Die Rolle des Komplementsystems bei Entstehung der altersabhängigen Makuladegeneration

Dissertation

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Meinen Eltern

Inhaltsverzeichnis

Abkürzungen ... III

Zusammenfassung ... IV

Summary ... VI

Einleitung ... 1

1 Charakteristika altersbedingter chronischer Erkrankungen ... **1**

2 Altersabhängige Makuladegeneration ... **2**

2.1 Anatomie und Immunologie des Auges ... *2*

2.2 Pathophysiologie der AMD ... *4*

2.2.1 Geografische Atrophie ... 5

2.2.2 Choroidale Neovaskularisation ... 5

2.3 Risikofaktoren ... *6*

3 Das Komplementsystem ... **8**

3.1 Die Kaskadenstruktur des Komplementsystems ... 9

3.1.1 Der alternative Weg ... 9

3.1.2 Der klassische Weg und der Lektinweg ... 10

3.1.3 Terminale Komplementprozesse ... 11

3.2 Die Regulation der Komplementkaskade ... 12

3.2.1 Komplementregulation auf Ebene von C3 ... 12

3.2.2 Komplementregulation im Auge ... 13

3.3 Die Faktor H-Proteinfamilie ... 13

3.3.1 Faktor H ... 14

3.3.2 Faktor H-ähnliches Protein 1 ... 16

3.3.3 Faktor H-verwandte Proteine ... 16

3.4 Faktor H-assoziierte Erkrankungen ... 17

3.4.1 Atypisch hämolytisch urämisches Syndrom ... 18

3.4.2 Die membranoproliferative Glomerulonephritis Typ-II ... 18

4 Das Komplementsystem und AMD ... **19**

4.1 Polymorphismen in Komplementproteinen und deren Einfluss auf die AMD ... 19

4.2 Neue komplementassoziierte Behandlungsmöglichkeiten ... 22

5 Fragestellung ... **24**

Übersicht der Manuskripte 25

Manuskripte 36

1 **The role of complement in age-related macular degeneration.**
Adv Exp Med Biol. 2010;703:9-24. 37

2 **The AMD-associated variation at position 62 does not, but the exchange at position 402 does affect surface regulation of the complement inhibitor FHL1.**
in Überarbeitung für das „Journal of Biological Chemistry“ 53

3 **Complement regulation at necrotic cell lesions is impaired by the AMD associated Factor H-H402 risk variant.**
in Überarbeitung für das „Journal of Immunology“ 69

4 **Factor H related protein 1 (CFHR-1) inhibits complement C5 convertase activity and terminal complex formation.**
Blood. 2009 Sep 17;114(12):2439-47 87

5 **An imbalance of human complement regulatory proteins CFHR1, CFHR3 and factor H influences risk for age-related macular degeneration.**
Hum Mol Genet. 2010 Dec 1;19(23):4694-704 96

6 **The C-terminal surface attachment region of Factor H targets the complement inhibitor compstatin to self surfaces and to sites of immunstress.**
in Vorbereitung für das „Journal of Immunology“ 107

Diskussion 126

Literaturverzeichnis 142

Anhänge i

Eigenständigkeitserklärung ii

Vorträge, Poster, Auszeichnungen iii

Stipendien und Patente v

Publikationsliste vi

Lebenslauf viii

Danksagung ix

Abkürzungen

Die physikalischen und chemischen Einheiten sind im SI-Standard angegeben. Die Bezeichnung einzelner Aminosäuren erfolgt im Ein-Buchstaben-Code. Es werden Standard-Abkürzungen für Chemikalien und ihre Verbindungen benutzt.

AMD	altersabhängige Makuladegeneration
ARPE-19	humane Zelllinie des retinalen Pigmentepithliums
aHUS	atypisch hämolytisch urämisches Syndrom
BM	Bruch's Membran
C4BP	*C4b-binding protein*
CD	*cluster of differentiation*
CFHR	*complement factor H-related protein*
CHO	*chinese hamster ovary cells*
CNV	choroidale Neovaskularisation
COMP_CFH15-20	Chimär aus Inhibitor Compstatin und den SCRs15-20
CR	*complement receptor*
CRP	C-reaktives Protein
DAF	*decay accelerating factor*
DEAP-HUS	*deficiency of cfhr plasma proteins and factor H-autoantibody positive HUS*
ELISA	*enzyme linked immunosorbent assay*
FHL1	*factor H-like protein 1*
GA	geografische Atrophie
GAG	Glykosaminoglykan
HUVEC	*human umbilical vein endothelial cells*
kDa	Kilodalton
MCP	*membrane cofactor protein*
mCRP	monomeres C-reaktives Protein
MPGN-II	membranoproliferative Glomerulonephritis Typ-II
MBL	*mannose binding lectin*
pCRP	pentameres C-reaktives Protein
RCA	*regulators of complement activation*
RGD	Zelladhäsionsmotiv aus Arginin-Glycin-Aspartat
RPE	retinales Pigmentepithel
SCR	*short consensus repeat*
SNP	*single nucleotide polymorphism*
TCC	*terminal complement complex*
TNF-α	Tumornekrosefaktor-α
VEGF	*vascular endothelial growth factor*
V62I	Valin- zu-Isoleucin-Austausch von Aminosäure 62
Y402H	Tyrosin- zu-Histidin-Austausch von Aminosäure 402
Δ*CFHR1/CFHR3*	Defizienz der *CFHR1-* und *CFHR3*-Gene

Zusammenfassung

Die altersabhängige Makuladegeneration (AMD) ist die häufigste Ursache für Erblindung in der westlichen Welt und betrifft etwa 50 Millionen Menschen. Die Erkrankung tritt ab dem 50. Lebensjahr auf, und ist charakterisiert durch die sukzessive Degeneration von Fotorezeptoren der Makula. Der Funktionsverlust der Makula –dem Ort des schärfsten Sehens– ist verbunden mit dem Verlust des zentralen Sehvermögens sowie einem reduzierten Kontrast- und Farbempfinden. Aufgrund der demografischen Entwicklung der nächsten Jahrzehnte, und den damit verbundenen steigenden Neuerkrankungen, stellt die AMD nicht nur eine medizinische, sondern auch sozioökonomische Herausforderung dar. Neben individuellen Risikofaktoren ist die AMD im Wesentlichen einer genetischen Prädisposition unterworfen. Im Jahr 2005 identifizierten genomweite Assoziationsstudien erstmals konkrete Risikogene. Eine zentrale Rolle in der AMD-Entstehung spielt dabei das Komplementsystem, in welchem Polymorphismen der Gene für *Faktor H*, *Faktor B*, *C2* und *C3* sowie die Defizienz der *CFHR1-/CFHR3*-Gene das individuelle Risiko beeinflussen. Das Komplementsystem des Menschen ist Teil der angeborenen Immunabwehr. Unter physiologischen Bedingungen spielen die von ihm ausgelösten Effektorfunktionen eine entscheidende Rolle in der Aufrechterhaltung der Gewebshomöostase sowie in der Eliminierung von Mikroorgansimen. Doch ein partieller oder vollständiger Funktionsverlust von Komplementregulatoren durch Polymorphismen, Mutationen oder Defizienzen ist Ursache zahlreicher Erkrankungen.

In der vorliegenden Promotionsarbeit wurden erstmals AMD-assoziierte Polymorphismen sowie Defizienzen des *Faktor H*-Genclusters dahingehend untersucht, inwieweit sie die Effektivität der Komplementregulation beeinflussen und dadurch das AMD-Risiko modifizieren. Die AMD-assoziierten Aminosäuresubstitutionen V62I und Y402H des Faktor H-/FHL1-Proteins wurden systematisch in Bezug auf Ligandenbindung und komplementregulative Funktionen verglichen. Die erhaltenen Ergebnisse zeigen, dass die Y402 → H402-Substitution, nicht aber die V62→I62-Variation, die Effektivität der beiden Regulatoren Faktor H sowie FHL1 beeinträchtigt. Die Variation von Y402 → H402 reduziert die Komplementregulation auf körpereigenen Oberflächen um 25 % und kann primär Zellschaden verursachen, d. h. AMD initiieren. Weiterhin konnte erstmals gezeigt werden, dass die Y402 → H402-Variation zusätzlich für die intraokularen Entzündungsereignisse in Gegenwart nekrotischer retinaler Zellen verantwortlich ist. Die AMD-assoziierten Faktor H-Risikovarianten beeinträchtigen die Opsonisierung nekrotischer Zellen und verhindern dadurch die anti-inflammatorische Eliminierung zellulären Debris durch Makrophagen. Die Arbeit gibt weiterhin Aufschluss darüber, warum die Defizienz der Komplementproteine CFHR1 und CFHR3 sich positiv auf den Krankheitsverlauf auswirkt. Beide Mitglieder der Faktor H-Familie wurden innerhalb dieser Arbeit erstmalig als Komplementregulatoren identifiziert. Zudem zeigt diese Promotionsarbeit, dass durch die hohe Sequenzidentität zwischen CFHR1, CFHR3 und Faktor H, der Regulator Faktor H von seinen Liganden verdrängt werden kann. Die Ergebnisse veranschaulichen, dass sich CFHR1, CFHR3 und Faktor H in einem empfindlichen Gleichgewicht befinden und die An- bzw. Abwesenheit der CFHR1-/CFHR3-Proteine die lokale, Faktor H-bedingte, Komplementregulation beeinflusst.

Zudem wurde im Rahmen dieser Arbeit ein neues komplementbasiertes Therapeutikum entwickelt, welches die oberflächenerkennenden Domänen SCR15-20 von Faktor H mit dem potenten, etablierten Komplementinhibitor Compstatin kombiniert. Der neue Inhibitor COMP_CFH15-20 blockiert spezifisch die Amplifikationsprozesse des Komplementsystems auf zellulären Oberflächen, und ist dadurch in der Lage, die, durch Polymorphismen oder Defizienzen, beeinträchtigte intraokulare Komplementregulation zu verbessern.

In ihrer Gesamtheit liefert die vorliegende Promotionsarbeit Erklärungen inwiefern Polymorphismen sowie Defizienzen des *Faktor H*-Genclusters die lokale, intraokulare Komplementregulation beeinträchtigen. Die Ergebnisse zeigen erstmals, welche komplementregulatorischen Prozesse in AMD-Patienten gestört sind. Damit liefert diese Promotionsarbeit einen wichtigen Beitrag zum Verständnis des Krankheitsmechanismus und definiert AMD eindeutig als Erkrankung, die durch unzureichende Kontrolle des Komplementsystems verursacht wird.

Summary

Age-related macular degeneration (AMD) is the most common form of blindness in western societies and affects approx. 50 million people worldwide. The disease affects older individuals (>50 years) and is characterized by a gradual degeneration of photoreceptors within the macula. This leads to a functional loss of the macula, which is the retinal spot responsible for high resolution vision. The loss of central vision is accompanied by impaired color and contrast sensitivity. Due to the demographic development in the next decades, and the associated increasing number of affected individuals, AMD is not only a medical but also a socioeconomic challenge. Besides several individual risk factors, there exists strong evidence that genetics has also a substantial contribution to the disease. In 2005, genome-wide linkage studies identified for the first time concrete risk genes for AMD. Polymorphisms within the *Factor H*, *Factor B*, *C2*, *C3* genes as well as deletion of the complement factor H-related genes *CFHR1* and *CFHR3* modify the individual disease risk. Thus, the complement system plays a central role in AMD development. The human complement system is part of the innate immunity. Under physiological conditions the induced effector functions are crucial in maintaining tissue homeostasis as well as in eliminating microorganisms. However, partial or complete loss of human complement regulators due to polymorphisms, mutations or deficiencies causes several diseases.

In the present thesis, AMD-associated polymorphisms as well as deficiencies of the *Factor H*-gene cluster were investigated. How they affect the efficiency of complement regulation and how they modify the AMD-risk. The AMD-associated amino acid substitutions V62I as well as Y402H of Factor H and FHL1 were systematically compared in terms of ligand binding and complement regulatory functions. The obtained results showed that only the Y402 → H402 variation, but not the V62→I62 variation affects the activity of Factor H and FHL1. The Y402 → H402 variation decreased the complement regulatory activity on cellular surfaces by 25 % and was able to primarily induce cell damage i.e. trigger AMD. In addition, the Y402 → H402 variation is also responsible for intraocular inflammation processes that occur in the presence of necrotic retinal cells. The risk variants of Factor H impair the opsonisation of necrotic cells and therefore inhibit an anti-inflammatory removal of cellular debris by macrophages. The thesis further reveals why the deficiency of the complement factor H-related proteins CFHR1 and CFHR3 has a protective effect on disease progression. Both, CFHR1 and CFHR3 were identified as novel human complement regulators. Due to the high sequence identity of the three regulators, CFHR1 and CFHR3 compete with Factor H for binding to several ligands. These findings allude to a critical balance between the complement regulators CFHR1, CFHR3 and Factor H in which the presence or absence of CFHR1/CFHR3 modulates the local Factor H-mediated complement activity.

The scope of this PhD work also comprises the development of a novel complement-based therapeutic, which consists of a combination of the surface recognition region of Factor H and the potent complement inhibitor compstatin. The novel inhibitor COMP_CFH15-20 specifically blocks the amplification steps of complement on cellular surfaces. The medical relevance of COMP_CFH15-20 would be the improvement of the intraocular complement

regulation which is, in most AMD patients, negatively affected by polymorphisms or deficiencies.

The present thesis explains how polymorphisms and deficiencies of the human *Factor H*-gene cluster modify the local, intraocular complement regulation. These findings show, for the first time, which complement regulatory processes are dysfunctional in AMD-patients. This thesis therefore provides a substantial contribution for a better understanding of the pathogenesis of AMD and clearly defines the disease as a disorder that is caused by an insufficiently controlled complement system.

Einleitung

1 Charakteristika altersbedingter chronischer Erkrankungen

Die Ätiologie von altersbedingten chronischen Erkrankungen ist komplex. Häufig fällt es schwer, konkrete Risikofaktoren zu definieren, da die jahrzehntelange Exposition gegenüber verschiedenen Umweltfaktoren oftmals nicht abschätzbare individuelle Auswirkungen zwischen einzelnen Individuen hat. Selten trägt ein einziger Umweltfaktor in relevantem Ausmaß zu dem komplexen Krankheitsgeschehen bei. Neben dem natürlichen Alterungsprozess und den damit verbundenen epigenetischen Veränderungen, haben sich dennoch individuelle Einflüsse wie das Rauchen, Übergewicht sowie die Art der Ernährung als Risikofaktoren für eine Vielzahl von altersbedingten Erkrankungen etabliert[1,2].

Zudem wird jeder Alterungsprozess von dem Zustand einer leichten, aber systemisch, stattfindenden Entzündung begleitet – ein immunologisches Phänomen, welches als „Entzündungsaltern" (englisch: *inflamm-aging*) bezeichnet wird[3]. Eine Vielzahl von internen und externen Stimuli resultiert dabei in einer chronischen Immunantwort insbesondere durch die angeborene Immunabwehr, was zu einer erhöhten Ausschüttung von pro-inflammatorischen Zytokinen führt[4]. Während dieser systemisch stattfindenden pro-inflammatorischen Prozesse kommt es anfangs zu noch asymptomatischen Gewebeschädigungen, deren Intensität aber stetig zunimmt. Das Phänomen des *inflamm-aging* steht dabei in direktem Zusammenhang mit altersbedingten Erkrankungen wie der altersabhängigen Makuladegeneration, Alzheimer, Arteriosklerose, Osteoporose oder *Diabetes mellitus* vom Typ 2[5,6]. Das Altern, oder vielmehr die damit assoziierten chronischen Erkrankungen, sind vermutlich die späte Konsequenz dafür, dass das menschliche Immunsystem nicht für das Erreichen eines hohen Lebensalters ausgelegt ist, d. h. aus evolutionärer Sicht keine Selektion gegen *inflamm-aging* stattfand[7]. Das Auftreten dieser Krankheiten hängt häufig vom Genotyp des Einzelnen ab. Demzufolge führen pro-inflammatorische Genotypen (die in der frühen Phase des Lebens von Vorteil waren, um Infektionen zu widerstehen) im Alter zu chronischen Entzündungsprozessen[8,9].

Aufgrund der späten Symptomatik sowie den zahlreichen individuellen Risikofaktoren, war es lange Zeit schwierig, konkrete Risikogene der einzelnen altersbedingten Erkrankungen zu identifizieren. Doch mit den Daten des *Human Genome Projects* änderten sich die Voraussetzungen für genetische Untersuchungen komplexer Erkrankungen[10]. Die wachsenden Datenbanken von Einzelnukleotidpolymorphismen (SNPs: „***s**ingle **n**ucleotide **p**olymorphisms*") des menschlichen Genoms ermöglichten die parallele Analyse von über 100 SNPs und führten zur Entdeckung zahlreicher Gene, die signifikant mit altersbedingten Erkrankungen assoziiert sind. So wurden Varianten in Genen des Komplementsystems mit der altersabhängigen Makuladegeneration[11-14], das Gen für Apolipoprotein E mit Alzheimer[15] sowie bis zu zehn unterschiedliche Gene für *Diabetes mellitus* vom Typ 2 als definitive Risikogene der entsprechenden Krankheit identifiziert[16].

Die vorliegende Arbeit befasst sich mit der altersabhängigen Makuladegeneration sowie dem Einfluss des Komplementsystems auf deren Pathogenese, so dass auf diese Punkte in den nächsten Kapiteln näher eingegangen wird.

2 Altersabhängige Makuladegeneration

Die **a**ltersabhängige **M**akula**d**egeneration (AMD) ist die häufigste Ursache für Erblindung des Menschen[17]. Vor allem in den Industrieländern Europas, Nordamerikas sowie Australien verursacht sie bis zu 32 % aller Neuerblindungen[18-20]. Die Erkrankung tritt ab dem 50. Lebensjahr auf, und ist charakterisiert durch das allmähliche Absterben von Fotorezeptoren der Makula. Die Makula ist jener Bereich der zentralen Netzhaut, mit der größten Fotorezeptordichte, deren sukzessive Degeneration zu einem schweren zentralen Sehverlust führt.

Zu Beginn verläuft der Krankheitsprozess der AMD langsam und asymptomatisch. Charakteristisch für das Anfangsstadium ist das Auftreten von Drusen, Ablagerungen von extrazellulärem Material unterhalb der Makula, die teilweise normale Erscheinungen eines natürlichen Alterungsprozesses sind[21]. Erhöht sich die Zahl und die Größe der Drusen, erfolgt der Übergang in ein fortgeschrittenes Krankheitsstadium, bei dem zwei Formen unterschieden werden: die geografische Atrophie und die neovaskuläre Form[22]. Die Zahl der Betroffenen wird in Deutschland auf etwa 4 Millionen, weltweit auf bis zu 50 Millionen, geschätzt[23,24]. Im Zuge der demografischen Entwicklung, einer besseren medizinischen Versorgung, einem stetig wachsenden Gesundheitsbewusstsein und der damit verbundenen hohen Lebenserwartung, ist mit einer deutlichen Zunahme der Betroffenen in den nächsten Dekaden zu rechnen. In den kommenden 20 Jahren wird demnach mit einer Verdopplung der Krankheitsfälle gerechnet, so dass dann 35 % der Bevölkerungen über 75 Jahre Anzeichen von AMD aufweisen werden[25].

Die genaue Ursache, welche zur Schädigung der Makula und der umgebenden retinalen Gewebe führt, ist bis heute noch nicht explizit definiert. Dennoch existieren zahlreiche Theorien, die den Pathomechanismus beschreiben. Eine chronische Immunantwort der angeborenen Immunabwehr, vor allem durch die unkontrollierte Aktivierung des Komplementsystems[26], lokale Entzündungsprozesse[27] sowie jahrelanger photooxydativer Stress[28] sollen entscheidend zum Krankheitsprozess beitragen.

