymerase (Thermo).

In order to obtain the right amplification of all the fragments in was necessary to introduce several changes in the protocols. cDNA was produced using both Oligo(dT) or specific primers: SCR-7R, SCR-14R, SCR-20R. For SCR 1-7 fragment the best results were obtained using cDNA retrotranscribed with the specific primer SCR-14R whereas for fragments

SCR 8-14 and SCR 15-20 cDNA produced with the specific primer SCR-20R was used. (Fig. 32)

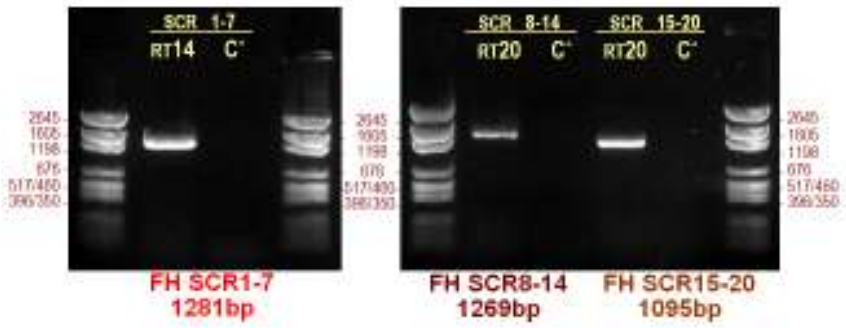


Fig. 32 Amplifications of FH fragments. Amplification from cDNA obtained with specific primers FH-SCR14-R (RT14) or FH-SCR20-R (RT20). C^- = negative control.

Finally for the amplification of the entire FH both cDNA retrotranscribed with Oligo(dT) or specific primer SCR-20R for a longer time gave good results. (Fig. 33)

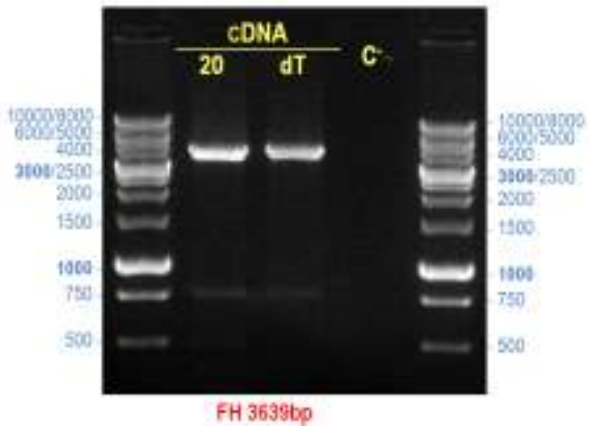


Fig. 33 Amplification of entire FH. Amplification from cDNA obtained with specific primer FH-SCR20-R (20) or OligodT (dT) primers. C^- = negative control.

All the amplifications with the proofreading polymerase were performed in CG buffer as the standard HF buffer gave poor results. This buffer is recommended for difficult templates that tend to form secondary structures.

Analysis of cDNA sequences with *RNAfold* program from the Vienna package [167] revealed the presence of secondary structures (Fig. 34)

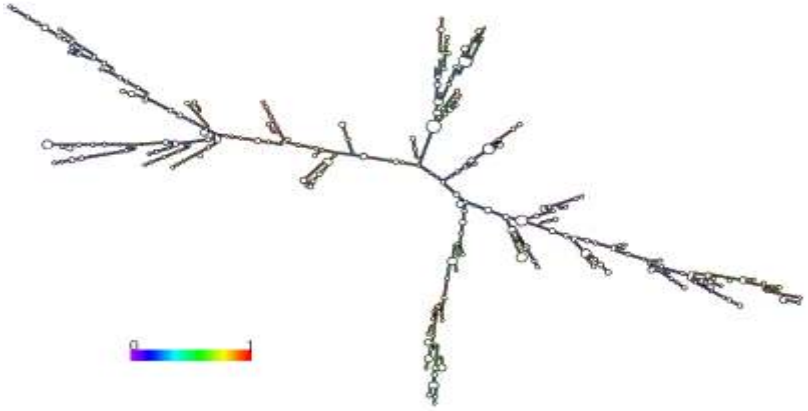


Fig. 34 Secondary structure of FH cDNA: graph obtained with RNAfold program

All the fragments were correctly amplified and quantified on an agarose gel (Fig. 35)