2.1 Anatomie und Immunologie des Auges

Die Hülle des menschlichen Auges besteht aus drei konzentrischen Schichten[29]. Der äußeren, gefäßarmen Sklera, dem Gefäßnetzwerk der Choroidea und der fotorezeptiven Retina (**Abbildung 1**). Die Retina besteht aus insgesamt 11 Schichten, die zusammen ein hochspezialisiertes Nervengewebe bilden. Den lichtempfindlichen Fotorezeptoren kommt dabei eine besondere Bedeutung zu, da sie den einfallenden Lichtreiz in einen Nervenimpuls umwandeln und über Nervenfasern zum Gehirn weiterleiten. Die höchste Dichte von Fotorezeptoren weist die Makula auf. Sie liegt exakt auf der optischen Achse in der zentralen Retina, und ist mit ihrem Zentrum der *Fovea centralis* verantwortlich für feinste Ortsauflösung und räumlich differenzierte Wahrnehmung[30]. Nur mit der Makula wird die größte Sehschärfe erreicht, die etwa beim Lesen benötigt wird. Eine Schädigung der Makula führt demnach nicht nur zum Verlust der zentralen Sehschärfe, sondern auch zu einer

verminderten Kontrastwahrnehmung, einem eingeschränkten Farbsehen und der Unfähigkeit, sich an veränderte Lichtverhältnisse anzupassen[31,32].

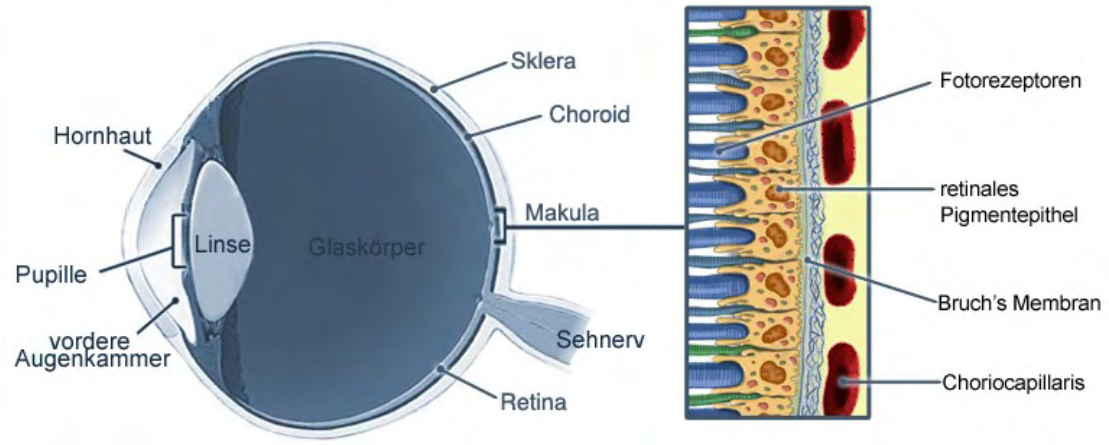

Abbildung 1: Der Aufbau des Auges. Das menschliche Auge wird –von außen nach innen– von der Sklera, der Choroidea und der Retina umhüllt. Über die Pupille trifft das Licht auf die Linse, wird gebündelt und fällt durch den Glaskörper auf die Makula, dem Bereich der Retina, mit den meisten Fotorezeptoren. In der Retina werden die optischen Signale umgewandelt und über den Sehnerv an das Gehirn geleitet. Die Fotorezeptoren sind apikal von Zellen des retinalen Pigmentepithels umschlossen, um Nährstoffe sowie Abbauprodukte über die Bruch's Membran von bzw. zu den Gefäßen der Choriocapillaris zu transportieren (Bildausschnitt rechts).

Das menschliche Auge, insbesondere die Bereiche der Hornhaut, des Glaskörpers sowie der Retina, zeichnen sich durch eine immunologische Sonderstellung aus. Das heißt, es existieren anatomische und zelluläre Besonderheiten, die eine physiologische Immunantwort im Auge reduzieren bzw. eliminieren[33]. Dieses sog. „Immunprivileg" des Auges hat zwei Hauptursachen. Zum einen führt jede Entzündung und die damit verbundene Einwanderung von Immunzellen zu einem Anschwellen des betroffenen Areals, und damit entweder zu einer Verschiebung der optischen Achse oder zu einer Trübung der Hornhaut. Zum anderen sind die neuronalen Elemente der gesamten Retina nicht regenerationsfähig und eine kollaterale Zerstörung während einer intraokularen Entzündung wäre fatal[34]. Beide Szenarien resultieren entweder in einer Verschlechterung oder in einem Verlust des Sehvermögens. Um sich vor den Konsequenzen einer lokalen Entzündungsreaktion zu schützen, ist ein Hauptmerkmal des immunprivilegierten Auges die fehlende Lymphdrainage[35]. Antigene verlassen das Auge hauptsächlich durch das Trabekelwerk, gelangen dadurch direkt in den venösen Blutkreislauf und erreichen als erstes Lymphorgan die Milz[36]. Zusätzlich existieren im Auge nur wenige antigenpräsentierende Zellen, die Blut-Retina-Schranke verhindert den Eintritt von Immuneffektorzellen, Kammerwasser hemmt die Proliferation bzw. Aktivierung von Makrophagen und die hohe Expression des FAS-(CD59-)Liganden führt zur Apoptose von FAS-exprimierenden Lymphozyten[37-40].

Um dennoch entstehenden zellulären und azellulären Debris effizient zu beseitigen, und vor mikrobiellen Infektionen geschützt zu sein, existiert im Auge ein sensibles Gleichgewicht zwischen der natürlichen Immunsuppression und einer geregelten Immunabwehr. Dafür besitzt das Auge ein aktives Komplementsystem[41]. Das Komplementsystem ist Teil der

angeborenen Immunabwehr und ist in der Lage, degenerierte Zellen für anti-inflammatorische Phagozytoseprozesse zu markieren sowie endogene Mikroorganismen zu eliminieren[42,43].

2.2 Pathophysiologie der AMD

Um dauerhaft die Umwandlung von Licht- in Nervenimpulse zu gewährleisten, werden Fotorezeptoren fortwährend mit Nährstoffen und Sauerstoff versorgt. Zu diesem Zweck sind die Außensegmente der Fotorezeptoren von den mikrovillären Fortsätzen des **r**etinalen **P**igment**e**pithels (RPE) umschlossen (**Abbildung 1**). *Tight junctions* gewährleisten, dass die benötigten Substrate nur durch aktiven Transport über das RPE von bzw. zu den Fotorezeptoren transportiert werden (= Blut-Retina-Schranke). Die weiteren Aufgaben des RPEs sind die kontinuierliche Phagozytose abgeschnürter Fotorezeptorsegmente sowie die Regeneration des Aldehyds all-*trans*-Retinal in das fotoaktivierte Isomer 11-*cis*-Retinal[44]. Die Basalmembran der RPE-Zellen bildet gleichzeitig die innerste Schicht der **B**ruch's **M**embran (BM). Die BM ist ein extrazellulärer Matrixkomplex aus kollagenhaltigen, elastischen Fasern, deren Hauptaufgabe in der Vermittlung des Stofftransports zwischen RPE und der Choriocapillaris besteht. Die Choriocapillaris ist Teil der Choroidea jenem Gefäßnetzwerk, welches der Versorgung der inneren Netzhautschichten und damit der Fotorezeptoren dient.

Altersbedingte Veränderungen in der RPE-BM-Choriocapillaris-Struktur bilden primär die Grundlage für die Entstehung der AMD. Die kontinuierlichen Phagozytoseprozesse des RPEs werden ineffizienter und unverdaute Stoffwechselendprodukte der phagozytierten Fotorezeptorsegmente werden in Form von Lipofuszin abgelagert[45]. Lipofuszin ist ein nicht weiter verwertbares Abfallprodukt, welches unter Einwirkung von Licht freie Radikale erzeugt, Zellen schädigt und den natürlichen Alterungsprozess weiter beschleunigt[46,47]. Neben der Akkumulation von Lipofuszin im Zytoplasma der RPE-Zellen, wird unverdautes Material auch in der BM abgelagert. Dadurch entstehen zum einen Drusen, und zum anderen kommt es zur Verdickung der BM durch Phospholipideinlagerungen und infolgedessen zu einer Diffusionsbarriere[48]. Über Jahre hinweg beeinträchtigen diese Veränderungen die zentrale Sehfunktion nicht. Ob und wann ein Übergang in die AMD stattfindet, ist vermutlich abhängig vom Genotyp des Einzelnen. Es wird aber davon ausgegangen, dass die Entstehung der AMD mit einem lokalen ggf. chronischen intraokularen Entzündungsprozess verbunden ist[27,49].

Das Auftreten kleiner Drusen wird zunächst als pigmentepitheliale Dysfunktion und nicht als Manifestation der AMD betrachtet. Zunahme der Drusen in Größe, Zahl und Konfluenz ist jedoch ein wichtiger Risikofaktor für die Entwicklung eines fortgeschrittenen Krankheitsstadiums[21]. Dieser Drusentyp ist das früheste, klinisch erkennbare Merkmal der AMD, und beeinträchtigt den regulären Stoffaustausch zwischen dem RPE und dem Choroid (**Abbildung 2**). Ein Drusendurchmesser von >100 µm kann das Ablösen des Pigmentepithels von der BM verursachen. Die Zusammensetzung von Drusen ist vielfältig, und umfasst Lipide, Kohlenhydrate, Proteine und komplette Fragmente von degenerierten RPE-Zellen[50,51]. Proteomanalysen und immunhistochemische Studien belegen, dass Drusen

immunreaktiv für Proteine des Komplementsystems sind, und zwar aus allen Stufen der Komplementkaskade[26,52]. Es wurden die zentrale Komplementkomponente C3 einschließlich ihrer Aktivierungs- (C3a, C3b) und Degradierungsprodukte (iC3b, C3d), Komplementregulatoren der Flüssig- und Festphase sowie terminale Komplementproteine in Drusen nachgewiesen. Weiterhin ist das **C**-reaktive Protein (CRP: *„**C**-**r**eactive **p**rotein“*) ein unspezifischer Entzündungsparameter, sowie Amyloid P in Drusen detektierbar[27]. Das Vorkommen all dieser Komponenten führte zu der Annahme, dass Drusen sekundäre Manifestationen eines primären RPE-Schadens sind, der vermutlich durch lokale Komplementaktivierung verursacht wird[53].

2.2.1 GEOGRAFISCHE ATROPHIE

Die beschriebenen Altersveränderungen in RPE-Zellen sowie das vermehrte Auftreten von großen, konfluenten Drusen resultieren in dem Krankheitsbild der **g**eografischen **A**trophie (GA)[54]. Diese Spätform der AMD ist charakterisiert durch ein flächiges Absterben von retinalen Pigmentepithelzellen und dem Entstehen von atrophen Arealen. Fotorezeptoren, welche über diesen Arealen liegen, degenerieren ebenfalls, da sie metabolisch von den RPE-Zellen abhängig sind und nicht mehr ausreichend mit Sauerstoff und anderen Substraten versorgt werden können. Dadurch kommt es zu einem langsamen, aber stetigem Verlust des zentralen Sehvermögens. Die GA macht etwa 90 % aller AMD-Fälle aus, resultiert aber nur bei 5 – 10 % der Betroffenen in einer Erblindung[17]. Die derzeitigen Therapiemöglichkeiten einer GA sind begrenzt und stellen nur präventive bzw. stabilisierende Ansätze dar. Jedoch sollen die selbstständige Kontrolle individueller Risikofaktoren wie das Einstellen des Rauchens, Diät oder die vermehrte Aufnahme von Antioxidantien (ß-Carotin, Zink, Vitamin E) das Risiko um die Hälfte reduzieren[55,56].

2.2.2 CHOROIDALE NEOVASKULARISATION

Die zweite –weitaus schwerwiegendere– Spätform der AMD ist die **c**horoidale **N**eo**v**askularisation (CNV). Sie ist gekennzeichnet durch das Einsprossen neu gebildeter choroidaler Kapillaren, über Schwachstellen der BM unter das RPE. Die neu gebildeten Gefäße sind porös, so dass Blut und Gewebsflüssigkeit austritt, was zu Schwellung und Flüssigkeitsansammlungen unterhalb der Makula führt[57]. Hierbei kommt es erstmals zu einer Verzerrung des wahrgenommenen Bildes. Über Wochen und Monate können die Blutgefäße im subpigmentepithelialen Raum proliferieren, bevor die RPE-Schicht reißt und sich die angesammelte Flüssigkeit im subretinalen Raum verteilt. Hierbei kommt es zu einem raschen Verlust des Sehvermögens, da während dieses Vorgangs RPE-Zellen und Fotorezeptoren irreversibel geschädigt werden. Obwohl die CNV bei nur etwa 10 % der Betroffenen auftritt, ist sie dennoch für mehr als 90 % der schweren Sehbeeinträchtigungen verantwortlich[58]. Zur therapeutischen Behandlung der CNV wird eine Kombination aus fotodynamischer Laserbehandlung und der intravitrealen Gabe von entzündungs- und gefäßwachstumshemmenden Präparaten angewandt[59-61]. Die Laserbehandlung resultiert in einem Veröden der neugebildeten Gefäße und die Verabreichung von anti-VEGFs blockiert hochspezifisch die Gefäßneubildung durch den *„**v**ascular **e**ndothelial **g**rowth **f**actor“* (VEGF).

Trotz vielversprechender Erfolge und einer signifikanten Steigerung der zentralen Sehschärfe kann das Fortschreiten der Krankheit bisher nur verlangsamt, jedoch nicht verhindert werden[62].

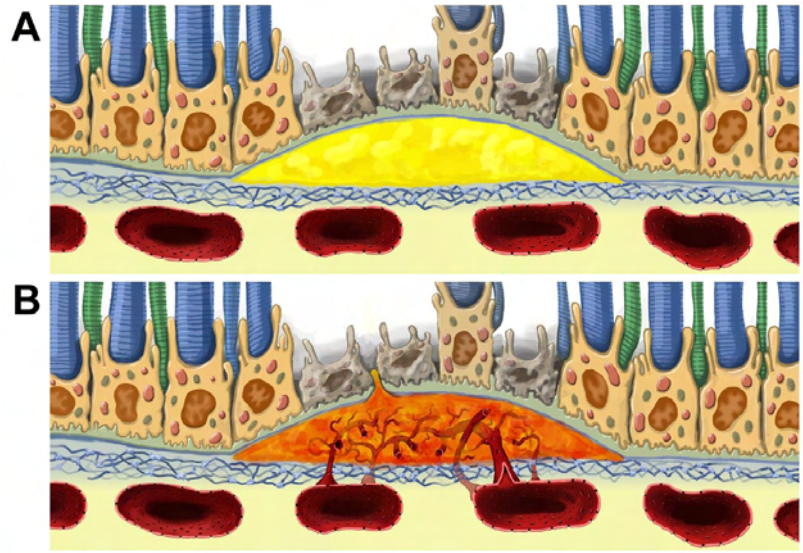

Abbildung 2: Spätformen der AMD. A) Die GA ist charakterisiert durch das vermehrte Auftreten von Drusen. Dadurch ist der Nährstofftransport zwischen der Choriocapillaris und dem RPE beeinträchtigt. Dies führt zum langsamen Absterben der RPE-Zellen und den darüber liegenden Fotorezeptoren. **B)** Die Spätform der CNV ist gekennzeichnet durch das Einwachsen von Kapillaren in den subpigmentepithelialen Raum. Aus undichten, porösen Gefäßen tritt Blut sowie Gewebsflüssigkeit aus, und resultiert in einer rapiden Zerstörung der Retina

2.3 Risikofaktoren

Mit zunehmendem Alter lassen sich in nahezu jedem Auge degenerative Veränderungen der Retina feststellen. Doch nur bei einem Teil der Betroffenen führen diese zu den pathologischen Manifestationen der AMD. Demzufolge existieren genetische und individuelle Faktoren, die das Risiko der AMD-Entstehung erhöhen und eine wichtige Rolle im Krankheitsprozess spielen (**Tabelle 1**).

Das Alter ist der wichtigste Risikofaktor. In allen bisherigen epidemiologischen Studien konnte nachgewiesen werden, dass die Prävalenz der AMD exponentiell mit dem Alter steigt. Liegt die Häufigkeit an einer der beiden Spätformen zu erkranken zwischen 55 - 64 Jahren noch bei 0.2 %, steigt sie in der Altersgruppe der >85-Jährigen auf 13 % an. Weiterhin ist das Auftreten von AMD abhängig von der ethnischen Herkunft[20]. Während mit 5.4 % die Prävalenz bei weißen Europäern, Amerikanern und Australiern relativ hoch ist, besitzen Personen afrikanischer Herkunft mit 2.4 % ein vergleichsweise geringes Risiko, leiden demzufolge weniger häufig unter AMD[63]. Ein weiterer hervorzuhebender Risikofaktor ist das Rauchen[64,65]. Bei starken Rauchern wurde ein 3-fach höheres Risiko für die Entwicklung des neovaskulären Spätstadiums festgestellt als bei Nichtrauchern. Dabei ist nicht primär das Nikotin der schädigende Faktor, sondern freiwerdende Sauerstoffradikale, welche auf Dauer die Retina schädigen. Weitere etablierte Faktoren der AMD-Entstehung schließen u. a. Bluthochdruck, Übergewicht sowie eine zu geringe Aufnahme von Antioxidantien oder Zink ein[18,66,67].

Der Nachweis der Erblichkeit einer AMD war lange Zeit schwierig, da aufgrund der späten Symptomatik und den zahlreichen individuellen Risikofaktoren, die Erstellung von

Familienstammbäumen erschwert war. Epidemiologische Studien sowie Untersuchungen von eineiigen Zwillingspaaren wiesen aber übereinstimmend darauf hin, dass die AMD im Wesentlichen einer genetischen Prädisposition unterworfen ist[68,69]. Mit der Verwendung von SNP-Datenbanken (SNP = „***s**ingle **n**ucleotide **p**olymorphism*“, welcher sich im Genpool einer Population durchgesetzt hat und häufig mit dem Auftreten bestimmter Krankheiten korreliert) ergaben sich neue Möglichkeiten, spezifische Risikogene für die AMD in großangelegten Fall-Kontroll-Studien zu identifizieren. Dadurch ist es im Jahr 2005 erstmalig gelungen, für die AMD ein konkretes Risikogen zu identifizieren[11,70]. Ein SNP in *Faktor H*, dem wichtigsten Regulator des alternativen Komplementweges, spielt demnach eine entscheidende Rolle. Der im Faktor H-Protein zum Aminosäureaustausch führende SNP erhöht das relative Risiko für die Entwicklung einer AMD 2 bis 4-fach für heterozygote Träger und 3 bis 7-fach für homozygote Träger[71]. Folglich trägt der Faktor H-Polymorphismus etwa 20-50 % zum AMD-Gesamtrisiko bei und erklärt somit einen wesentlichen Teil der genetischen Prädisposition. Durch genetische Studien, deren Focus nun speziell auf Gene von Komplementproteinen gerichtet war, wurden in weniger als zwei Jahren weitere Polymorphismen innerhalb des *Faktor H*-Genclusters[12,72] sowie der Komplementkomponenten *Faktor B*[13], *C2*[13] und *C3*[14] signifikant mit AMD assoziiert. Ein zweiter wichtiger AMD-assoziierter Genlocus wurde auf Chromosom 10 identifiziert. Er beinhaltet die beiden Risikogene, „***a**ge-**r**elated **m**aculopathy **s**usectibility gene **2**“(ARMS2)* und *„**h**igh-**t**emperature **r**equired factor **A1**“* (*HTRA1*), die entweder zusammen oder einzeln das AMD-Risiko beeinflussen[73,74].

Tabelle 1: genetische und individuelle Risikofaktoren der AMD

TYP	
genetisch	Faktor H, CFHRs, Faktor B, C2, C3, ARMS2, HTRA2,
individuell	Alter, Rauchen, ethnische Herkunft, Übergewicht, Bluthochdruck, Art der Ernährung

ARMS2:*„**a**ge-**r**elated **m**aculopathy **s**usectibility gene **2**“, CFHRs: „**c**omplement **f**actor **H**-**r**elated proteins“ HTRA1: „**h**igh-**t**emperature **r**equired factor **A1**“*

Da die Kombination der jeweiligen Risikovariationen aller Komplementproteine bis zu 75 % zum AMD-Gesamtrisiko beitragen, ist davon auszugehen, dass das Komplementsystem und durch die Proteine Faktor H, Faktor B und C3 –insbesondere der alternative Aktivierungsweg– eine Hauptrolle in der AMD-Entstehung spielt[75,76]. Im folgenden Kapitel soll deshalb das humane Komplementsystem genauer vorgestellt werden.