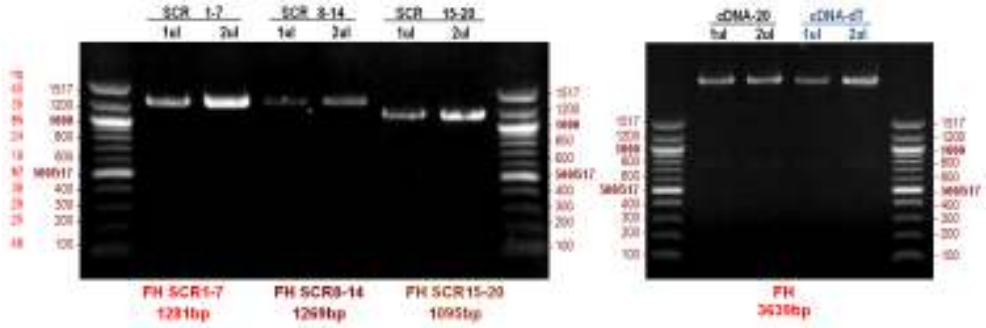


Fig. 35 Quantification of PCR products

The amplified fragments were inserted into the pFastBac™/HBM-TOPO® vector (Life Technologies) using TOPO-cloning and used to transform competent *E. coli*. Clones were analysed by colony PCR to check the presence and the right orientation of the fragments. Two parallel PCR reactions were performed using one primer on the vector together with a forward or a reverse primer specific for the fragment. The clones that give

amplification in the PCR Forward but not in the PCR Reverse possess the fragment in the correct orientation (Fig. 36).

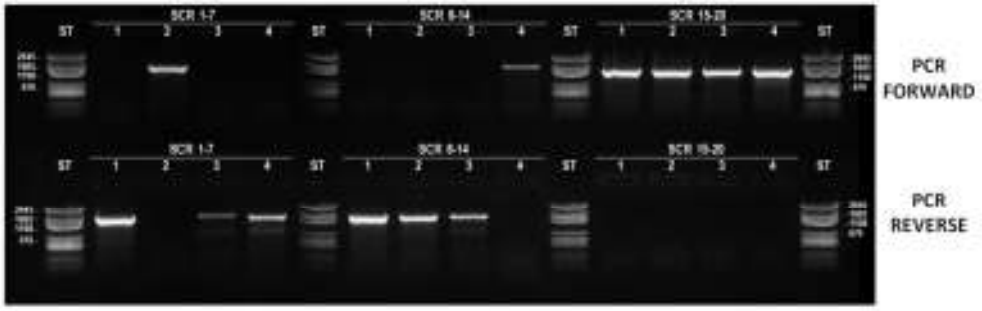


Fig. 36 Colony PCR

Plasmid DNA was extracted from positive clones and sent to a sequence service, sequences were analysed with CLC Sequence Viewer (CLC bio). Some clones were found to possess the correct sequences and selected for the following steps.

Notably, the sequence of FH and of the SCR 1-7 fragment has been shown to possess the 402T allele (TAT codon) which is associated with a low risk of AMD, whereas the sequence present on NCBI database possesses the high risk allele 402H (CAT codon) (Fig. 37).

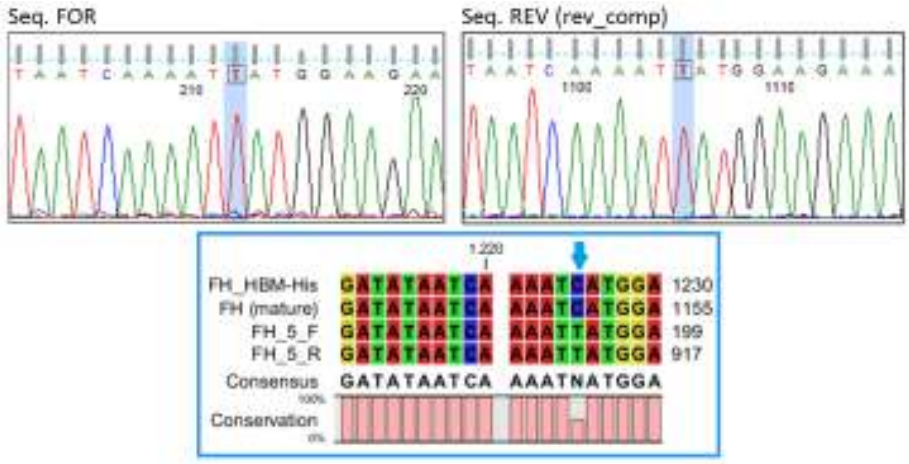


Fig. 37 Sequencing results: the clones possess the low risk allele 402T.
(Legend: FH_HBM-His = sequence of FH from NCBI inserted into the vector, FH (mature) = sequence of FH from NCBI, FH_5_F and FH_5_R = Forward and Reverse sequences of clone n. 5)

DISCUSSION

Complement Factor H (FH) is a crucial regulator of complement activation. Mutations in the CFH gene are frequently associated with a number of human diseases, furthermore it has an important role in infectious diseases as many pathogens are able to recruit it on their surface to protect themselves from complement attack.

However, the study of this protein is complicated by the presence of other homologous proteins belonging to the "FH protein family". The high amino acid identity among the family members is a cause of cross-reactivity for antibodies. Antibodies raised against FH often detect also different FHRs and antibodies generated against FHRs can cross-react with FH or other FHRs. This cross-reactivity is thus a challenge for correct quantification and/or purification of FH from plasma.

The aim of this study was mainly to develop some quantitative and functional FH-specific assays, using a monoclonal antibody (MAb 5H5) produced in our laboratory that was shown to be specific for FH. Another important focus was the production of recombinant FH in both an eukaryotic and a prokaryotic expression system, in order to study the importance of protein glycosylation and to further and more precisely refine structure-function relationships, as well as to develop safer therapeutic tools: in fact, current therapies for FH defects only rely on blood derivatives.

By the use of high-density hybridoma cultures with CELLine Bioreactor, it was possible to produce a consistent amount of MAb 5H5. This antibody was the starting point for the development of FH-specific assays.

The first use of MAb 5H5 was the purification of FH using the antibody conjugated with Sepharose resin. Thanks to the high affinity of MAb 5H5 for FH, it was possible to set up a "one-step" purification procedure, which

gives a highly pure protein without any FHRs contamination, starting from total human serum.

The availability of highly purified FH was an important feature for the development of FH-specific assays.

An ELISA assay for specific quantification of serum FH using the purified MAb 5H5 in solid phase together with a chicken polyclonal antibody to FH, also produced in our lab, and 5H5-affinity purified FH as a standard was developed. A correct quantification of FH levels in samples from a number of diseases due to complement defects is a crucial point. Methods for the quantification of serum FH are often not specific as they recognise both FH and FHRs proteins and are therefore unreliable. The first quantifications based on polyclonal antibodies overestimated FH plasma concentration that were reported to be in the range of 115–685 μg/ml and sometimes even to 1 mg/ml, more recent studies using monoclonal antibodies have established mean FH concentrations around 233 μg/ml (in young adults) and 269 μg/ml (in elderly individuals) [143].

The assay developed turned out to be specific and reliable and was then used to quantify FH from different samples. It was first used to quantify FH serum levels from aHUS patients revealing a FH deficit in four patients. Subsequently it was also used to quantify FH levels in different samples from AMD patients such as plasma, aqueous and vitreous humour.

A micromethod to purify FH from small amounts of sample was developed using 5H5-coupled Sepharose resin and further improved by the use of Micro Bio-Spin™ Columns (Bio-Rad) which allow for a faster procedure.

This method resulted to be really efficient enabling to obtain enough material for structural and functional studies which require the use of purified proteins.

Most importantly, it has the advantage of reducing the amount of serum needed for FH purification. This is an important feature considering the fact

that FH-related diseases, e.g. aHUS, affect mainly children and the material available is poor.

FH can be cleaved by a plasmatic protease into two fragments that are held together by a disulphide bond, this can be mimicked in vitro by trypsin.

The ability of MAb 5H5 to recognise both the intact and the trypsin-cleaved form of FH, was demonstrated by IP and further exploited to set up an integrity assay to be used on different samples. The analysis on aHUS patients revealed that they all possess the intact form, whereas analysis on chromatography purified FH revealed the presence of the cleaved form.

The question whether the cleaved FH form has a reduced activity is still controversial. The cut is within the SCR6 domain and separates the domains with cofactor activity from the rest of the molecule, even if the structure is still held together by disulfide bridges.

It has been shown that the cofactor activity resides in the 38 KDa N-terminal fragment and that this fragment is also able to work alone [168].

However, the data available in the literature on the cofactor activity of the cleaved form are different and often conflicting. Some works show that the cofactor activity in the fluid phase seems to be increased by the cleavage, whereas the cofactor activity for the degradation of membrane-bound C3b is certainly decreased [169].