3 Das Komplementsystem

Das Komplementsystem des Menschen ist Teil der angeborenen Immunabwehr. Unter physiologischen Bedingungen werden durch das Komplementsystem tote Zellen (nekrotisch, apoptotisch) und Mikroorganismen (Bakterien, Pilze) markiert, durch professionelle Phagozyten erkannt und entfernt. Das Komplementsystem spielt also eine entscheidende Rolle in der Aufrechterhaltung der Gewebshomöostase sowie in der Eliminierung von Mikroorgansimen[42,43]. Darüber hinaus verbindet das Komplementsystem die angeborene mit der adaptiven Immunabwehr, indem es die Antikörperantwort von B-Lymphozyten und das immunologische Gedächtnis verstärkt[77]. Ende des 19. Jahrhunderts wurde das Komplementsystem als hitzeempfindlicher Bestandteil des Blutplasmas durch den belgischen Biologen Jules Bordet beschrieben sowie der Ausdruck „Komplement“ von Paul Ehrlich eingeführt[78]. Heute, mehr als 110 Jahre nach den ersten Entdeckungen, ist die zentrale Rolle des Komplementsystems innerhalb der angeborenen Immunabwehr besser bekannt, und komplizierte Zusammenhänge zwischen der adaptiven Immunabwehr sind detaillierter –aber nicht vollständig– verstanden. Ziel der kommenden Jahre wird sein, die genauen Mechanismen zu identifizieren mit denen das Komplementsystem die adaptive Immunität beeinflusst, um Autoimmunerkrankungen zu verstehen und ggf. behandelbar zu machen.

Das Komplementsystem umfasst eine Gruppe von mehr als 50 löslichen sowie membrangebundenen Proteinen. Diese reagieren kaskadenartig miteinander, um entweder modifizierte, körpereigene Zellen zu markieren und entzündungsfrei zu entfernen oder aber um Entzündungsprozesse auszulösen, damit Mikroorganismen effizient eliminiert werden. Hauptbildungsort der löslichen Komplementproteine sind die Hepatozyten der Leber. Dabei wird eine inaktive Vorstufe des Proteins ins Plasma ausgeschüttet, bei Bedarf durch enzymatische Spaltung lokal aktiviert, so dass in der Regel zwei funktionell aktive Spaltprodukte entstehen. Das kleinere der beiden Fragmente, ein Anaphylatoxin, wird mit dem Buchstaben „a“, das größere Fragment, meist eine Serinprotease, mit dem Buchstaben „b“ gekennzeichnet. Im Verlauf der Komplementaktivierung entstehen durch die proteolytische Spaltung der löslichen Komplementkomponenten C3, C4, C5 die Anaphylatoxine C3a, C4a und C5a, die Opsonine C3b und C4b sowie C5b[79]. Die Anaphylatoxine bewirken bei Makrophagen und Monozyten die Freisetzung der proinflammatorischen Zytokine **T**umor**n**ekrose**f**aktor-α (TNF-α), Interleukin 1 oder Interleukin 6[80]. Dadurch wird lokales Gewebsendothel aktiviert und Leukozyten können, u. a. durch die Expression von P-Selektin, an Endothelzellen adhärieren[81]. Des Weiteren aktiviert C5a neutrophile Zellen und bewirkt bei Mastzellen die Freisetzung von Histamin, welches die Permeabilität der Blutgefäße am Ort der Komplementaktivierung erhöht. Bereits adhärierte Leukozyten migrieren nun, durch den Prozess der Diapedese, aus dem Blutstrom in das umgebende Gewebe, um dort Infektionen zu bekämpfen. Die zielgerichtete Migration der Leukozyten zum Ursprungsort der Komplementaktivierung erfolgt durch die chemotaktische Wirkung der Anaphylatoxine C3a, C4a und C5a[82].

Während die Anaphylatoxine an der Reaktionsstelle freigesetzt werden, um eine lokale Entzündungsreaktion auszulösen, verbleiben die Fragmente C3b, C4b und C5b auf der Zelloberfläche, opsonisieren diese und bilden die Bestandteile der C3- und der C5-Konvertasen. Opsonisierte, körpereigene tote Zellen sowie Immunkomplexe werden von den Komplementrezeptoren CR1, CR3 und CR4 (CR: „*complement receptor*“) auf Makrophagen erkannt, gebunden, aufgenommen und anschließend in die Leber transportiert, wo sie letztendlich eliminiert werden[83]. Der Prozess der komplementvermittelten Entfernung von körpereigenem Zellmaterial läuft entzündungsfrei ab und verhindert die chronische Ablagerung von zellulären Bestandteilen sowie die Ausprägung von Autoimmunerkrankungen[84]. Auf der Oberfläche von Mikroorganismen mündet die Komplementaktivierung in den terminalen Komplementweg. An dessen Ende steht die Bildung eines lipophilen Membranangriffskomplexes, welcher in der osmotischen Lyse von Mikroorganismen resultiert[85].

Das Komplementsystem beeinflusst auch die adaptive Immunabwehr. Opsonisierte Zellen sowie Immunkomplexe werden, wie beschrieben, effizient von Makrophagen sowie dendritischen Zellen über CR1, CR3 und CR4 erkannt und aufgenommen. Dabei handelt es sich bei diesen Zelltypen um professionell antigenpräsentierende Zellen, denen eine Schlüsselrolle bei der Initiierung der adaptiven Immunabwehr zukommt. Die Aktivität der B-Zellen, die dritte professionelle antigenpräsentierende Zelle, wird ebenfalls durch Proteine des Komplementsystems direkt beeinflusst. Durch Komplementaktivierung wird die Anforderung für die Aktivierung einer B-Zelle herabgesetzt, was eine verstärkte B-Zell-Antwort zur Folge hat[77,86]. Weiterhin induziert das Komplementsystem, durch die Anaphylatoxine C3a und C5a sowie C3-Aktivierungsprodukte, eine T-Zellantwort und trägt zu einer effizienten $CD4^+$ sowie $CD8^+$ Immunantwort bei[87].

3.1 Die Kaskadenstruktur des Komplementsystems

Die Aktivierung des Komplementsystems erfolgt über drei Wege: den Alternativen-, den klassischen- und den Lektinweg (**Abbildung 3**). Die Grundstruktur aller drei Komplementwege ist identisch und folgt dem Schema der Aktivierung, gefolgt von der Formation einer C3-Konvertase, Spaltung von C3, Bildung einer C5-Konvertase, Spaltung von C5 und letztendlich die Bildung des terminalen Komplementkomplexes. Dabei ist die Aktivierung des zentralen Moleküls C3 entscheidend, um eine effiziente Beladung degenerierter Zellen oder eines Krankheitserregers mit C3b zu gewährleisten. In allen drei Wegen kommt es deshalb zur Amplifikation dieses zentralen Schrittes.

3.1.1 Der alternative Weg

Der alternative Weg ist der evolutionär älteste Weg der Komplementaktivierung. Die Aktivierung erfolgt kontinuierlich auf niedrigem, kontrolliertem Niveau und ist unabhängig von speziellen Initiatoren[88]. Ursache der kontinuierlichen Aktivierung ist die spontane Hydrolyse der zentralen Komplementkomponente C3. Dabei wird im C3-Molekül eine interne Thioesterbindung durch H_2O angegriffen, was zu einer Konformationsänderung und damit zur Bildung von $C3(H_2O)$ führt[89]. $C3(H_2O)$ ermöglicht die Anlagerung des Zymogens

Faktor B. Die Serinprotease Faktor D spaltet Faktor B in die beiden Fragmente Ba und Bb, wobei das Bb-Fragment nun selbst eine aktive Serinprotease darstellt, und an C3(H_2O) gebunden bleibt[90]. Der neu entstandene C3(H_2O)Bb Enzymkomplex, ist eine C3-Konvertase und spaltet C3 in das Opsonin C3b und das Anaphylatoxin C3a. Die Abspaltung von C3a exponiert die hochreaktive Thioesterbindung in C3b, die in weniger als 100 µs kovalent an Amino- bzw. Hydroxygruppen von benachbarten Zelloberflächen bindet[91]. Das, an zelluläre Oberflächen fixierte C3b, interagiert erneut mit Faktor B, welches wiederum durch Faktor D gespalten und aktiviert wird. So entsteht die oberflächengebundene C3-Konvertase des alternativen Weges, C3bBb. C3bBb spaltet C3, reaktives C3b lagert sich auf zellulären Oberflächen ab und der Prozess der Faktor B-Bindung und Aktivierung beginnt von vorn. Dieser sogenannte *„Amplification Loop“* bewirkt eine fortwährende Aktivierung des alternativen Weges und resultiert in einer effektiven Opsonisierung von Oberflächen mit C3b-Proteinen.

3.1.2 Der klassische Weg und der Lektinweg

Die Initiierung des klassischen- und des Lektinweges ist, im Gegensatz zum alternativen Komplementweg, abhängig von spezifischen Aktivatoren. Der klassische Weg wird durch die C1q-Bindung an Antigen-Antikörper-Komplexe oder aber antikörperunabhängig durch direkte Bindung von C1q an modifizierte körpereigene Zellen, über das CRP, Polyanionen oder DNA aktiviert[92]. Das Initiatorprotein C1q ist mit den inaktiven Serinproteasen C1s sowie C1r assoziiert. Interagiert C1q mit einem spezifischen Liganden auf der Zieloberfläche kommt es zu einer Konformationsänderung innerhalb des Proteinkomplexes und zu einer kaskadenartigen Aktivierung der beiden assoziierten Serinproteasen. Die aktiven Serinproteasen spalten die Komplementkomponente C4 in die Fragmente C4a und C4b. Die Abspaltung des Anaphylatoxins C4a bewirkt die Exposition einer Thioesterbindung in C4b, wodurch C4b kovalent mit Amino- bzw. Hydroxygruppen der Zieloberfläche interagiert[93,94]. An C4b lagert sich C2, welches ebenfalls gespalten und aktiviert wird. Das größere C2a-Fragment bleibt an C4b gebunden, und es entsteht die C3-Konvertase des klassischen Weges: C4bC2a.

Der Lektinweg ähnelt dem klassischen Komplementweg, ist jedoch unabhängig von C1q und Antigen-Antikörper-Komplexen. Die Aktivierung des Lektinweges erfolgt durch Mannosestrukturen auf mikrobiellen Oberflächen[95]. Dabei erkennt Mannose-bindendes Lektin (MBL*: „**m**annose **b**inding **l**ectin“*) Pathogen-assoziierte molekulare Muster (PAMPs: *„**p**athogen **a**ssociated **m**olecular **p**atterns“*), die sich im Laufe der Evolution zwischen Mikroorganismen erhalten haben, aber nicht auf autologen Zellen vorkommen. Die Bindung von MBL an PAMPs, aktiviert MBL-assoziierte Serinproteasen und resultiert in der Spaltung und Aktivierung der Komplementkompontenen C4 sowie C2[96]. Mit C4bC2a entsteht im Lektinweg die gleiche C3-Konvertase, wie auch im klassischen Weg. Ablagerung von C3b durch den klassischen und/oder den Lektinweg erleichtert die Erkennung modifizierter körpereigener Zellen bzw. eines Krankheitserregers durch Makrophagen, ist aber auch die Grundlage für die parallele Initiierung des alternativen Weges durch den *„Amplification Loop“*.

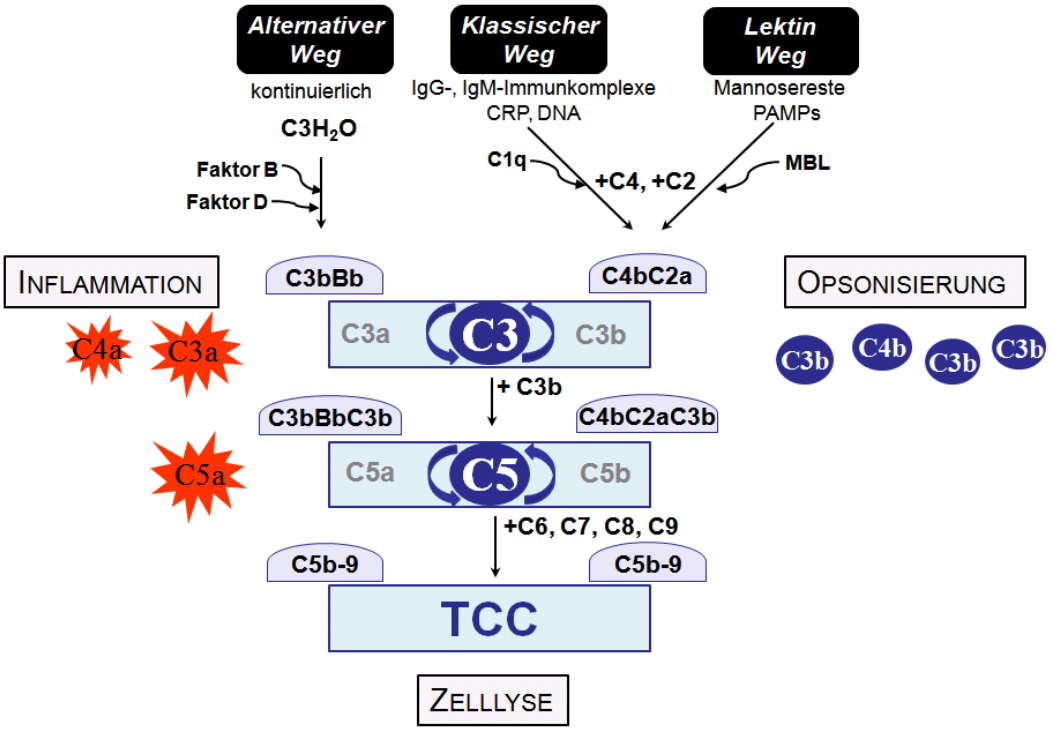

Abbildung 3: Die Komplementkaskade. Die drei Wege der Komplementaktivierung unterscheiden sich hinsichtlich ihrer Aktivierung, konvergieren aber auf Ebene der C3-Konvertase. Durch Anlagerung eines zusätzlichen C3b-Moleküls an die entsprechende C3-Konvertase, entstehen die C5-Konvertasen des alternativen bzw. des klassischen und des Lektinweges. Die Spaltung von C5 leitet die terminalen Komplementprozesse ein, führt zur Zusammenlagerung des membranangreifenden Komplexes (TCC) und damit zur Lyse der Zielzelle **(Zelllyse)**. Durch die Anaphylatoxine C3a, C4a und C5a werden Leukozyten chemotaktisch zum Ursprungsort der Komplementaktivierung gelockt und eine lokale Entzündungsreaktion entsteht **(Inflammation)**. Die Opsonine C3b, C4b binden an die Oberfläche von modifizierten Zellen oder Mikroorgansimen, und markieren diese für die Phagozytose **(Opsonisierung)**.

3.1.3 TERMINALE KOMPLEMENTPROZESSE

Alle drei Komplementaktivierungswege führen zur Bildung einer C3-Konvertase und damit zur Opsonisierung von zellulären Oberflächen mit C3b. Bindet C3b an den entsprechenden C3bBb- oder C4bC2a-Enzymkomplex, entsteht mit C3bBbC3b die C5-Konvertase des alternativen Weges, oder mit C4bC2aC3b die des klassischen und des Lektinweges. C5 bindet über C3b an diese Enzymkomplexe und wird daraufhin in C5b und den potenten Entzündungsmediator C5a gespalten. Aktiviertes C5b lagert sich jedoch nicht, wie C3b oder C4b über Thioesterbindungen an die Zelloberfläche an, sondern verbleibt an der C5-Konvertase und löst eine nichtenzymatische Zusammenlagerung der Komplementkomponenten C6, C7, C8 und C9 aus. Es entsteht der terminale Komplementkomplex (TCC: *„terminal complement complex“*)[97]. Konformationsänderungen innerhalb einzelner Komponenten resultieren in den amphiphatischen Eigenschaften des TCC der sich in die Phospholipidmembran inseriert, deren Integrität zerstört und zur osmotischen Lyse der Zielzelle führt[98].

3.2 Die Regulation der Komplementkaskade

Die Komplementkaskade ist ein effektives System der menschlichen Immunabwehr, welches durch sein enormes Amplifikationspotenzial eindringende Mikroorganismen innerhalb von Sekunden eliminiert, noch bevor die adaptive Immunabwehr initiiert wird. Die Aktivierung ist jedoch zeitlich, räumlich sowie in ihrer Intensität begrenzt, um gesunde, körpereigene Zellen vor den Auswirkungen eines aktivierten Komplementsystems zu schützen[79]. Weiterhin bedarf es einer geregelten Komplementaktivierung auf nekrotischen sowie apoptotischen Zellen[84,99]. Auf diesen modifizierten körpereigenen Zellen, ist eine Aktivierung des Komplementsystems nur bis zur Ebene von C3 erwünscht, um C3b-Ablagerung sowie iC3b-Formation zu generieren. Die Interaktion von oberflächengebundenem C3b/iC3b mit CR1-, CR3-, sowie CR4-exprimierenden Makrophagen ermöglicht eine effiziente anti-inflammatorische Phagozytose und damit die Aufrechterhaltung der Gewebshomöostase[100]. Obwohl die Regulation des Komplementsystems alle Ebenen, beginnend von der Initiierung, über die C3- und C5-Konvertasen, bis zu den terminalen Komplementprozessen umfasst, hat vor allem die Reglementierung der C3-Konvertasen zentrale Bedeutung. Denn die Steuerung der C3-Konvertasen schützt zum einen intakte körpereigene Zellen vor der Amplifikation einer ungewollten Komplementaktvierung, und ermöglicht zum anderen eine effiziente Phagozytose degenerierter, körpereigner Zellen.

3.2.1 Komplementregulation auf Ebene von C3

Die C3-Konvertasen aller drei Aktivierungswege resultieren in C3b-Generierung, und dadurch zu einer fortwährenden Aktivierung des alternativen Weges durch den *„Amplification Loop"*. Die reaktive Thioestergruppe des C3b-Moleküls interagiert willkürlich mit allen Zelloberflächen und diskriminiert nicht zwischen Aktivatoroberflächen (z. B. Mikroorgansimen) und Nichtaktivatoroberflächen (z. B. körpereigene, gesunde Zellen)[101]. Ebendiese Unterscheidung in „Selbst" und „Fremd" ist jedoch essenziell, um intakte, körpereigene Zellen vor den Konsequenzen einer Komplementaktivierung zu schützen[102]. Daher wird die Aktivität der C3-Konvertasen auf drei verschiedene Arten modifiziert[88]. Erstens kann die Bindung von Faktor B an C3b oder C2 an C4b kompetitiv durch die Flüssigphaseregulatoren Faktor H sowie dem C4b-bindenden Protein (C4BP: *„**C4b**-binding **p**rotein"*) gehemmt werden. Das Vorhandensein von Sialinsäureresten speziell auf körpereigenen Zellen verstärkt die Bindung ebendieser Regulatoren an jedes C3b- bzw. C4b-Molekül, so dass Faktor B bzw. C2 kompetitiv verdrängt werden und die Formation der entsprechenden C3-Konvertase erschwert wird. Hat sich mit C3bBb bzw. C4bC2a bereits eine C3-Konvertase gebildet, beschleunigen die Regulatoren CD55 (DAF: *„**d**ecay **a**ccelerating **f**actor"*) CR1, Faktor H, das Faktor H-ähnliche Protein (FHL1: *„**f**actor **H**-**l**ike protein **1**"*) sowie C4BP deren Zerfall. Die Fähigkeit bestimmter Regulatoren, die C3-Konvertase zu dissoziieren, wird als *„decay-accelerating activity"* bezeichnet. Drittens katalysiert die Serinprotease Faktor I, in Gegenwart von verschiedenen Kofaktoren die enzymatische Spaltung der α-Kette von C3b sowie C4b, und generiert die Inaktivierungsprodukte iC3b und iC4b. Als Kofaktoren agieren die membranständigen Proteine CR1 sowie CD46 (MCP: *„**m**embrane **c**ofactor **p**rotein"*) und die im Plasma gelösten Regulatoren Faktor H, FHL1 und

C4BP. Die Kofaktoraktivität der Flüssigphaseregulatoren kann weiterhin in Gegenwart von Zink[103], **m**onomerem **CRP** (mCRP)[100] oder dem Faktor H-verwandten Protein 3[104] verstärkt werden und resultiert in einer erhöhten iC3b-Ablagerung. Durch Interaktion mit den Rezeptoren CR1 – CR4 auf Makrophagen vermittelt iC3b seine Funktion, und initiiert die Phagozytose von nekrotischen und apoptotischen Zellen sowie deren Elimination aus dem System[99]. Die Existenz löslicher Regulatoren wie Faktor H oder auch C4BP ist insbesondere auf körpereigenen Strukturen essenziell, auf denen membranständige Regulatoren wie CR1, MCP und DAF nicht exprimiert werden. Ein Fehlen oder ein Funktionsverlust v. a. von Faktor H, dem Regulator des alternativen Komplementweges, resultiert in der Ausprägung systemischer Erkrankungen, die durch unkontrollierte Komplementaktivierung ausgelöst werden[105,106].