The presence of the cleaved form could also be due to an artefact of the purification methods.

During the various steps, necessary for FH purification with biochemical methods, the plasmatic protease inhibitor is first removed thus enabling the plasmatic protease to cut FH.

With affinity purification methods, such as the method developed here based on MAb 5H5, the presence of the cleaved form is generally not observed. Thus, the integrity test is an important tool to assess the presence of the cleaved form especially in those FH preparations obtained

with biochemical methods that are used to prepare FH for substitution therapies.

The ability of MAb 5H5 to bind with the same affinity both the intact and the cleaved form of FH is probably due to the fact that this antibody recognises a structural epitope that remains the same in the two forms but is lost after reduction of disulphide bridges. In fact, MAb 5H5 recognises FH only under unreducing conditions. This feature is probably also the reason why MAb 5H5 is specific for FH and does not recognise any of the FHRs proteins.

The epitope recognised by MAb 5H5 has not yet been identified; being a conformational mapping the epitope is challenging. Recombinant proteins that are discussed later, will be probably useful for this purpose.

Finally, MAb 5H5 was used to develop two functional assays to assess both the N-terminal cofactorial activity and C-terminal protective activity of FH.

The “cofactor assay” is based on the fact that FI can cut C3b in vitro only in the presence of a functional FH; if CFH is not functional C3b cannot be cleaved. The test was set up using purified C3b and FI together with different amounts of affinity purified FH. The test was used to screen different serum samples or fractions derived from a biochemical purification of FH and revealed a reduced activity in samples which underwent different chromatography steps.

This reduced activity is probably due to the long procedure used to purify FH, consisting in two sequential chromatography steps, that somehow modify the protein. It doesn’t appear to be related to the presence of the cleaved form, but it is probably due to other modifications that occur in the protein during the procedure.

The “Haemolysis test” is useful to assess the protective activity of FH that is dependent on the C-terminal part of the molecule. This test is based on the fact that FH is able to protect erythrocytes from complement attack; in the absence of FH or in the presence of a defective FH erythrocytes undergo

lysis which can be measured spectrophotometrically following the release of haemoglobin. The test will be performed making use of FH-depleted serum, which causes lysis of the erythrocytes, and 5H5-affinity purified FH from patient's sera in order to assess the ability of those FH to restore protection.

For the moment the assay has been set up using a normal control serum in order to test all the conditions. It was first necessary to test different buffer preparations as the correct buffer needed for this kind of test is not commercially available in Italy. FH is an important inhibitor of complement activation and its depletion from serum causes the indiscriminate activation of complement leading to the consumption of complement proteins. To avoid this activation that would render FH-depleted serum useless for this test, it is necessary to produce it in the presence of EDTA that blocks both the CP and the AP. However, the presence of EDTA will inhibit erythrocytes lysis thus precluding the possibility to perform the "Haemolysis test". For these reasons it was necessary to perform the test in the presence of different concentrations of EDTA in order to establish the minimum concentration that still prevents complement to be activated in serum but allows the test to function. The set up of the last conditions needed to perform the final test is still under development.

This test will be useful to assess N-terminal functionality especially in aHUS patients in which most mutations are located in the last two SCRs (SCR 19-20) and cause haemolysis. The genetic characterisation of this group of aHUS patients has already been performed whereas functional data at protein level are still missing. Therefore, the functional data obtainable with this test will be fundamental to complement the characterisation of these patients and their relatives performed by DNA sequencing, thus allowing for a detailed description of the structure-function relationship.

The other aim of this project was the cloning and expression of FH in both an eukaryotic and a prokaryotic system.

As FH is a glycoprotein and a correct glycosylation might be important for all its functions, I planned to use a modified insect expression system in which the glycosylation pattern is very similar to that of humans. Unglycosylated proteins to be used as a control will be produced in *E. coli*. FH is a big soluble protein with 20 SCR domains of about 60 aa each that bear different functions. I decided to clone separately both the entire protein and three main fragments containing approximately 7 SCR each (SCR 1-7, SCR 8-14, SCR 15-20).

Four clones containing respectively the entire FH and the three fragments were obtained and confirmed by sequencing to possess the correct sequences. These clones will be used to produce the recombinant proteins into the two different systems.