3.2.2 KOMPLEMENTREGULATION IM AUGE

Obwohl, oder gerade weil, das Auge einen immunologischen Sonderstatus besitzt, welcher das Ausmaß physiologischer Immunantworten reduziert, weist das Auge ein funktionsfähiges Komplementsystem auf. Die Aktivierung der Komplementkaskade findet durch den alternativen sowie klassischen Weg, jedoch nicht durch den Lektinweg, statt[41]. Vor allem die kontinuierlich stattfindende Aktivierung des alternativen Komplementweges erlaubt im Falle einer Infektion eine sofortige Bekämpfung des eingedrungenen Mikroorganismus sowie die Entfernung von zellulären Debris. Doch muss die Komplementkaskade im Auge feinreguliert werden, um eine komplementvermittelte intraokulare Entzündung zu verhindern[107]. Dafür exprimiert der RPE-Choroid-Komplex sowohl membranständige (MCP, DAF, CD59)[108] als auch gelöste Regulatorproteine (Faktor H, FHL1)[109,110]. Auf degenerierten, drusennahen RPE-Zellen ist die Expression der membranständigen Regulatoren MCP sowie DAF stark reduziert, so dass die Flüssigphaseregulatoren Faktor H und FHL1 deren Fehlen kompensieren müssen. Da weiterhin das C4BP-Protein nicht intraokular synthetisiert wird, liegt es an Faktor H und FHL1 eine komplementvermittelte Entzündungsreaktion zu verhindern[76].

3.3 Die Faktor H-Proteinfamilie

Faktor H, der essenzielle Regulator des alternativen Komplementweges, ist Namensgeber einer Proteinfamilie, die aus sieben, strukturell verwandten Mitgliedern besteht (**Abbildung 4**)[111]. Die Mitglieder der Faktor H-Familie sind auf Chromosom 1, Bereich 1q32, innerhalb des *RCA*-Genclusters kodiert (RCA: „***r**egulators of **c**omplement **a**ctivation*“). Das *Faktor H*-Gen, mit einer Größe von 94 kb und 23 Exons, ist eng gekoppelt an die fünf eigenständigen Gene der Faktor H-verwandten Proteine 1-5 (CFHRs: „***c**omplement **f**actor **H**-**r**elated proteins*“)[112]. Das FHL1-Protein wird vom selben Gen wie Faktor H kodiert, entsteht jedoch während eines alternativen Spleißvorgangs[113]. Alle Proteine der Faktor H-Familie werden hauptsächlich von den Hepatozyten der Leber gebildet und von dort in den Blutkreislauf sezerniert. Aber auch andere Zelltypen wie Lymphozyten, Fibroblasten, Neuronen oder retinale Pigmentepithelzellen können Faktor H und FHL1 synthetisieren, um so deren lokale Konzentration bei Bedarf zu erhöhen. Die gesamte Proteinfamilie ist aus

globulären Proteindomänen den sog. „*short consensus repeats*“ (SCRs) aufgebaut. Jedes SCR besteht aus etwa 60 Aminosäuren, die in fünf antiparallelen ß-Faltblättern angeordnet sind. Zusammen mit vier hochkonservierten Cysteinresten, welche untereinander zwei Disulfidbrücken ausbilden, ergibt sich die typische globuläre Tertiärstruktur der SCRs[114]. Die Anzahl dieser Proteindomänen variiert stark zwischen den Mitgliedern der Faktor H-Familie, doch durch zahlreiche Duplikationen innerhalb des *RCA*-Genclusters weisen die SCRs einiger Familienmitglieder untereinander eine Sequenzidentität von 30 - 100 % auf[115].

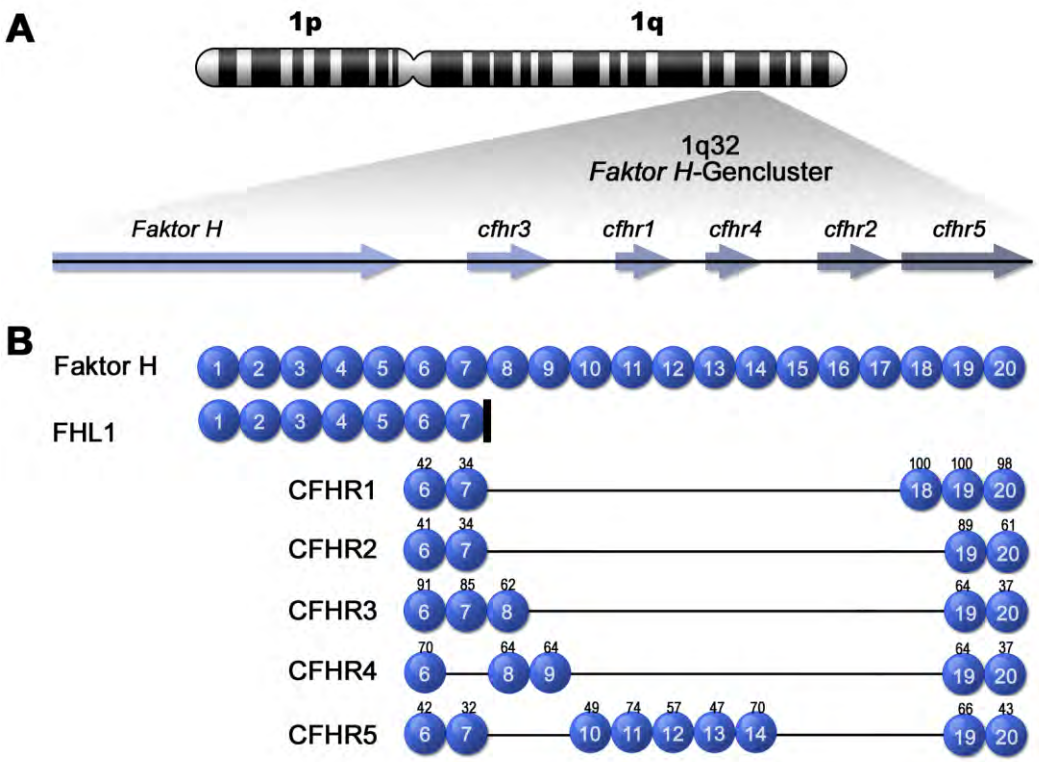

Abbildung 4: Die Mitglieder der Faktor H-Familie. A) Die sechs Gene des *Faktor H*-Genclusters sind auf dem langen Arm des Chromosoms 1 kodiert und aufeinanderfolgend angeordnet. *Faktor H* sowie die fünf *CFHRs* werden durch eigenständige Gene (Pfeile) kodiert, wohingegen FHL1 aus einem alternativen Spleißvorgang des *Faktor H*-Gens entsteht. **B)** Die sekretierten Plasmaproteine bestehen alle aus globulären SCR-Domänen, die sich in ihrer Anzahl unterscheiden. Durch evolutionäre Duplikationsvorgänge weisen einzelne SCRs eine hohe Sequenzidentität zueinander auf. Die Sequenzen von Faktor H und der CFHRs zeigen vor allem konservierte Regionen in den SCRs 6-7 sowie 19-20 von Faktor H. Die SCRs der Proteine sind basierend auf der maximalen Homologie vertikal angeordnet und die prozentuale Sequenzidentität zu Faktor H als Zahlenwert angegeben.

3.3.1 FAKTOR H

Das 150 kDa-Glykoprotein Faktor H wurde 1965 erstmalig isoliert[116]. Das sezernierte Protein besteht aus einer einzigen, langgestreckten Polypeptidkette von 20 aufeinanderfolgenden SCRs. Die einzelnen SCRs sind über flexible Linkerregionen, von drei bis acht Aminosäuren miteinander verbunden, so dass Faktor H eine Vielzahl von Konformationen annehmen kann[117]. Die Konzentration von Faktor H im Plasma variiert, je nach Alter und Gesundheitszustand, zwischen 400 – 800 µg/ml. Das Rauchen, einer der wichtigsten

individuellen Risikofaktoren der AMD, reduziert die physiologische Faktor H-Konzentration[118]. Die lokale Expression von Faktor H in RPE-Zellen wird hauptsächlich durch Interferon-γ über den STAT1-Signalweg induziert. Oxydativer Stress, verursacht durch Rauchen oder altersbedingte Lipofuszinablagerungen des RPEs, supprimiert die Interferon-γ-vermittelte Faktor H-Expression, so dass weniger lokales Faktor H zur Verfügung steht[110].

Faktor H ist der wichtigste Regulator des alternativen Weges in der Flüssigphase. Es besitzt drei Hauptfunktionen: 1. Es ist Kofaktor für die Faktor I vermittelte Inaktivierung von C3b, 2. Es konkurriert mit Faktor B um die Bindung an C3b und inhibiert auf diese Weise die Bildung der C3-Konvertase C3bBb und 3. Es beschleunigt den Zerfall eines bereits generierten C3bBb-Komplexes, so dass die C3-Konvertase ihre Aktivität verliert. Alle drei Mechanismen setzen eine Interaktion mit C3b voraus. Zu diesem Zweck besitzt Faktor H distinkte Bereiche, die mit verschiedenen Aktivierungsformen von C3b interagieren[119]. Die erste Bindestelle wurde im N-Terminus des Proteins lokalisiert und befindet sich in den SCRs 1-4. Sie ist die regulatorische Domäne des Proteins und verantwortlich für Kofaktoraktivität und die beschleunigte Dissoziation der C3-Konvertase. Über die SCRs 12-14 bindet Faktor H an C3c. Der C-Terminus bindet C3d. Neben der Regulation des alternativen Komplementweges im Plasma ist Faktor H zusätzlich in der Lage, dessen spontane Aktivierung auf körpereigenen Zellen zu regulieren. Dazu interagiert Faktor H mit negativ geladenen Strukturen der Zelloberfläche wie Sialinsäureresten oder Glykosaminoglykanen, und erhöht damit die Affinität für bereits gebundenes C3b[120,121]. Innerhalb des Faktor H-Moleküls sind die SCRs 16-20 für die Bindung an Sialinsäure verantwortlich. Mit **G**lykos**a**mino**g**lykanen (GAG) intergiert Faktor H über SCR 7, SCR 13 und SCR 20. Die Bindung von Faktor H an Zellmembranen oder die extrazelluläre Matrix ist essenziell, um bei der Komplementaktivierung zwischen Aktivator- und Nichtaktivatoroberflächen zu unterscheiden[106]. Die Interaktionen von Faktor H sind jedoch nicht nur auf Zelloberflächen und C3b beschränkt **(Abbildung 5)**. Gebunden an mCRP besitzt Faktor H zusätzlich anti-inflammatorische Funktion und ist involviert in die Erkennung, Markierung und Aufnahme von nekrotischen und apoptotischen Körperzellen durch Makrophagen. Über die Domänen SCR 7, SCR 8-11 und SCR 19-20 bilden sich stabile Faktor H - mCRP-Komplexe, welche die Freisetzung von TNF-α oder Interleukin-8 durch Makrophagen, und damit eine Entzündungsreaktion inhibieren[100]. Darüber hinaus bindet Faktor H über CR3 an neutrophile Granulozyten oder über L-Selektin an Leukozyten[122].

Die Eigenschaft von Faktor H die Komplementaktivierung zu regulieren, haben sich zahlreiche humanpathogene Mikroorganismen zu eigen gemacht. Diese Pathogene akquirieren Faktor H über spezielle Oberflächenproteine an ihre Membran, verhindern dadurch die Opsonisierung ihrer Oberfläche mit C3b und folglich ihre Lyse und Phagozytose[111]. Diese Strategie der Immunevasion wird speziesübergreifend von humanpathogenen Pilzen (z. B. *Candida albicans)*[123], grampositiven *(*z. B. *Streptococcus pneumoniae)*[124] sowie gramnegativen Bakterien (z. B. *Haemophilus influenzae)*[125], Parasiten *(*z. B. *Echinococcus granulosus)* und Viren *(*z. B. Westnilvirus*)* ausgeübt.

3.3.2 FAKTOR H-ÄHNLICHES PROTEIN 1

Das 42 kDa große FHL1-Protein entsteht aus einer Spleißvariante des Faktor H-Transkripts und erreicht eine Plasmakonzentration von bis zu 50 µg/ml. FHL1 besteht aus 431 Aminosäuren[113]. Die ersten 427 bilden sieben Proteindomänen, welche identisch mit den sieben N-terminalen SCRs von Faktor H sind (**Abbildung 5**). Die restlichen vier Aminosäuren: Serin, Phenylalanin, Threonin und Leucin (SFTL) befinden sich am C-Terminus und sind spezifisch für FHL1. Durch die Anwesenheit der SCRs 1-4 besitzt FHL1 die gleichen komplementregulatorischen Funktionen wie Faktor H, d. h. es bindet C3b und verfügt dadurch über Kofaktoraktivität für Faktor I sowie die Fähigkeit, die Dissoziation der C3-Konvertase zu beschleunigen[126]. Über SCR 7 interagiert FHL1 mit Zelloberflächen und mCRP. Weiterhin vermittelt ein RGD-Motiv in SCR 4 die Bindung von FHL1 an die Integrine adhärenter Zellen[127]. Obwohl Faktor H ebenfalls über die RGD-Domäne verfügt, ist diese Eigenschaft für FHL1 einzigartig. Damit kann FHL1 sowohl die komplementregulatorischen Funktionen von Faktor H ausführen und verstärken, aber auch unabhängig von Faktor H an Prozessen des Immunsystems teilnehmen.

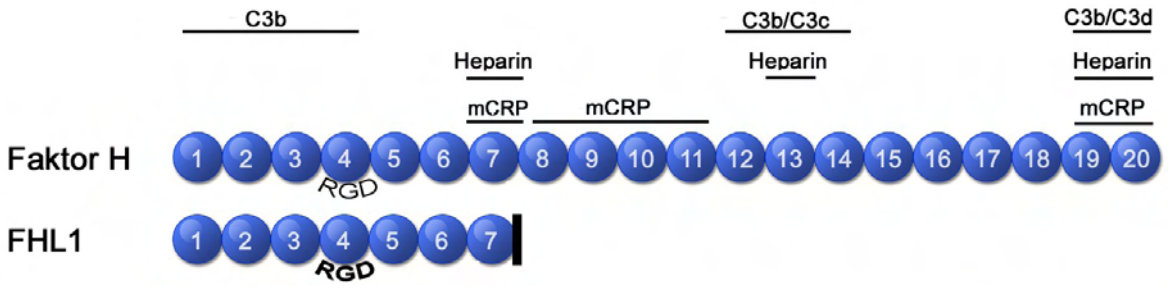

Abbildung 5: Lage der funktionellen Proteindomänen von Faktor H und FHL1. Faktor H ist aus 20 SCRs aufgebaut, von denen die sieben N-terminalen Domänen identisch mit denen des FHL1-Proteins sind. Die vier charakteristischen C-terminalen Aminosäuren SFTL von FHL1 sind durch einen vertikalen schwarzen Balken am Ende des Proteins dargestellt. Die Bindedomänen der Liganden sind mit horizontalen Balken gekennzeichnet. C3b bzw. dessen Inaktivierungsprodukte C3c und C3d werden über die SCRs 1-4, 12-13 sowie 19-20 gebunden, negativ geladene Oberflächenstrukturen, wie z. B. Heparin über die SCRs 7, 13 sowie 19-20, und mCRP interagiert mit den SCRs 7, 8-11 und 19-20. Das RGD-Motiv in SCR 4 ist nur in FHL1 (fettgedruckt), nicht aber in Faktor H, aktiv.

3.3.3 FAKTOR H-VERWANDTE PROTEINE

Es existieren insgesamt fünf CFHR-Proteine, deren Plasmakonzentration sich über einen Bereich von 5 – 100 µg/ml erstreckt. Die CFHRs sind aus vier, fünf bzw. neun SCR-Proteindomänen aufgebaut, und weisen insbesondere mit den SCRs 6-7 sowie SCRs 19-20 von Faktor H eine hohe Sequenzidentität auf. Demzufolge besitzen sie mit C3b, Heparin, CRP oder diversen pathogenen Oberflächenproteinen die gleichen Liganden wie Faktor H[112].

CFHR1 wurde erstmalig 1991 beschrieben und besteht aus fünf SCRs[128]. Die Konzentration im Plasma wird auf etwa 100 µg/ml geschätzt. Ein Großteil der CFHR1-Proteine ist jedoch mit Lipoproteinpartikeln assoziiert, so dass das tatsächlich verfügbare CFHR1 im Plasma vermutlich mehr als die geschätzten 100 µg/ml beträgt. Es existieren zwei

Glykosylierungsformen von CFHR1: das einfach glykosylierte 37 kDa Protein CFHR1-α und das zweifach glykosylierte CFHR1-β mit einer Mobilität von 43 kDa. Die drei C-Terminalen SCRs 3-5 weisen eine nahezu identische Sequenz zu den C-terminalen SCRs 18-20 von Faktor H auf (100 %, 100 %, 98 %)[129]. Dies, und die Interaktion von CFHR1 mit C3b sowie Heparin, lässt eine Kompetition der beiden Komplementproteine um die Binding dieser Liganden vermuten. Obwohl dem CFHR1-Protein sowohl die Kofaktoraktivität für die Serinprotease Faktor I sowie zerfallsbeschleunigende Aktivität für die C3-Konvertase fehlt, wurde es kürzlich als ein neuer Regulator der Komplementkaskade identifiziert.

CFHR3 besteht ebenfalls aus fünf aufeinanderfolgenden SCRs und zeigt im N-Terminus eine hohe Homologie zu Faktor H. Im Plasma existieren bis zu vier glykosylierte Isoformen, so dass die Plasmakonzentration von CFHR3 zwischen 50 – 80 µg/ml liegt. Dem CFHR3-Protein fehlt, wie dem CFHR1-Protein, sowohl die Funktion der Kofaktoraktivität als auch die Fähigkeit, den C3bBb-Komplex zu dissoziieren. Dennoch wurde in Gegenwart von CFHR3 eine verstärkende Kofaktoraktivität von Faktor H beschrieben[104]. Die spezifische Funktion von CFHR3 ist jedoch bisher unbekannt.

CFHR2, CFHR4 und CFHR5 sind ebenfalls Plasmaproteine und Bestandteile von Lipoproteinpartikeln. Die Funktion von CFHR2 ist bisher noch unbekannt. CFHR4 spielt eine Rolle bei der Opsonisierung nekrotischer Zellen[130] und CFHR5 besitzt Kofaktoraktivität, d. h. es beeinträchtigt die Aktivität der C3-Konvertase, indem es die C3a-Bildung inhibiert[131].

3.4 Faktor H-assoziierte Erkrankungen

Die stark vernetzte Struktur des Komplementsystems und dessen enormes Amplifikationspotenzial sind ideal, um zelluläre Debris oder Mikroorganismen in kürzester Zeit zu eliminieren. Doch kleinste Veränderungen in der Konzentration von Komplementkomponenten, deren partieller oder vollständiger Funktionsverlust durch Polymorphismen, Mutationen sowie Defizienzen, sind die Ursache zahlreicher komplementassoziierter Erkrankungen[75]. Der Funktionsverlust v. a. von Proteinen der Faktor H-Familie resultiert in einer gestörten Regulation des alternativen Komplementweges[132]. Dessen Natur der Aktivierung erfordert eine fortwährende Regulation, um die spontane Komplementaktivierung im Plasma und auf körpereigenen Zellen zu reduzieren. Die Fähigkeit von Faktor H bereits in der Flüssigphase des Serums C3b bzw. die C3-Konvertase C3(H_2O)Bb zu binden, und dadurch die Amplifikation der Komplementkaskade zu verringern, macht es zu einem der wichtigsten Regulatoren des Komplementsystems. Faktor H ist insbesondere in jenen Geweben essenziell, denen membranständige Regulatoren wie CR1, MCP und DAF fehlen. Ein Beispiel dafür ist die Basalmembran der Nierenglomeruli, die Bruch's Membran der Retina oder die exponierte extrazelluläre Matrix. Auf diesen Oberflächen führt ein Funktionsverlust von Faktor H zu massiven Schäden aufgrund einer verminderten Komplementregulation. In Verbindung mit Defekten im Faktor H Protein sind bisher drei Krankheitsbilder beschrieben worden: die AMD[71], das **a**typisch **h**ämolytisch **u**rämische **S**yndrom[133] (aHUS) und die **m**embrano**p**roliferative **G**lomerulo**n**ephritis Typ**II**[134] (MPGN-II).

Das aHUS und die MPGN-II sind Erkrankungen der Niere. Trotz vermeintlich gleicher Ursache, dem Funktionsverlust von Faktor H durch Mutationen, kommt es bei aHUS und MPGN-II zur Ausprägung unterschiedlicher Krankheitsbilder, die im Folgenden kurz beschrieben werden.

3.4.1 ATYPISCH HÄMOLYTISCH URÄMISCHES SYNDROM

Das aHUS ist eine chronische Erkrankung, welche durch die vermehrte Lyse von Erythrozyten (hämolytische Anämie), die Schädigung von Gefäßen (Mikroangiopathie) und einer reduzierten Thrombozytenzahl (Thrombozytopenie) gekennzeichnet ist. In schweren Fällen resultieren diese Symptome in einem akuten Nierenversagen. Bis heute sind über 100 verschiedene Mutationen im *Faktor H*-Gen beschrieben, die signifikant mit aHUS assoziiert sind. Ein Großteil, d. h. ca. 60 % aller bisher beschriebenen Faktor H-Mutationen, betrifft die SCRs 19-20[135,136]. Die Mutationen vermindern die Bindung von Faktor H an Zelloberflächen und/oder C3b, und infolgedessen dessen komplementregulatorischen Funktionen[137].

DEAP-HUS (**de**ficiency of cfhr plasma proteins and factor H-**a**utoantibody **p**ositive HUS) ist eine Sonderform des aHUS und charakterisiert durch das Fehlen der Plasmaproteine CFHR1 und CFHR3 sowie dem gleichzeitigem Auftreten von Faktor H-Autoantikörpern[138]. Die Autoantikörper blockieren die C-terminale Region von Faktor H, resultieren in einer reduzierten Faktor H-Zellbindung und einem damit einhergehendem Endothelschaden[139,140].

3.4.2 DIE MEMBRANOPROLIFERATIVE GLOMERULONEPHRITIS TYP-II

Die MPGN-II ist eine Erkrankung, die durch komplementhaltige Ablagerung (*dense deposits*) innerhalb der glomerulären Basalmembran charakterisiert ist und vorwiegend bei Kindern diagnostiziert wird. Die sukzessive Verdickung der glomerulären Basalmembran resultiert in einer massiven Schädigung der Nierenglomeruli. Die charakteristischen *dense deposits* enthalten C3-Aktivierungsprodukte und sind in ihrer Struktur ähnlich den Drusen der AMD[141,142]. Häufig entwickeln MPGN-II-Patienten auch retinale Drusen, die im Spätstadium ernsthafte Auswirkungen auf das zentrale Sehvermögen haben[143]. Die, der MPGN-II, zugrunde liegende unzureichende Kontrolle des alternativen Komplementweges wird entweder durch die vollkommene Abwesenheit von Faktor H oder aber durch Mutationen innerhalb des Faktor H-Proteins verursacht[144]. Weiterhin existiert bei einer Vielzahl der Patienten ein Autoantikörper, welcher gegen die C3-Konvertase C3bBb gerichtet ist, diese stabilisiert und in der Folge die beschleunigte Dissoziation durch Faktor H verhindert[145].

Aufgrund der zahlreichen anatomischen Gemeinsamkeiten der glomerulären Basalmembran mit der RPE-BM-Choriocapillaris-Struktur, dem Vorhandensein von Drusen sowie den neu entdeckten AMD-assoziierten Faktor H-Polymorphismen liegt die Vermutung nahe, dass AMD wie MPGN (oder aHUS) eine Krankheit ist, die durch unzureichende Kontrolle des alternativen Komplementweges verursacht wird.

4 Das Komplementsystem und AMD

Bereits 15 Jahre vor der Identifikation von Faktor H als AMD-Risikogen, häuften sich Hinweise, dass lokale Entzündungsreaktionen in Verbindung mit Komplementaktivierung eine entscheidende Rolle in der AMD-Pathogenese spielen[26,27]. Komplementkomponenten aus allen Ebenen der Kaskade sowie das Akute-Phase-Protein CRP, wurden nahe bzw. in Drusen lokalisiert[50,146]. Drusen galten daher als sekundäre Manifestationen eines primären RPE-Schadens, dessen Ursache unbekannt war[53].

Komplementaktivierung hat die Generation der Anaphylatoxine C3a und C5a zur Folge. Neben dem Nachweis dieser bioaktiven Fragmente in Drusen, wirken beide Anaphylatoxine aktiv auf das lokale Netzhautgewebe. C3a sowie C5a induzieren die VEGF-Expression von RPE-Zellen und rekrutieren durch ihre chemotaktische Wirkung Makrophagen zum RPE-Choroid-Komplex[147]. Intraokulare Komplementaktivierung hat demnach Einfluss auf die CNV, eine AMD-Spätform, sowie die damit verbundene, charakteristische Angiogenese der Kapillaren des Choroids. Die Produkte einer Komplementaktivierung sowie das Akute-Phase-Protein CRP können zudem in erhöhtem Maße auch im Plasma von AMD-Patienten detektiert werden[148,149]. Die Anwesenheit dieser Komponenten im Blutkreislauf induziert, dass der Entzündungsprozess der AMD nicht nur auf die Retina limitiert ist, sondern systemisch. In welchem Ausmaß eine lokale Reaktion im Auge Einfluss auf systemische Komplementaktivierung hat, ist jedoch bisher noch unklar.

Die Annahme, dass das Komplementsystem eine potenziell primäre Ursache für die Symptome der AMD ist, etablierte sich nachdem in großangelegten Fall-Kontroll-Studien, Polymorphismen zunächst in Faktor H, kurz darauf auch in den Komponenten Faktor B, C2 sowie C3 mit AMD assoziiert wurden. Die bis dato identifizierten Auswirkungen der einzelnen Polymorphismen, auf Protein- und ggf. funktioneller Ebene, werden im nächsten Abschnitt genauer erläutert.

4.1 Polymorphismen in Komplementproteinen und deren Einfluss auf die AMD

Das *Faktor H*-Gen war das erste konkrete Risikogen, welches mit AMD assoziiert wurde[11,70,71]. Die beschriebene Variation ist lokalisiert im Exon 9 des *Faktor H*-Gens und führt an Nukleotidposition 1277 zu einem Austausch eines Thymins durch ein Cytosin. Dies erzeugt sowohl im Faktor H-, aber auch im FHL1-Protein, einen Austausch der Aminosäure T**y**rosin (Y) durch **H**istidin (H) an Position 402 in SCR 7 (= Y402H-Polymorphismus, **Abbildung 6**). Über Proteindomäne 7 interagiert Faktor H mit GAGs humaner Zelloberflächen, mit mCRP sowie diversen Oberflächenproteinen humanpathogener Mikroorganismen[150]. Unter Verwendung aufgereinigter oder rekombinanter Faktor H-Varianten band die H402-Risikovariante mit geringerer Intensität an Oberflächen der RPE-Zellen, der BM oder der Choriokapillaris[109,151,152]. Ob und wie stark dabei die lokale Komplementregulation beeinträchtigt wird, war unklar. Ebenfalls interagiert die H402-Risikovariante vermindert mit mCRP[153,154]. Die funktionellen Konsequenzen dieser reduzierten mCRP-Interaktion waren bis dato unbekannt und sind, wie auch die Auswirkungen der reduzierten Zellbindung,

Gegenstand der vorliegenden Promotionsarbeit. Die Assoziation mit CRP ist vor allem dahingehend interessant, da CRP einer der wichtigsten Biomarker einer lokalen sowie systemischen Entzündungsreaktion ist und erhöhte Werte sowohl in Drusen als auch im Plasma von AMD-Patienten nachweisbar sind. Weiterhin zeigen homozygote Träger der HH402-Variation 2.5-fach erhöhte CRP-Werte im RPE-Choroid-Komplex als homozygote Träger der Nichtrisikovariation YY402[155]. Genetische Veränderungen von CRP sind selbst nicht mit AMD assoziiert[156]. Neben dem Y402H-Polymorphismus wurden in Faktor H bis zu 50 weitere SNPs identifiziert, die das AMD-Risiko sowohl signifikant erhöhen, aber auch reduzieren[72]. Größtenteils sind diese SNPs in Introns, d. h. den nicht kodierenden Abschnitten der DNA, lokalisiert, so dass ein funktioneller Einfluss auf das Faktor H-Protein, zwar nicht ausgeschlossen, aber schwer nachweisbar ist. Es existiert eine weitere Faktor H-Variation, die das Risiko der AMD modifiziert. Dabei wird im Exon 2 des *Faktor H*-Gens an Nukleotidposition 257 ein Guanin durch Adenin ausgetauscht[146]. Auf Proteinebene führt dies zu einer **V**alin (V) zu **I**soleucin (I)-Substitution an Position 62 von SCR1, wobei Isoleucin einen schützenden Effekt auf die AMD-Entstehung ausübt (= V62I-Polymorphismus, **Abbildung 6**). SCR 1 ist Teil der C3b-Binderegion von Faktor H und essenziell für die Ausübung der Kofaktor- sowie der zerfallsbeschleunigten Aktivität für die C3-Konvertase C3bBb. Bisher existiert nur eine Studie, welche eine erhöhte C3b-Interaktion für die protektive I62-Faktor H-Variante beschreibt[157]. Inwieweit der Y402H- und der V62I-Polymporphismus die komplementregulatorische Funktion von Faktor H und FHL1 beeinflussen, und ob sich beide Polymorphismen evtl. gegenseitig beeinflussen ist eine weitere Fragestellung mit der sich diese Promotionsarbeit auseinandersetzt.

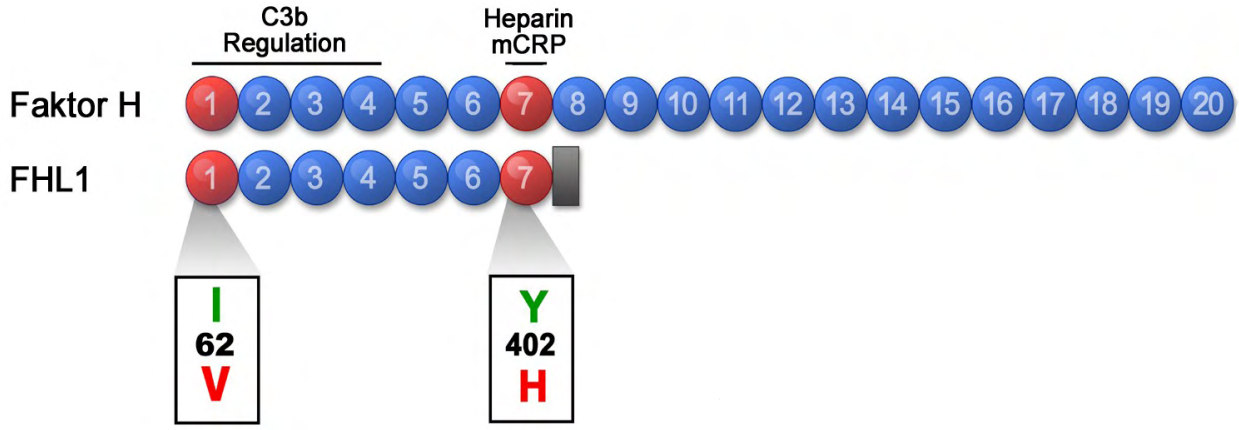

Abbildung 6: Die beiden zentralen AMD-assoziierten Aminosäuresubstitutionen in Faktor H und FHL1. Die AMD-relevanten Aminosäurevariationen in SCR 1 und SCR 7 betreffen sowohl Faktor H als auch dessen alternatives Spleißprodukt FHL1. Der Valin (V) – Isoleucin (I)-Austausch findet im SCR 1 an Aminosäureposition 62 statt. Die Gegenwart von I62 (grün) ist mit einem reduzierten AMD-Risiko verbunden. SCR 1 ist Teil der C3b-Binderegion sowie der regulatorischen Domäne SCR 1-4 und beteiligt an der Kofaktoraktivität sowie der *decay accelerating activity* von Faktor H/FHL1. Der Tyrosin (Y) – Histidin (H)-Austausch findet im SCR 7 an Aminosäureposition 402 statt. In SCR7 befinden sich die Binderegionen für die Liganden Heparin und mCRP. Das homozygote Vorkommen von H402 (rot) ist mit einem 7-fach erhöhten AMD-Risiko assoziiert.

Ein dritter, hochgradig mit AMD-assoziierter, Polymorphismus innerhalb des *Faktor H*-Genclusters betrifft die CFHR-Region (**Abbildung 7**). Die vollständige Deletion der *CFHR1*- und *CFHR3*-Gene, und die damit verbundene Abwesenheit der entsprechenden Proteine im Plasma ist mit einem reduzierten AMD-Risiko verbunden[12]. CFHR1 und CFHR3 sind Mitglieder der Faktor H-Familie und können über ihre SCRs mit C3b, Heparin sowie mit GAGs interagieren[158]. Durch zahlreiche evolutionäre Duplikationsprozesse innerhalb des *Faktor H*-Genclusters weisen sie eine hohe Sequenzidentität mit Faktor H auf, so dass eine komplementregulative Funktion vermutet wird. Wie jedoch das Fehlen der Plasmaproteine das Risiko der AMD-Entstehung reduzieren soll, wenn es gleichzeitig ein signifikanter Risikofaktor für die DEAP-HUS Sonderform ist, war fraglich. Die hohe Sequenzidentität von CFHR1, CFHR3 und Faktor H ermöglicht theoretisch eine Verdrängung von Faktor H durch die beiden CFHR-Proteine, und wurde in dieser Arbeit als Hypothese für den protektiven Einfluss der CFHR1-/CFHR3-Deletion bearbeitet.

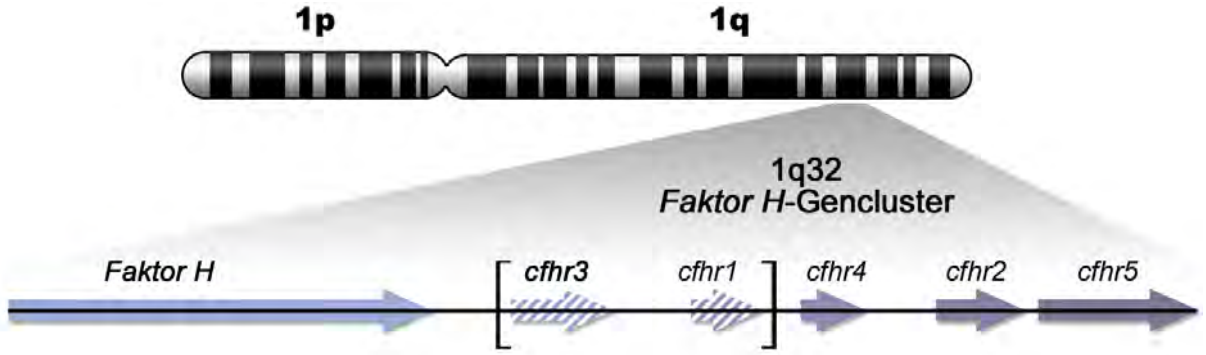

Abbildung 7: Deletion von CFHR1 und CFHR1 im *Faktor H*-Gencluster. Das Fehlen eines 84 kbp großen genomischen Fragmentes auf Chromosom 1 *downstream* des *Faktor H*-Gens, resultiert in dem Verlust der Gene für *CFHR1* und *CFHR3* (schraffierte Pfeile). Die daraus folgende vollständige Abwesenheit der CFHR1- und CFHR3-Proteine im Plasma hat einen schützenden Effekt auf AMD-Entstehung. Das Gen für *Faktor H* sowie die Gene von *CFHR2*, *CFHR4* und *CFHR5* sind durch die Deletion nicht betroffen.

Zusätzlich zu den Proteinen des *Faktor H*-Genclusters sind auch Polymorphismen der klassischen Komplementkomponente C2 (E318D) sowie des alternativen Proteins Faktor B (R32Q, R32W, L9H) mit AMD assoziiert[13] (**Tabelle 2**). Alle vier Varianten haben einen schützenden Effekt auf die AMD-Entwicklung. Da sich die einzelnen Polymorphismen jedoch in einem nahezu vollständigem *„linkage disequilibrium"* befinden, ist das Auffinden der kausalen Variante erschwert[159]. Genetische und funktionelle Studien zeigen jedoch, dass der protektive Effekt hauptsächlich durch die Faktor B-Variationen zustande kommt. Dabei interagiert die R32-Variante stärker mit C3b als die W32- oder Q32-Variante, und bildet eine stabilere C3-Konvertase des alternativen Komplementweges[160]. Die L9H-Variation von Faktor B beeinträchtigt vermutlich die Sekretion des Faktor B-Proteins. Der schützende Effekt resultiert dabei in einer reduzierten Ausbildung der C3-Konvertase und einer damit verbundenen herabgesetzten Komplementaktivierung. Ein seit langem bekannter Aminosäureaustausch in der zentralen Komplementkomponente C3 (R80G) ist ebenfalls mit einem erhöhten AMD-Risiko assoziiert[14]. Der Austausch eines positiv geladenen Arginis mit einem neutralen Glycin im C3b-Teil des C3-Moleküls verändert die Ladung des Proteins[161].

Dies, sowie die unmittelbare Nähe der Substitution zu der reaktiven Thioestergruppe, beeinträchtigt vermutlich die Interaktion von C3b mit Oberflächen oder Liganden.

Tabelle 2: Übersicht über etablierte AMD-Risikovarianten von verschiedenen Komplementkomponenten

Gen	Polymorphismus	Protein	Auswirkungen
Faktor H	rs1061170	Y402 → H402	reduzierte Zell- und CRP-Bindung
Faktor H	rs800292	V62 → I62	erhöhte C3b-Interaktion
cfhr1 / *cfhr3*	Deletion	abwesend	unbekannt
Faktor B	rs547154	R32 → Q32 / W32	instabilere C3-Konvertase: C3bBb
Faktor B	rs4151667	L9 → H9	modifizierte Sekretion von Faktor B
C2	rs9332739	E318 → D318	unbekannt
C3	rs2230199	R80 → G80	Ladungsänderung des C3-Proteins

rs:„*reference SNP*“

Polymorphismen in den nicht kodierenden DNA-Abschnitten des *Faktor I*-Gens sind sowohl mit einem erhöhten als auch reduzierten Risiko der AMD-Entstehung verbunden[162]. Studien, die ggf. funktionelle Konsequenzen dieser Variationen auf die Aktivität der Serinprotease beschreiben, existieren noch nicht. Weiterhin sollen Varianten in den Genen der Komplementkompontenen *C5*, *C7* sowie *CFHR5* das AMD-Risiko beeinflussen[163,164]. Diese Studien sind noch nicht wiederholend bestätigt worden, so dass diese genetischen Varianten –bis jetzt– noch keine etablierten Risikofaktoren der AMD sind. Nichtsdestotrotz bestätigen sie zusätzlich, dass das humane Komplementsystem zentral am Krankheitsprozess der AMD beteiligt ist. Ob die lokalen Entzündungsreaktionen im RPE-Choroid-Komplex durch ein unzureichend kontrolliertes Komplementsystem verursacht werden, bei der die genannten Polymorphismen unabhängig oder gemeinsam agieren, muss weiter geprüft werden.