The recombinant proteins produced will be useful for different purposes. First of all they can be used to screen which parts of FH are involved in interaction with proteins of known pathogens and to explore interaction with other pathogens. Secondly their ability to restore the function of a defective FH or to protect against FH autoantibodies will be analysed. The final goal is to use them for therapy in many diseases caused by CFH defects for which the only possible treatment is currently plasma exchange. Furthermore, these recombinant fragments will also be used to try to identify the so far elusive conformational epitope recognised by MAb 5H5.

CONCLUSIONS

The study of complement factor H (FH) is complicated by the presence of FHR proteins that share a high degree of similarity.

Thanks to the use of a monoclonal antibody (MAb 5H5) produced in our lab, and shown to be specific for FH, it was possible to develop several FH-specific tests.

A fast and efficient method for the purification of FH was developed and further scaled down to be applicable to small samples. A home-made ELISA for the correct quantification of FH in biological fluids, without any interference from FHR proteins, was developed and used to screen different samples. A test for the assessment of FH structural integrity, based on the fact that FH can be cleaved by a protease into a probably less active form, was also developed. This test used to screen both FH purified from patient's sera and samples deriving from different purification procedures.

Finally two functional assays to test both the C-terminal cofactorial activity and the N-terminal protective activity of FH were developed.

The "cofactor assay", based on the fact that FI can cut its substrate C3b only in the presence of a functional FH, has been set up and was used to screen different samples. This test was important especially to assess functionality of some FH preparations obtained with a biochemical purification method, that were intended to be used for therapeutic purposes.

The second one, the "haemolysis test" based on the fact that a functional FH is able to protect sheep erythrocytes from haemolysis, is now in its fine tuning phase. This test is a modification of a test normally used to assess AP activation in sera. For the test a FH-depleted serum from a normal subject will be used together with FH purified from patients.

The developed tests that have been used in the present work on aHUS patients and FH preparations produced for therapeutic purposes, will be used in the future also for a bigger screening on AMD patients for whom a pilot study just started. The study of AMD patients will be part of a larger project that will involve other methods such as the study of retinal epithelial cells which have been shown to produce FH locally in the eye [86] and the genetic screening of some SNPs which have been shown to correlate with the development of the disease [138].

Another aim of this project was the cloning of the entire FH and some fragments for the expression into both a prokaryotic and a eukaryotic system in order to produce both glycosylated and unglycosylated proteins. The clones have been produced and are ready to be introduced into the expression hosts.

The recombinant proteins produced will be useful for different purposes. First of all they can be used to screen which parts of FH are involved in interaction with proteins of known pathogens and to explore interaction with other pathogens. Secondly their ability to restore the function of a defective FH or to protect against FH autoantibodies will be analysed. The final goal is to use them for therapy in many diseases caused by FH defects for which the only possible treatment is currently plasma exchange

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APPENDIX

A. Factor H ELISA

BUFFERS:

Coating buffer: TBS, pH 7.65

Wash buffer: TBS/Tween 0.1%

Overcoating buffer: 3% BSA in TBS/Tween 0.5% (TTBS)

Sample buffer: TBS-BSA 1%, Tween 0.1%

PROTOCOL

1. *Coating*:
   - Dilute MAb 5H5 stock solution (3 mg/ml) to 1:2000 in *Coating buffer* to give a final concentration of 1.5 µg/ml
   - Dispense 100 µl/well in a microtiter plate and incubate o/n at 4°C

Wash the plate three times with *Wash buffer*

2. *Overcoating:*
   - Dispense 250 µl/well of *Overcoating buffer*
   - Incubate 1 h at 37°C in an Eppendorf Thermomixer

Wash the plate three times with *Wash buffer*

3. *Samples:*
   - Dilute the samples, control and standard in *Sample buffer*
     - **Standard Curve:**
       5H5-affinity purified FH (1 µg/µl)
       - prepare 5 two-fold serial dilutions starting from 250 ng/ml
       - Dispense 100 µl/well to the assigned microassay wells in triplicate
     - **Samples:**
       Plasma or sera
       - prepare 3 dilutions: 1:2.000, 1:4.000 and 1:8.000
       - Dispense 100 µl/well to the assigned microassay wells in duplicate
     - **Positive Control:**
       5H5-affinity purified FH (174 µg/ml)
       - prepare 3 dilutions: 1:2.000, 1:4.000 and 1:8.000

- Dispense 100 µl/well to the assigned microassay wells in duplicate

- Reserve two wells for blank, 100 µl/well of *Sample buffer*
- Incubate 1 h at 37°C in an Eppendorf Thermomixer