4.2 Neue komplementassoziierte Behandlungsmöglichkeiten

Bisher existieren noch keine zugelassenen Präparate für die Behandlung der AMD, die direkt das Komplementsystem beeinflussen. Wie bereits erwähnt, wird die GA vorbeugend therapiert, und die Behandlung der CNV basiert auf Laserbestrahlung sowie der Gabe von monoklonalen Antikörpern gegen VEGF. Das Fortscheiten der Krankheit kann daher nur verlangsamt, jedoch nicht aufgehalten werden. Die sukzessive Aufdeckung des Komplementsystems als vermeintliche Ursache der degenerativen Veränderungen im subretinalen Raum macht es nun zu einem potenziell therapeutischen Angriffspunkt.

Das bisher vielversprechendste, in der Entwicklung befindliche, Therapeutikum ist das 1.5 kDa große, zyklische Peptid Compstatin[165]. Compstatin bindet natives C3, inhibiert dessen Spaltung in C3a und C3b und blockiert die Amplifikation aller drei Komplementwege. Der Inhibitor zeichnet sich durch seine geringe Toxizität und die hohe Spezifität für humanes C3 aus, Eigenschaften die in zahlreichen *in-vitro-* sowie *in-vivo*-Studien bestätigt wurden[166]. Momentan sind erste klinische Studien der Phase 1 erfolgreich abgeschlossen, in denen die Verträglichkeit und Sicherheit weiter überprüft wurden[167]. Compstatin wird dabei intravitreal verabreicht, lagert sich in Form eines Gelpolsters ab und wird langsam über viele Monate in geringen Dosen abgegeben. Ein Rückgang der Drusenformation sowie ein Fall mit kompletter Auflösung eines Makulaödems wurden beschrieben.

Andere Möglichkeiten die Komplementaktivierung im Auge zu kontrollieren, umfassen den Einsatz von humanisierten Antikörpern gegen C5 (Eculizumab) oder Faktor D (TNX-234)[168], Hybridproteine, die die Eigenschaften von zwei Komplementregulatoren kombinieren (CR2-Faktor H)[169] sowie die Gabe biologisch aktiver rekombinanter Regulatoren (C1-Inhibitor, Faktor H)[170]. Zentrale Aufgabe zukünftiger, komplementbasierter Therapeutika wird dabei die Regulierung der C3-Aktivierung sein, um die nachfolgende Generation von Anaphylatoxinen, Opsoninen sowie TCC-Formation zu verhindern. Gleichzeitig muss aber eine systemische Inhibierung vermieden werden, um die ursprünglichen Aufgaben des Komplements (Opsonierung degenerierter Zellen, Abwehr von Mikroorganismen), weiterhin im immunprivilegiertem Auge zu ermöglichen.

5 Fragestellung

Zahlreiche genetisch basierte Studien lieferten die Grundlage zu der Annahme, dass die altersabhängige Makuladegeneration eine Erkrankung ist, welche durch unzureichende Kontrolle des alternativen Komplementweges verursacht wird. Die genauen Auswirkungen der kürzlich identifizierten Polymorphismen in den Proteinen Faktor H, FHL1, Faktor B, C2 und C3 bzw. deren Einfluss auf die Krankheitsentstehung sind nahezu unbekannt. Ebenfalls ist die Tatsache, warum das vollständige Fehlen der Plasmaproteine CFHR1 und CFHR3, schützend auf die AMD-Entstehung wirkt, ungeklärt.

Ziel dieser Arbeit war es daher, die funktionellen Auswirkungen der Y402H- und der V62I-Variation von Faktor H bzw. FHL1 zu charakterisieren. Dafür sollten die entsprechenden Faktor H-Varianten aus genotypisierten AMD-Patienten isoliert sowie rekombinante FHL1-Varianten hergestellt werden. Der darauffolgende systematische Vergleich aller Proteinvarianten in Bezug auf Ligandenbindung und deren komplementregulative Funktion in Flüssigphase sowie auf Oberflächen sollte zeigen, inwiefern die Funktionen von Faktor H und FHL1 durch die Aminosäurevariationen beeinträchtigt sind. Vor allem sollte überprüft werden, ob die Y402H-Variation Einfluss auf anti-inflammatorische Funktion von Faktor H hat, und in der Folge die Markierung sowie Aufnahme von nekrotischen, retinalen Debris beeinträchtigt ist. Darüber hinaus sollten die bis dato unbekannten Proteinfunktionen von CFHR1 und CFHR3 analysiert werden. Die hohe Sequenzidentität beider Proteine zu Faktor H, legte die These nahe, dass sowohl CFHR1 als auch CFHR3 mit Faktor H um die Bindung an gemeinsame Liganden konkurriert. Daher sollten Verdrängungsassays zeigen, ob die Anwesenheit von CFHR1 und CFHR3 die komplementregulative Funktion von Faktor H beeinflusst. Die Ergebnisse sollten dann eine konkrete Aussage ermöglichen, inwiefern das Fehlen von zwei Mitgliedern der Faktor H-Familie einen positiven Effekt auf die AMD-Entwicklung ausüben kann. Damit soll diese Arbeit insgesamt ein besseres Verständnis dafür liefern, ob und inwieweit AMD-assoziierte Polymorphismen und Defizienzen innerhalb der Faktor H-Familie den Krankheitsverlauf der AMD beeinflussen.

Zudem sollte, im Rahmen dieser Arbeit, ein neues komplementbasiertes Therapeutikum entwickelt werden. Der oberflächenbindende C-Terminus von Faktor H sollte mit dem etablierten Komplementinhibitor Compstatin vereint werden. Ziel war es, einen Inhibitor zu kreieren, welcher die komplementinhibitorischen Eigenschaften von Compstatin gezielt an Oberflächen rekrutiert und dabei –wie Faktor H– Aktivator von Nichtaktivatoroberflächen unterscheidet. Die kombinierten Eigenschaften des Chimärs sollten anschließend in Gegenwart von komplementaktivem Plasma auf Oberflächen studiert und mit nativem Compstatin verglichen werden. Die neuen Erkenntnisse sowie Möglichkeiten des Chimärs sollen als Grundlage für die Entwicklung eines neuen therapeutischen Wirkstoffes dienen, der bei Faktor H-assoziierten Krankheiten, wie der AMD, zum Einsatz kommen kann.

Übersicht der Manuskripte

Die vorliegende Dissertation basiert auf sechs Manuskripten. Die Auflistung der Manuskripte erfolgt nach inhaltlichen Gesichtspunkten und nicht nach dem Zeitpunkt der Veröffentlichung.

The role of complement in age-related macular degeneration.

Peter Zipfel, **Nadine Lauer**, Christine Skerka

Advances in Experimental Medicine and Biology Inflammation and Retinal Disease: Complement Biology and Pathology. 2010;703:9-24. Review

Inhalt der Publikation

Der Review beschreibt die Rolle des Komplementsystems bei der Entstehung der altersabhängigen Makuladegeneration. Er greift dabei das derzeit allgemeinakzeptierte Konzept auf, bei welchem eine unzureichend regulierte Komplementaktivierung zu lokalen, intraokularen Entzündungsreaktionen führt und infolgedessen pathophysiologische Veränderungen im subretinalen Raum entstehen. Dabei werden sowohl die bisher bekannten, AMD-assoziierten Polymorphismen der Komplementproteine Faktor H, FHL1, C2, Faktor B, C3, als auch das Fehlen der Komplementregulatoren CFHR1 und CFHR3, erläutert und in Beziehung zueinander gesetzt. Der Review betrachtet damit in der Gesamtheit, wie eine potenzielle Überaktivierung des Komplementsystems zu einer lokalen, chronischen Entzündung führt und dies direkt zur Entwicklung und/oder dem Fortschreiten der AMD beiträgt.

Umfang und Inhalt des Eigenanteils sowie Beiträge der Koautoren

Nadine Lauer war an der Planung des Reviews beteiligt und hat die Abschnitte zwei sowie drei geschrieben. Des Weiteren hat Nadine Lauer die Abbildungen konzipiert und erstellt.

Peter F. Zipfel und **Christine Skerka** haben den Review geplant und die Abschnitte eins sowie vier verfasst.

The AMD-associated variation at position 62 does not, but the exchange at position 402 does affect surface regulation of the complement inhibitor FHL1.

Nadine Lauer, Julia Böhme, Hans-Martin Dahse, Christine Skerka, Peter F. Zipfel

Manuskript in Überarbeitung für das „*Journal of Biological Chemistry*"
Eingangsbestätigung: 28.03.2011

Inhalt des Manuskriptes

Der Komplementfaktor H besitzt zahlreiche hochsignifikante Risikoallele, die eine entscheidende Rolle bei der Disposition zur AMD spielen. Das Manuskript vergleicht die funktionellen Auswirkungen der Polymorphismen V62I und Y402H, welche sowohl in Faktor H als auch FHL1 vorkommen. Mittels *in-vitro*-Mutagenese wurden dabei vier FHL1 Varianten generiert: FHL1_$V_{62}Y_{402}$, FHL1_$I_{62}Y_{402}$, FHL1_$V_{62}H_{402}$, und FHL1_$I_{62}H_{402}$. Vergleichende Analysen zeigen, dass die Variation an Aminosäureposition 62 keine Auswirkung auf die komplementregulatorische Aktivität von FHL1 hat. Dementgegen verändert die Substitution an Position 402 das Bindeverhalten von FHL1 an zellulären Oberflächen und verhindert infolgedessen eine adäquate Inhibierung des alternativen Komplementweges.

Umfang und Inhalt des Eigenanteils sowie Beiträge der Koautoren

Nadine Lauer hat die folgenden Experimente geplant, durchgeführt, ausgewertet und interpretiert: Expression und Aufreinigung der rekombinanten FHL1-Proteine, Kultivierung der Sf9-, CHO- und ARPE-19-Zelllinien, Generierung von FHL1/Faktor H-defizienten humanen Serum, Protein-Protein-Interaktion mittels ELISA, vergleichende FHL1-Bindung an ARPE-19-Zellen mittels Durchflusszytometrie, Kofaktorassays in Flüssigphase, Hämolyseversuche auf Schaferythrozyten sowie die Bestimmung der Vitalität von CHO-Zellen. Ebenfalls war Nadine Lauer am Schreiben der Publikation beteiligt.

Julia Böhme hat die *in-vitro*-Mutagenese durchgeführt.

Hans-Martin Dahse war an der Auswertung des Vitabilitätsassays auf CHO-Zellen beteiligt.

Christine Skerka und **Peter F. Zipfel** waren maßgeblich an der Konzeption der Studie sowie am Schreiben der Publikation beteiligt. Des Weiteren waren sie verantwortlich für die Betreuung und Finanzierung des Forschungsprojektes.

Complement regulation at necrotic cell lesions is impaired by the AMD associated Factor H - H402 risk variant.

Nadine Lauer, Michael Mihlan, Andrea Hartmann, Ursula Schlötzer-Schrehardt, Claudia N. Keilhauer, Hendrik PN. Scholl, Peter Charbel-Issa, Frank Holz, Bernhard H. F. Weber, Christine Skerka, Peter F. Zipfel

Manuskript in Überarbeitung für das *„Journal of Immunology"*
Eingangsbestätigung: 02.02.2011

Inhalt des Manuskriptes

Dieses Manuskript charakterisiert die funktionellen Auswirkungen des hochsignifikanten Faktor H-Risikoalleles Y402H auf die Faktor H – mCRP-Komplexbildung. Es wird gezeigt, dass die protektive Faktor H-Y402-Variante starke Komplexe mit mCRP eingeht, und dadurch vermehrt an nekrotische retinale Pigmentepithelzellen rekrutiert wird. Die komplementregulierenden sowie anti-entzündlichen Funktionen von Faktor H werden dabei signifikant durch mCRP verstärkt. Konfokale Laserscanmikroskopie zeigt weiterhin, dass mCRP vorzugsweise an stark geschädigten Membranbestandteilen generiert wird und Faktor H spezifisch an jene Stellen rekrutiert. Für die Faktor H-H402-Risikovariante ist die Komplexbildung mit mCRP, und die damit einhergehende erhöhte Faktor H-Aktivität an nekrotischen Membranläsionen, signifikant reduziert. Damit stellt das Manuskript erstmals eine Hypothese für die Ablagerung von degeneriertem, nekrotischem RPE-Zellmaterial im subretinalen Raum auf, erläutert die möglicherweise daraus resultierenden intraokularen Entzündungsreaktionen und pathophysiologischen Veränderungen.

Umfang und Inhalt des Eigenanteils sowie Beiträge der Koautoren

Nadine Lauer hat folgende Experimente geplant, durchgeführt, ausgewertet und interpretiert: Expression und Aufreinigung der rekombinanten FHL1-Proteinvarianten, Kultivierung der Sf9-Insektenzelllinie sowie einer primären und einer immortalisierten RPE-Zelllinie, Generierung von mCRP, Protein-Protein-Interaktion mittels ELISA, vergleichende Bindestudien von Faktor H – mCRP-Komplexen an nekrotische Zellen mittels Durchflusszytometrie sowie konfokaler Laserscanmikroskopie, Kofaktorassays in Flüssig- und Festphase sowie die Peptidspot-Analyse. Ebenfalls war Nadine Lauer am Schreiben der Publikation beteiligt.

Michael Mihlan war an der Konzeption der Studie beteiligt und hat den Zytokinassay durchgeführt, ausgewertet und interpretiert.

Andrea Hartmann hat humanen Faktor H aus AMD-Patienten isoliert und aufgereinigt.

Ursula Schlötzer-Schrehardt hat Gewebeschnitte vom subretinalen Raum aus AMD-Patienten sowie gesunden Kontrollpatienten immunhistochemisch auf das Vorhandensein von mCRP und pCRP überprüft.

Claudia N. Keilhauer hat AMD-Patienten aus Würzburg betreut, behandelt und Blutproben genommen.

Hendrik PN. Scholl, Peter Charbel-Issa und **Frank Holz** haben AMD-Patienten aus Bonn betreut, behandelt und Blutproben genommen.

Bernhard H. F. Weber hat genomische DNA der AMD-Patienten aus Würzburg sowie Bonn extrahiert, sequenziert und genotypisiert.

Christine Skerka und **Peter F. Zipfel** waren maßgeblich an der Konzeption der Studie, an der Interpretation der Ergebnisse sowie am Schreiben der Publikation beteiligt. Des Weiteren waren sie verantwortlich für die Betreuung und Finanzierung des Forschungsprojektes.

Factor H-related protein 1 (CFHR-1) inhibits complement C5-convertase activity and terminal complex formation.

Stefan Heinen, Andrea Hartmann, **Nadine Lauer**, Ulrike Wiehl, Hans-Martin Dahse, Sylvia Schirmer, Katharina Gropp, Tina Enghardt, Reinhard Wallich, Steffi Hälbich, Michael Mihlan, Ursula Schlötzer-Schrehardt, Peter F. Zipfel, Christine Skerka

Blood. 2009 Sep 17;114(12):2439-47.

Inhalt der Publikation

Die Publikation identifiziert das Komplementprotein CFHR1 erstmals als einen Inhibitor der C5-Konvertase des alternativen sowie des terminalen Komplementweges. CFHR1 bindet C5 sowie C5b6 über den N-Terminus und verhindert dadurch die C5b-Ablagerung auf humanen Zelloberflächen, blockiert die Formation der C5-Konvertase sowie den darauffolgenden Aufbau des TCC. Die Funktion von CFHR1 ist demnach verschieden von Faktor H, welches ausschließlich auf C3-Ebene fungiert. Aufgrund der hohen C-terminalen Sequenzidentität binden sowohl CFHR1 als auch Faktor H an zelluläre Oberflächen, wobei CFHR1 in der Lage ist, Faktor H von diesen kompetitiv zu verdrängen.

Umfang und Inhalt des Eigenanteils sowie Beiträge der Koautoren

Nadine Lauer hat folgende Experimente geplant, durchgeführt und interpretiert: Kofaktoraktivität von CFHR1, Verdrängung von Faktor H durch CFHR1 auf Heparin sowie die Generierung von Faktor H-defizienten humanen Serum.

Stefan Heinen erzeugte CFHR1/Faktor H-depletiertes humanes Serum und hat folgende Experimente geplant, durchgeführt, ausgewertet sowie interpretiert: Hämolyseversuche und Analyse des TCC-Zerfalls in Gegenwart von CFHR1.

Andrea Hartmann hat rekombinantes CFHR1-Protein exprimiert und aufgereinigt sowie CFHR1 aus humanem Serum isoliert. Des Weiteren hat sie Hämolyseversuche, Durchflusszytometrie, Mikroskopie, ELISA-Experimente und Immunpräzipitation geplant, durchgeführt und interpretiert.

Ulrike Wiehl hat die Hämolyseversuche auf Schaferythrozyten sowie den Zerfall der C3- und C5-Konvertase mit aufgereinigten Proteinen geplant, durchgeführt und interpretiert.

Hans-Martin Dahse hat den Vitabilitätsassay mit humanen Zellen durchgeführt.

Sylvia Schirmer klonierte sowie exprimierte die CFHR1-Deletionskonstrukte und hat ELISA-Experimente mit den rekombinanten Proteinen geplant, durchgeführt und interpretiert**.**

Katharina Gropp hat die Experimente zur Konvertasestabilität und Protein-Protein-Interaktionsstudien geplant, durchgeführt und interpretiert.

Tina Enghardt hat die Bindung von CFHR1 an humane Zellen mittels Durchflusszytometrie geplant und durchgeführt.

Reinhard Wallich generierte den monoklonalen CFHR1-Antikörper JhD10.

Steffi Hälbich hat die Experimente zur Oberflächenplasmonenresonanzspektroskopie geplant, durchgeführt und interpretiert.

Michael Mihlan stellte komplementaktives CFHR1/CFHR3-defizientes humanes Plasma zur Verfügung und war bei der Erstellung des Manuskriptes beteiligt.

Ursula Schlötzer-Schrehardt hat Gewebe aus Nieren und Augen immunhistochemisch auf CFHR1-Expression überprüft.

Peter F. Zipfel und Christine Skerka waren maßgeblich an der Konzeption der Studie sowie an der Interpretation der Ergebnisse beteiligt. Des Weiteren waren sie verantwortlich für die Betreuung und Finanzierung des Forschungsprojektes und haben die Publikation geschrieben.

An imbalance of human complement regulatory proteins CFHR1, CFHR3 and factor H influences risk for age-related macular degeneration (AMD).

Lars G. Fritsche*, **Nadine Lauer***, Andrea Hartmann, Selina Stippa, Claudia N. Keilhauer, Martin Oppermann, Manoj K. Pandey, Jörg Köhl, Peter F. Zipfel, Bernhard H. F. Weber, Christine Skerka

Human Molecular Genetics. 2010 Dec 1;19(23):4694-704.
* Gleichberechtigte Erstautorschaft

Inhalt der Publikation

Die Publikation beschäftigt sich mit der Frage, wie das Fehlen der Komplementproteine CFHR1 und CFHR3 einen schützenden Effekt auf die AMD-Entstehung induzieren kann. Dabei wurde in einer Fall-Kontroll-Studie von 530 AMD-Patienten gezeigt, dass die CFHR1-/-CFHR3-Defizienz (Δ*CFHR1* / *CFHR3)* unabhängig von zahlreichen signifikanten Faktor H-Risikoallelen (u. a. Y402H) auftritt, und demnach die Funktionen der beiden Komplementproteine eine Rolle in der AMD-Pathogenese spielen. Das CFHR3-Protein wurde erstmals als ein neuer Komplementregulator identifiziert, welcher Faktor I-Kofaktoraktivität besitzt, C5a-Generierung blockiert und damit die C5a-vermittelte Chemotaxis neutrophiler Granulozyten reduziert. Sowohl CFHR1 als auch CFHR3 verdrängen Faktor H von C3b und modifizieren infolgedessen die Regulation auf C3-Ebene. Die Abwesenheit von CFHR1 und CFHR3 erhöht demnach die lokale Regulation durch Faktor H, was einen positiven Effekt in der intraokularen Komplementregulation zur Folge hat.

Umfang und Inhalt des Eigenanteils sowie Beiträge der Koautoren

Nadine Lauer hat folgende Experimente geplant, durchgeführt und interpretiert: Generierung von Faktor H-defizienten humanen Serum, Verdrängung von Faktor H durch CFHR1 bzw. CFHR3 von C3b, C3-Konvertasestabilität in Gegenwart von CFHR1 und CFHR3, Hämolyseversuche mit Hühnererythrozyten, Bestimmung der Serumkonzentration von CFHR3.

Lars G. Fritsche hat genomische DNA der AMD-Patienten sowie der Kontrollgruppe extrahiert, sequenziert und genotypisiert. Des Weiteren hat er den MLPA-Assay (MLPA: *„**m**ultiplex **l**igation-**d**ependent **p**robe **a**mplification“*) durchgeführt, ausgewertet und interpretiert.

Andrea Hartmann hat die rekombinanten Proteine CFHR1 und CFHR3 exprimiert und aufgereinigt. Des Weiteren hat sie Hämolyseversuche, ELISA-Experimente und Kofaktorassays geplant, durchgeführt und interpretiert.

Selina Stippa hat die C3-Konvertaseaktivität in CFHR1 / CFHR3-defizientem Serum nach Zugabe von CFHR1, CFHR3 und Faktor H bestimmt.

Claudia N. Keilhauer hat AMD-Patienten aus Würzburg betreut, behandelt und Blutproben genommen.

Martin Oppermann generierte den monoklonalen CFHR3-Antikörper 4H9.

Manoj K. Pandey und **Jörg Köhl** haben Versuche zur Chemotaxis neutrophiler Granulozyten geplant, durchgeführt und interpretiert.

Peter F. Zipfel war am Schreiben der Publikation beteiligt.

Bernhard H. F. Weber und **Christine Skerka** waren maßgeblich an der Konzeption der Studie sowie an der Interpretation der Ergebnisse beteiligt. Des Weiteren waren sie verantwortlich für die Betreuung und Finanzierung des Forschungsprojektes und haben die Publikation geschrieben.

The C-terminal surface attachment region of Factor H targets the complement inhibitor compstatin to self surfaces and to sites of immunstress.

Nadine Lauer, Daniel Ricklin, Stefan Heinen, Apostolia Tzekou, John D. Lambris, Peter F. Zipfel

Manuskript in Vorbereitung für das *„Journal of Immunology"*

Inhalt des Manuskriptes

In diesem Manuskript wird ein neuartig entwickelter Komplementregulator vorgestellt, welcher die hohe C3-/C3b-Affinität des systemisch wirkenden Komplementinhibitors Compstatin mit den Zellbindeeigenschaften von Faktor H kombiniert. Dabei wurde die DNA-Sequenz von Compstatin mit der DNA-Sequenz des C-terminalen Faktor H-Fragmentes SCR15-20 gekoppelt und das Hybridprotein COMP_CFH15-20 generiert. COMP_CFH15-20 inhibiert die Komplementaktivierung sowohl auf Aktivator- als auch auf Nichtaktivatoroberflächen, wobei seine inhibitorische Funktion insbesondere lokal auf Zelloberflächen gerichtet ist. Gebunden an lebende sowie nekrotische CHO- und HUVEC-Zellen ist die Aktivität von COMP_CFH15-20 signifikant stärker als die des Inhibitors Compstatin allein. Demnach erlaubt der neuartig generierte Regulator eine kontrollierte Komplementregulation speziell an Stellen von erhöhtem Immunstress bzw. Zell- und Gewebeschäden, und verhindert demzufolge einige Nebenwirkungen von systemisch wirkenden Komplementregulatoren.

Umfang und Inhalt des Eigenanteils sowie Beiträge der Koautoren

Nadine Lauer hat folgende Experimente geplant, durchgeführt, ausgewertet und interpretiert: Expression und Aufreinigung des COMP_CFH15-20-Hybridproteins, Kultivierung der Sf9-, CHO- und HUVEC-Zelllinien, Generierung von Faktor H-defizienten humanem Serum, Protein-Protein-Interaktion mittels ELISA, Bindestudien des Hybridproteins an lebende sowie nekrotische Zellen mittels Durchflusszytometrie und konfokaler Laserscanmikroskopie, Hämolyseassays auf Kaninchen- und Schaferythrozyten und darauffolgende C5a-Bestimmung bzw. C5b-Ablagerung sowie die Bestimmung der Vitalität von CHO Zellen. Ebenfalls war Nadine Lauer am Schreiben der Publikation beteiligt.

Daniel Ricklin hat die Experimente zur Oberflächenplasmonenresonanzspektroskopie geplant, durchgeführt und interpretiert.

Stefan Heinen klonierte sowie exprimierte das COMP_CFH15-20 Hybridprotein und hat Hämolyseversuche geplant, durchgeführt und interpretiert.

Apostolia Tzekou hat Experimente zur Oberflächenplasmonenresonanzspektroskopie durchgeführt.

John D. Lambris hat aktives sowie inaktives Compstatin als Kontrollpeptide zur Verfügung gestellt.

Peter F. Zipfel war maßgeblich an der Konzeption der Studie sowie an der Interpretation der Ergebnisse beteiligt. Des Weiteren war er verantwortlich für die Betreuung und Finanzierung des Forschungsprojektes und hat die Publikation geschrieben.

Manuskripte

1 The role of complement in age-related macular degeneration.

Advances in Experimental Medicine and Biology Inflammation and Retinal Disease: Complement Biology and Pathology. 2010;703:9-24. Review

Chapter 2
The Role of Complement in AMD

Peter F. Zipfel, Nadine Lauer, and Christine Skerka

Abstract Age related macular degeneration (AMD) is a common form of blindness in the western world and genetic variations of several complement genes, including the complement regulator Factor H, the central complement component C3, Factor B, C2, and also Factor I confer a risk for the disease. However deletion of a chromosomal segment in the Factor H gene cluster on human chromosome 1, which results in the deficiency of the terminal pathway regulator CFHR1, and of the putative complement regulator CFHR3 has a protective effect for development of AMD. The Factor H gene encodes two proteins Factor H and FHL1 which are derived from alternatively processed transcripts. In particular a sequence variation at position 402 of both Factor H and FHL1 is associated with a risk for AMD. A tyrosine residue at position 402 represents the protective and a histidine residue the risk variant. AMD is considered a chronic inflammatory disease, which can be caused by defective and inappropriate regulation of the continuously activated alternative complement pathway. This activation generates complement effector products and inflammatory mediators that stimulate further inflammatory reactions. Defective regulation can lead to formation of immune deposits, drusen and ultimately translate into damage of retinal pigment epithelial cells, rupture of the interface between these epithelial cells and the Bruch's membrane and vision loss. Here we describe the role of complement in the retina and summarize the current concept how defective or inappropriate local complement control contributes to inflammation and the pathophysiology of AMD.

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1 Age-Related Macular Degeneration

1.1 The Disease

Age related macular degeneration (AMD) is the leading cause of blindness in the elderly population, especially in the Western World. Today, more than 20 Mio individuals over 50 years of age show early signs of this sight-threatening disease (Congdon et al. 2004; Pascolini et al. 2004). The actual demographic development predicts an increase in the number of elderly people and thus a higher number of people at risk by about 50 % (Friedman et al. 2004).

In the early stage of the disease immune deposits, which are termed drusen, develop between the Bruch's membrane (BM) and adjacent retinal pigment epithelial cell (RPE) layer (Bird et al. 1995). Ongoing progression of the inflammatory reactions and the disease enhances size and the number of drusen and result in two severe forms: geographic atrophy (GA) leads to death of macula surrounding photoreceptors that overly degenerated RPE cells. Choroidal neovascularisation (CNV) is characterized by the growth of blood vessels into the retinal layer which leak fluid or bleed. Both forms result in a complete loss of central vision (de Jong 2006).

AMD is a multifactorial disease which is caused by several genetic factors, by environmental factors and disease susceptibility is also influenced by age and ethnic background (Vingerling et al. 1995; Klein et al. 2004).

1.2 AMD: A Chronic Inflammatory Disease

The pathogenesis of AMD is subject of intensive research and recent reports showed that early as well as advanced stages of AMD are caused by defective complement activation and local inflammation (Johnson et al. 2001). At present additional concepts for AMD pathophysiology are discussed, which however are not exclusive to the initial complement and inflammation hypothesis. These explanations are based on inefficient or defective transport/diffusion of nutrients from the choroids via the Bruch's membrane to the photoreceptors, or inappropriate reverse transport of waste products from photoreceptors to the choroids (Zarbin 1998). Consequently debris may accumulate along the interface of the RPE cells and the Bruch's membrane, providing an activator surface that allows complement activation. Also in this concept defective or inefficient complement regulation may cause further amplification of the complement cascade and inflammation. Both concepts may explain how inappropriate control and inhibition of spontaneous or triggered complement activation causes progression and amplification of the complement cascade and consequently the generation of inflammatory activation compounds in form of C3a and in particular of the potent inflammatory marker C5a (Scholl et al. 2008). If low level complement activation persists over weeks, months and even years, already a minor change in the composition of one regulatory component may

cause deregulation and damage. This continuous imbalance can then progress into pathophysiology, in manifestation of drusen and degeneration of the RPE cell layer and of the overlying photoreceptors.

AMD is associated with complement activation or deregulation of the spontaneously initiated alternative complement pathway leading to local release of inflammatory activation products and to local inflammation, which relate to the pathogenesis of the disease (Donoso et al. 2006). This concept is further confirmed by immunohistochemical analyses and proteome assays, which identified proteins and components associated with complement activation and inflammation in drusen. Multiple complement components, regulators, complement activation products and inflammatory proteins are identified in drusen, including C3, C3d, the terminal components C5, C6, C7, C8 and C9, terminal complement regulators vitronectin and clusterin, apolipoproteins apoA1, apoA4 and apoE as well as thrombospondin, serum amyloid A (SAP-A) and SAP-P (Anderson et al. 2002; Crabb et al. 2002; Li et al. 2006a). This composition demonstrates the association of complement and complement regulators in the process of drusen formation and likely also in the associated inflammatory reactions. The activated complement systems triggers further events and which ultimate cause formation of drusen, cell damage of the RPE cells and visual loss (Hageman et al. 2001).

Most or all of these proteins that are identified by immunohistochemistry and proteome analyses are expressed locally, e.g. by RPE cells and are present in the choroids. Thus explaining or confirming a local role of complement and inflammation in the pathogenesis of AMD. Immunohistochemical analyses also identified the inflammatory marker C-reactive protein (CRP) in drusen and in deposits formed along the Bruch's membrane (Anderson et al. 2002; Laine et al. 2007; Skerka et al. 2007). Based on immunoblot assays the local level of C-reactive protein (CRP) in the choroidal stroma was about 2.5-fold higher in individuals with the Factor H risk variant (Johnson et al. 2006). These increased CRP levels in the choroids of individuals at risk are indicative for a chronic inflammation. Upon inflammation, infection or tissue damage CRP plasma levels increase substantially, from minimal, undetectable levels to plasma concentrations of more than 500 mg/l (Pepys and Hirschfield 2003). CRP, is a member of the pentraxin protein family and this 125 kDa is composed of five identical subunits, which are stabilized by calcium ions (Volanakis 2001; Casas et al. 2008). In addition a monomeric 25 kDa form of CRP exists (Potempa et al. 1987; Ji et al. 2007; Mihlan et al. 2009). The exact biological functions of pCRP and mCRP are currently unclear.

2 Age-Related Macular Degeneration: A Genetic Disorder

Population based analyses, twin studies (Hammond et al. 2002; Seddon et al. 2005) and familial aggregation analyses (Seddon et al. 1997) suggested that AMD is a heritable disease and that the majority of late AMD cases has a specific genetic background. Over the last years evidence accumulated that AMD is caused by

genetic factors. Initiated by the Human Genome Project in 2005, four independent genome-wide linkage studies identified two major chromosomal loci that confer major risk for this retinal disease and that account for approximately 50% of cases (Edwards et al. 2005; Haines et al. 2005; Klein et al. 2005; Rivera et al. 2005). One chromosomal region is located in human chromosome 1q31, which includes the Factor H gene cluster, and the second region is located on human chromosome 10q26 which covers the two closely located genes ***ARMS2*** (**a**ge-**r**elated **m**aculopathy **s**usectibility **2**) and ***HTRA1*** (**h**igh-**t**emperature **r**equired factor **A1**) (Dewan et al. 2006; Yang et al. 2006).

The region on human chromosome 1q31 includes the gene representing the complement regulator **Factor H** and the five CFHRs (**C**omplement **Factor H** **R**elated genes) CFHR1 to CFHR5 (Rodriguez de Cordoba et al. 2004). Within the *Factor H gene* numerous relevant single nucleotide polymorphisms (SNPs) were identified by SNP genotyping, including regions within the promoter-, the coding- and also within the non coding intronic regions (Li et al. 2006b). The major risk variant SNP rs1061170 is located in exon 9, and translates on the protein level at position 402 of both Factor H and FHL1 in the exchange of the amino acid tyrosine (Y) to the risk variant histidine (H). The H402 variant increases the risk for AMD about twofold to fourfold for heterozygotes cases and about threefold to sevenfold for homozygote individuals. Subsequent genetic studies confirmed the high association of the Y402H polymorphism in population and different ethnic groups worldwide (Souied et al. 2005; Okamoto et al. 2006; Seitsonen et al. 2006; Simonelli et al. 2006), and revealed that the H402 risk variant confers a similar risk for development of drusen, and the two severe forms of GA and CNV (Magnusson et al. 2006). Additional SNPs associated with exudative age related macular degeneration were identified in the promoter region of Factor H (position -257; SNP rs3753394); in exon 2, resulting in the exchange at residue 62 of isoleucine (I) to valine (V) (I62V; SNP rs800292), in exon 7 changing the codon but maintaining the alanine (A) residue (A307A; SNP rs1061147) and two which are located within the intervening sequence between exon 15 and exon 16 (SNPs rs380390 and rs1329428) (Chen et al. 2006).

A Chromosomal deletion of a 84-kbp fragment directly downstream of the *Factor H gene*, that includes the genes coding for CFHR1 and CFHR3 has a protective effect in AMD (Hughes et al. 2006; Spencer et al. 2008a; Schmid-Kubista et al. 2009). Case control studies revealed the complete absence of both genes comprise 0.8–1.1% of cases and 2.6–5.7% of the age-matched controls (Hageman et al. 2006).

Additional complement genes associated and linked to AMD include the classical pathway component **C2,** as well as the alternative pathway protein **Factor B** which are both located in close arrangement on human chromosome 6p21 (Gold et al. 2006). The L9H and R32Q variant in Factor B are in nearly complete linkage disequilibrium with the E318D or rs547154 SNP9 in intron 10, respectively of C2. All four polymorphisms are highly protective for AMD. In addition a common polymorphism, i.e. rs2230199 in the ***C3*** *gene*, which is encoded on chromosome 19p13 is strongly associated with AMD (Yates et al. 2007; Spencer et al. 2008b). This variation of C3 that results on the protein level in an arginine (R) to glycine (G) exchange

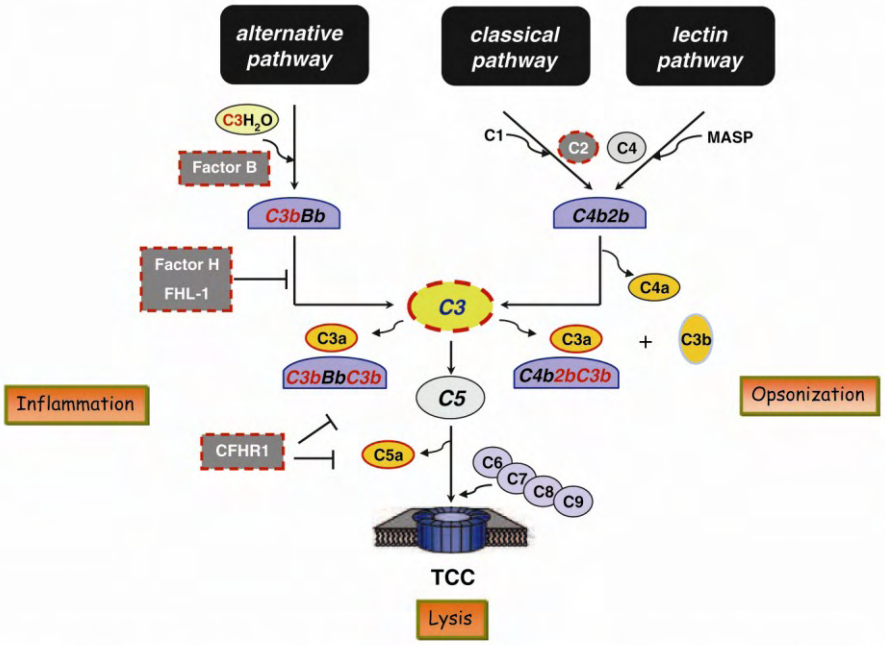

Fig. 1 Complement genes associated with AMD. The complement cascade is initiated by three separate pathways: the alternative, the lectin and the classical pathway. The three major effort functions of the activation completed cascade result in inflammation (effector compounds C3a and C5a), lysis (terminal complement complex, TCC) and opsonization (effector compound surface deposited C3b). Complement proteins that are associated with AMD are indicated by *red dashed lines* include the regulators of the alternative pathway convertase C3bBb: Factor H and FHL1, the C5 convertase and TCC inhibitor CFHR1, Factor B, C3 and C2. The enzymes that are generated and assembled upon complement cascade activation are shown with a blue background

at position 102 (R102G) increases the risk for AMD about twofold for heterozygote and about threefold for homozygote individuals. The association of complement genes in the pathogenesis of AMD further strengthens the concept that the human complement system is involved in the pathogenesis of AMD (Fig. 1).

The second disease associated locus on human chromosome 10q26 includes two separate genes. ***ARMS2*** (also termed LOC387715) which encodes a hypothetical protein. The AMD-associated SNP rs10490924 on exon 1 of the *ARMS2 gene* reveals an estimated risk of 2.6 for heterozygous und 7.0 for homozygous individuals (Rivera et al. 2005). The SNP results in a non-synonymous A69S alteration in the corresponding protein (Fritsche et al. 2008). Recent studies confirmed that this polymorphism in the ARMS2 gene is highly associated with AMD. The second high-susceptibility gene within the 10q26 region comprises the SNP rs11200638 within the ***HTRA1*** gene that is suggested to present a promoter variant of the heat shock serine protease (Dewan et al. 2006; Yang et al. 2006).

3 Effect of the Reported SNPs for Protein Function

3.1 Factor H and Other Complement Proteins

The genetic associations summarized above demonstrate that Factor H, FHL1, CFHR1, CFHR3, C2, Factor B and C3 are involved with AMD development and they show that complement plays a key role in the disease. Obviously sequence exchanges in complement genes and in the encoded complement proteins and regulators disturb the delicate balance of complement activation vs. inhibition and result in activation and inflammation (Zipfel and Skerka 2009).

3.1.1 Factor H and FHL1

The human *Factor H gene* encodes two proteins, **Factor H**, which is composed of 20 SCR domains, and the **Factor H-like protein** (FHL1) (Zipfel and Skerka 1999; Jozsi and Zipfel 2008). FHL1 is derived from an alternatively spliced transcript and the secreted protein is composed of the first seven N-terminal domains of Factor H including codon 402 in SCR 7 and exhibits a unique C-terminal extension of four amino acids (Misasi et al. 1989). Both Factor H and FHL1 are complement regulators that act on the level of C3 convertase and control formation and fate of C3 and C3 convertase C3bBb (Pangburn et al. 1977; Kuhn and Zipfel 1996). Both regulators have multiple binding sites for C3b, Heparin, C-reactive protein (CRP) and binding sites for cellular and biological surfaces. In this context SCR 7 mediates binding to heparin, cell surfaces and CRP (Jarva et al. 1999; Giannakis et al. 2003).