Wash the plate three times with *Wash buffer*

4. *Primary antibody:*
   - Dilute the **Chicken anti-FH** antibody 1:25.000 in *Sample buffer*
   - Dispense 100 µl/well
   - Incubate 1 h at 37°C in an Eppendorf Thermomixer

Wash the plate three times with *Wash buffer*

5. *Secondary antibody:*
   - Dilute the **anti-chicken IgY-HRP** antibody (Promega) 1:5.000 in *Sample buffer*
   - Dispense 100 µl/well
   - Incubate 1 h at 37°C in an Eppendorf Thermomixer

Wash the plate three times with *Wash buffer*

6. *Develop:*
   - Prepare the *Substrate Solution*: Warm the reagent to room temperature before use. Mix equal volumes (5 ml) of TMB Peroxidase Substrate and Peroxidase Substrate Solution B (KPL) immediately prior to use.

     (*Note*: TMB Peroxidase Substrate contains 3,3',5,5'- tetramethylbenzidine at a concentration of 0.4 g/L in an organic base. Peroxidase Substrate Solution B contains H_20_2 at a concentration of 0.02% in a Citric Acid buffer.)
   - Dispense 100 µl/well
   - Incubate at 37°C in an Eppendorf Thermomixer
   - Read the plate in Microplate Reader (Model 680 Bio-Rad) at 595 nm, after 20 and 40 min.
7. *Stop:*
   - Stop the reaction with 100 µl/well of *Stop Solution* (2N Sulphuric Acid), solution will turn yellow
   - Read the plate in Microplate Reader (Model 680 Bio-Rad) at 450 nm

B. Haemolysis test

Buffers:

- **V-AP Buffer (Veronal) 1X**: Veronal 1X + 5 mM $MgCl_2$ + 10 mM EGTA + 0.1% gelatin
  Veronal Buffer 5X (Lonza): Barbitol 15.58 mM, Sodium Barbitol 9.07 mM, NaCl 727.24 mM, $CaCl_2$ 1.26 mM, $MgCl_2$ 4.13 mM
- **H-AP Buffer (Hepes) 1X***:* Hepes 1X + 6 mM $MgCl_2$ + 10 mM EGTA + 0.1% gelatin
  Hepes Buffer (5X): HEPES 21 mM, NaCl 725 mM
- ***N-Saline***: 154 mM NaCl

Preparation of Sheep Erythrocytes (E_{sh}) for Alternative Pathway Assay

1. Under aseptic conditions, remove 0.5 ml packed Sheep Erythrocytes from stock stored in Alsever's solution (Sigma).
2. Wash in Alsever's solution (several times)
3. Count Erythrocytes and transfer a part into a new tube with AP buffer
   (NOTE: Erythrocytes were divided into two different tubes containing the two AP buffers: **V-AP** and **H-AP**)
4. Wash in AP buffer
5. Resuspend in the same buffer to the required concentration for assay (10^8/ml)

Protocol

1. Prepare one set of disposable 1,5ml tubes for serum dilutions and one set of disposable 2ml tubes for the assay. Include in the assay-rack tubes for 0% controls and for total-lysis controls. Place tubes on ice. (NOTE: two different set for the two different AP buffers were prepared)
2. Prepare six serial 1.5-fold dilutions of serum sample starting from 1:4 in ice-cold ***AP buffer***, mixing well after each dilution before doing the next.
3. Add 100 µl of $\mathbf{E_{Sh}}$ suspension (in AP buffer at 10^8/mL) to each assay tube.

4. Transfer 100 µl of each dilution to the appropriate tube in the assay rack. Place 100 µl ***AP buffer*** in the background-lysis control tubes.
5. Incubate 60 min at 37°C with occasional shaking to keep the cells in suspension.
6. To all serum tubes and background-lysis control tubes add 300 µl ice-cold ***N-saline*** to stop the reaction, to total-lysis control tubes add 400 µl H_20.
7. Centrifuge 5 min at 1000 × *g*, 4°C, to pellet the cells.
8. Read the OD_{415} of each supernatant (200 µl in duplicate wells)

Calculations:

1. Subtract background-lysis control absorbance from each value to obtain corrected absorbances (Ab_c)
2. Calculate fractional haemolysis in each well relative to the 100% lysis wells: Fractional haemolysis (y) = (Ab_c serum/Ab_c 100%)

PRODOTTI SCIENTIFICI DEL DOTTORANDO

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