The AMD-associated tyrosine to histidine exchange at position 402 of both Factor H and FHL1 affect the binding intensity to heparin and CRP (Clark et al. 2006; Laine et al. 2007; Sjoberg et al. 2007; Skerka et al. 2007; Yu et al. 2007; Ormsby et al. 2008). The protective variants of both Factor H and FHL1, carrying Y402, bind stronger and the risk variants with H402 bind with lower affinity to their ligands. This differences in binding are explained by the alteration of the distribution of positively charged amino acids by the H402 which are essential for a proper heparin binding over SCR 7 (Prosser et al. 2007; Ormsby et al. 2008). However, differences in heparin affinity of the two allotypes were not reported consistently, what is explained to be glycosaminoglycan specific (Herbert et al. 2007).

The reduced heparin binding of the risk variant translates into lower binding to cellular surfaces, e.g. of RPE cells and results in an inefficient complement regulation at the cell surface (Skerka et al. 2007).

The exchange at amino acid position 402 also affects interaction with the inflammatory marker CRP. Using either intact Factor H, recombinant FHL1 or recombinant fragment SCRs 6–8 the protective variants always showed stronger binding, and the risk variants showed consistently reduced binding to CRP. Individuals who are homozygous for the risk form H402 show 2.5-fold elevated CRP immunoreactivity throughout the choroid and extracellular deposits along the Bruch's membrane as

demonstrated by immunohistochemistry (Johnson et al. 2006). Conversely, AMD patients that carry the protective Y402 phenotype show lower CRP immunoreactivity in the choroidal stroma, drusen as well as basal deposits. Deposition of CRP in drusen or subRPE deposits is a biomarker for chronic inflammation in RPE/chroid complex (Ross et al. 2007). This suggests that the Y402H polymorphism affects Factor H-mediated CRP function and plays a role in local ocular inflammation and cellular injury of RPE cells provoked by complement activation.

One major function of the activated complement system is to identify and tag modified self-cells such as apoptotic particles and necrotic cells, to allow non-inflammatory clearance (Zipfel and Skerka 2009). CRP marks damaged cells and tissues by binding to constituents such as DNA or phosphocholine that are exposed on injured cells. CRP binds the inhibitor Factor H (Ji et al. 2006a, b). Binding of this central soluble complement regulator is relevant on the surface of apoptotic and necrotic cells. CRP activates the classical complement pathway resulting in formation of the C3 convertase which generates C3b and causes C3b surface deposition and opsonization. However, by binding the inhibitor Factor H further progression of the cascade, amplification, C5 convertase formation and terminal pathway activation is inhibited (Gershov et al. 2000; Mihlan et al. 2009). Recently we showed that the monomeric form of CRP, mCRP binds Factor H and increases Factor H inhibitory action on the surface of apoptotic self cells (Mihlan et al. 2009). Factor H has three binding sites for mCRP and one binding site is located in the AMD-associated SCR 7 of both Factor H and FHL1 (Mihlan et al. 2009). Consequently uptake and removal of apoptotic particles was enhanced and bound Factor H showed a potent anti-inflammatory effect.

Recently also a functional difference was reported for the N-terminal located isoleucin (I) to valine (V) exchange at amino acid position 62 of Factor H and FHL1. The I62 variant is associated with the protective form for AMD and most likely has a higher thermal stability (Hocking et al. 2008). In addition Tortajada et al. showed that the protective I62 variant exhibits an increased affinity for C3b and enhanced cofactor activity in the Factor I-mediated cleavage of fluid phase and surface bound C3b (Tortajada et al. 2009). However in the structural model of the co crystal generated with the N-terminal region of Factor H and C3b, residue 62 in SCR 1 of Factor H is positioned outside the interaction interface of the two proteins (Wu et al. 2009).

3.1.2 Complement Factor H Related Proteins

Deletion of an 84-kbp fragment in the human Factor H gene cluster, which is positioned directly downstream of the Factor H gene has a protective effect for AMD development. The homozygous deletion of the chromosomal fragment results in the complete absence of the two plasma proteins **CFHR1** and **CFHR3** (Zipfel et al. 2007). CFHR1 is a regulator of both the C5 convertase and of the terminal pathway and thus controls reactions downstream of the C3 convertase, which is controlled by Factor H (Heinen et al. 2009). A CFHR1 related function is proposed for the CFHR3 protein (Hellwage et al. 1999).

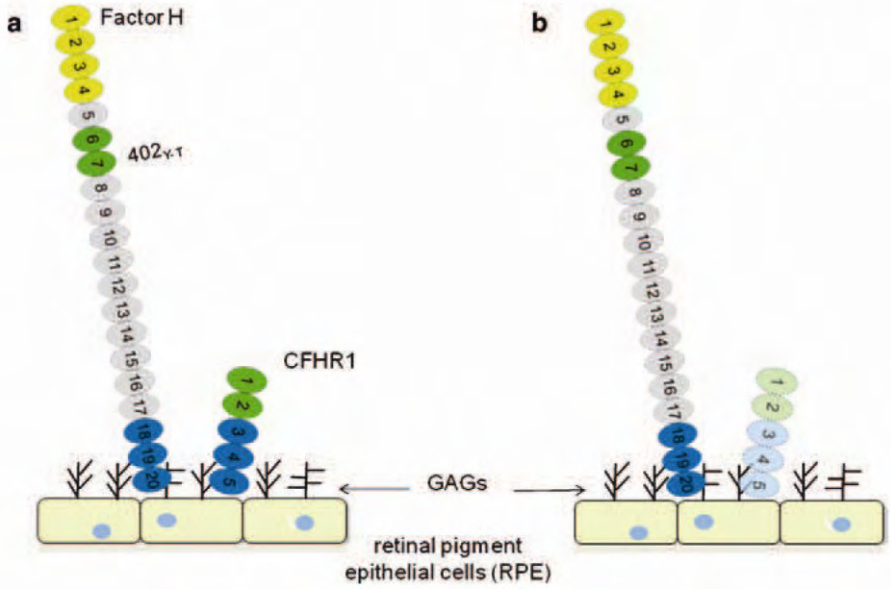

Fig. 2 Factor H and CFHR1 have identical C-terminal surface binding regions and bind to the same sites on the cell surface. (**a**) Factor H is composed of 20 SCR domains and CFHR1 by five domains. The three C-terminal SCRs of Factor H and that of CFHR1 (shown in *blue color*) show almost sequence identity and bind to the same ligands on the cell surface. Both proteins are present in plasma and in vitreous fluid and bind simultaneously to the same sites and surfaces. The relevant amino acid exchange at position 402 of Factor H is in domain 7 and is indicated in the Factor H model. (**b**) Genetic data show that chromosomal deletion of the *CFHR1* gene, which results in the absence of CFHR1 in vitreous fluid and in plasma has a protective effect in AMD. In the absence of CFHR1 more binding sites for Factor H are available on the surface of retinal pigment epithelial cells, resulting in increased Factor H binding and in a stronger local protection

CFHR1 and Factor H share almost identical C-termini. The three C-terminal domains of CFHR1 (i.e. SCRs 3–5) and that of Factor H (i.e. SCRs 18–20) show sequence identity of >98% (Skerka et al. 1991). The C-terminal region of Factor H is central for surface recognition and cell binding and this activity is shared by CFHR1 (Jozsi and Zipfel 2008). In consequence CFHR1 and Factor H bind simultaneously to the same sites on the cell surface. In the situation of CFHR1 deficiency more binding sites for Factor H are available and accessible (Heinen et al. 2009) (Fig. 2).

3.1.3 Other Complement Proteins Associated with AMD: C2, Factor B and C3

Polymorphisms in complement component **C2** (E318D) and **Factor B** (L9H, R32Q) are protective for AMD development (Gold et al. 2006). C2 is a component of the classical complement pathway, and Factor B is involved in alternative pathway

activation. C2 as well as Factor B are expressed in the neural retina, RPE and choroid. Factor B is also identified in ocular drusen. Genetic and functional data suggest that the protective effect is most likely mediated by mutations in the Factor B gene than mutations in the C2 gene. While the AMD associated C2 variants are either conservative change or an intronic SNP, the Q32 variant of Factor B has reduced hemolytic activity (Lokki and Koskimies 1991). Furthermore is the L9H exchange located within the signal peptide and may affect secretion and processing of Factor B. The AMD associated Factor B variants modulate activation of the alternative complement pathway and therefore may lead to an overall complement deregulation.

The AMD-associated polymorphism in **C3** results in exchange of arginine (R) to glycine (G) at amino acid position 102 (Yates et al. 2007). This exchange affects the mobility of the protein and results in a "fast" C3F (fast) and a "slow variant" C3s (slow) (Botto et al. 1990). C3F, the risk variant for AMD, is also associated with renal disease including IgA nephropathy, as well as MPGN-II (Wyatt et al. 1987; Abrera-Abeleda et al. 2006) and has been reported to influence long-term success of renal transplants (Brown et al. 2006). R102 forms together with neighbored or adjacent amino acid residues a positively charged spot on the surface of the C3 molecule, which in C3b is in close proximity with negatively charged amino acids close to the thioester-containing domain (Janssen et al. 2006; Yates et al. 2007). Substitution of positively charged R102 to neutral G102 likely weakens the interaction between charged surfaces and potentially influences thioester activity. Therefore the AMD associated risk variant G102 may affect C3b-Factor H complex formation resulting in less efficient C3b inactivation on (retinal) surfaces. Consequently C3 convertase action is increased and more activations products in form of C3b, iC3b, C3d are deposited onto ocular cell surfaces and generate more anaphylatoxin C3a (Johnson et al. 2001; Nozaki et al. 2006).

3.2 Gene Products of the Chromosome 10q26: ARMS-2 and HRTA1

Chromosome 10q26 encodes two genes that are not related to complement and that are strongly associated with risk for AMD development. The AMD-associated polymorphisms of **ARMS2** (rs10490924; A69S) and **HTRA1** (rs11200638, promoter polymorphism) are in strong linkage disequilibrium that their possible effects are indistinguishable in statistical analysis (Jakobsdottir et al. 2005). Apparently the SNP in the ARMS2 genes shows stronger association with AMD than the HTRA1 gene. This may explain the high association of chromosome 10q26 with AMD (Kanda et al. 2007).

The ARMS gene has an open reading frame which encodes an evolutionary new protein of 12 kDa with so far unknown biological function. A deletion–insertion polymorphism resulting in loss of the polyadenylation signal in ARMS2 RNA is strongly associated with AMD and directly affects transcript formation. Consequently in homozygous individuals no ARMS mRNA is detected. In individuals with the

protective A69 variant the protein is expressed in the retina, placenta and weakly also in the kidney, lung and heart. The exact localization of AMRS2 is still not defined. Different studies report either a cytosolic or mitochondrial localization within retinal cells (Kanda et al. 2007; Fritsche et al. 2008; Wang et al. 2009). The ARMS2 protein seems associated with microtubules of the cytoskeletion and with mitochondrial outer membranes. However independent of the exact cellular location of ARMS2, the S69 variant seems not expressed and the absence of this protein leads to progression of AMD. Thus, identification of the functional biological role of ARMS2 will provide new insights in AMD development.

The HTRA1 gene on chromosome 10q26 encodes a serine protease that is expressed in murine and human RPE cells (Oka et al. 2004). HTRA1 likely regulates degradation of extracellular matrix proteoglycans. This activity can facilitate access of other degradative matrix enzymes, such as collagenases and matrix metalloproteinases, to their substrates (Grau et al. 2006). The SNP rs11200638 within the promoter region of the HTRA1 gene is strongly associated with development of CNV. Apparently the risk variation affects expression levels of the protein and in eyes of donors, homozygous for the risk variant RPE show increased expression by factor of 1.7, as compared to the protective variant (Dewan et al. 2006; Yang et al. 2006). Such higher HTRA1 protein levels may alter the integrity of Bruch's membrane, favoring invasion of choroid capillaries across the extracellular matrix, as described for the wet form of AMD. Together, these findings suggest a potential new mechanism for AMD pathogenesis, independent of the complement-mediated AMD progression.

4 Lessons Learned from Rare Disorders (HUS, MPGN)

Defective and inappropriate complement regulation can cause AMD. In addition to this frequent retinal disease AMD, two rare kidney disorders, in from of atypical Hemolytic Uremic Syndrome (aHUS) and Membrano Proliferative Glomerulo-Nephritis (MPGN II, also termed dense deposit disease) are also associated with Factor H gene mutations, CFHR1/CFHR3 deficiency as well as mutations/polymorphisms in genes coding for complement regulators C3, Factor B and Factor I (Thurman and Holers 2006; Zipfel et al. 2006). Thus related genetic defects suggest similar or highly related pathophysiological mechanisms. Thus defective local complement activation results in related local damaged, which however manifest in different organs (Zipfel 2009).

Gene mutations have been reported for the rare renal disorders aHUS (Perez-Caballero et al. 2001) and MPGN II (Appel et al. 2005). Very related mechanisms can affect the endothelial lining of the glomerulus (aHUS) or the glomerular basement membrane, as well as the retinal cell and surface layer the Bruch's membrane. This similarity suggests that the fine tuned, coordinated action of multiple complement components is essential to maintain the delicate balance of the complement system and to prevent inflammation and cell damage. An exchange of one single residue

in the DNA, RNA or amino acid sequence can already affect protein expression and protein function. Such an exchange can affect the coordinated action and the progression of the cascade and the delicate balance between activation and inhibition of the complement cascade (Skerka and Zipfel 2008).

The knowledge how gene defects in complement components are translated into local defective complement regulation allowed defining appropriate therapy for these rare disorders aHUS and MPGN II. The related pathomechanisms of AMD, atypical HUS and MPGN II is relevant to design and use complement and other anti-inflammatory agents to locally control complement activation in these different organs.

5 Outlook

Based on genetic, functional, and immunohistological data evidence has accumulated that AMD is caused by deregulated local complement activation which develops into chronic inflammation. As related mechanisms of defective complement control results in damage of renal tissues a common link between diseases is emerging which were initially considered unrelated disorders. This allows prediction of additional disease associated genes and allows defining novel targets for complement inhibition.

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References

Abrera-Abeleda MA, Nishimura C, Smith JL, Sethi S, McRae JL, Murphy BF, Silvestri G, Skerka C, Jozsi M, Zipfel PF, Hageman GS, Smith RJ (2006) Variations in the complement regulatory genes factor H (CFH) and factor H related 5 (CFHR5) are associated with membranoproliferative glomerulonephritis type II (dense deposit disease). J Med Genet 43(7):582–589

Anderson DH, Mullins RF, Hageman GS, Johnson LV (2002) A role for local inflammation in the formation of drusen in the aging eye. Am J Ophthalmol 134(3):411–431

Appel GB, Cook HT, Hageman G, Jennette JC, Kashgarian M, Kirschfink M, Lambris JD, Lanning L, Lutz HU, Meri S, Rose NR, Salant DJ, Sethi S, Smith RJ, Smoyer W, Tully HF, Tully SP, Walker P, Welsh M, Wurzner R, Zipfel PF (2005) Membranoproliferative glomerulonephritis type II (dense deposit disease): an update. J Am Soc Nephrol 16(5):1392–1403

Bird AC, Bressler NM, Bressler SB, Chisholm IH, Coscas G, Davis MD, de Jong PT, Klaver CC, Klein BE, Klein R et al (1995) An international classification and grading system for age-related maculopathy and age-related macular degeneration. The International ARM Epidemiological Study Group. Surv Ophthalmol 39(5):367–374

Botto M, Fong KY, So AK, Koch C, Walport MJ (1990) Molecular basis of polymorphisms of human complement component C3. J Exp Med 172(4):1011–1017

Brown KM, Kondeatis E, Vaughan RW, Kon SP, Farmer CK, Taylor JD, He X, Johnston A, Horsfield C, Janssen BJ, Gros P, Zhou W, Sacks SH, Sheerin NS (2006) Influence of donor C3 allotype on late renal-transplantation outcome. N Engl J Med 354(19):2014–2023

Casas JP, Shah T, Hingorani AD, Danesh J, Pepys MB (2008) C-reactive protein and coronary heart disease: a critical review. J Intern Med 264(4):295–314

Chen LJ, Liu DT, Tam PO, Chan WM, Liu K, Chong KK, Lam DS, Pang CP (2006) Association of complement factor H polymorphisms with exudative age-related macular degeneration. Mol Vis 12:1536–1542

Clark SJ, Higman VA, Mulloy B, Perkins SJ, Lea SM, Sim RB, Day AJ (2006) His-384 allotypic variant of factor H associated with age-related macular degeneration has different heparin binding properties from the non-disease-associated form. J Biol Chem 281(34):24713–24720

Congdon N, O'Colmain B, Klaver CC, Klein R, Munoz B, Friedman DS, Kempen J, Taylor HR, Mitchell P (2004) Causes and prevalence of visual impairment among adults in the United States. Arch Ophthalmol 122(4):477–485

Crabb JW, Miyagi M, Gu X, Shadrach K, West KA, Sakaguchi H, Kamei M, Hasan A, Yan L, Rayborn ME, Salomon RG, Hollyfield JG (2002) Drusen proteome analysis: an approach to the etiology of age-related macular degeneration. Proc Natl Acad Sci U S A 99(23):14682–14687

de Jong PT (2006) Age-related macular degeneration. N Engl J Med 355(14):1474–1485

Dewan A, Liu M, Hartman S, Zhang SS, Liu DT, Zhao C, Tam PO, Chan WM, Lam DS, Snyder M, Barnstable C, Pang CP, Hoh J (2006) HTRA1 promoter polymorphism in wet age-related macular degeneration. Science 314(5801):989–992

Donoso LA, Kim D, Frost A, Callahan A, Hageman G (2006) The role of inflammation in the pathogenesis of age-related macular degeneration. Surv Ophthalmol 51(2):137–152

Edwards AO, Ritter R 3rd, Abel KJ, Manning A, Panhuysen C, Farrer LA (2005) Complement factor H polymorphism and age-related macular degeneration. Science 308(5720):421–424

Friedman DS, O'Colmain BJ, Munoz B, Tomany SC, McCarty C, de Jong PT, Nemesure B, Mitchell P, Kempen J (2004) Prevalence of age-related macular degeneration in the United States. Arch Ophthalmol 122(4):564–572

Fritsche LG, Loenhardt T, Janssen A, Fisher SA, Rivera A, Keilhauer CN, Weber BH (2008) Age-related macular degeneration is associated with an unstable ARMS2 (LOC387715) mRNA. Nat Genet 40(7):892–896

Gershov D, Kim S, Brot N, Elkon KB (2000) C-Reactive protein binds to apoptotic cells, protects the cells from assembly of the terminal complement components, and sustains an antiinflammatory innate immune response: implications for systemic autoimmunity. J Exp Med 192(9):1353–1364

Giannakis E, Jokiranta TS, Male DA, Ranganathan S, Ormsby RJ, Fischetti VA, Mold C, Gordon DL (2003) A common site within factor H SCR 7 responsible for binding heparin, C-reactive protein and streptococcal M protein. Eur J Immunol 33(4):962–969

Gold B, Merriam JE, Zernant J, Hancox LS, Taiber AJ, Gehrs K, Cramer K, Neel J, Bergeron J, Barile GR, Smith RT, Hageman GS, Dean M, Allikmets R (2006) Variation in factor B (BF) and complement component 2 (C2) genes is associated with age-related macular degeneration. Nat Genet 38(4):458–462

Grau S, Richards PJ, Kerr B, Hughes C, Caterson B, Williams AS, Junker U, Jones SA, Clausen T, Ehrmann M (2006) The role of human HtrA1 in arthritic disease. J Biol Chem 281(10):6124–6129

Hageman GS, Hancox LS, Taiber AJ, Gehrs KM, Anderson DH, Johnson LV, Radeke MJ, Kavanagh D, Richards A, Atkinson J, Meri S, Bergeron J, Zernant J, Merriam J, Gold B, Allikmets R, Dean M (2006) Extended haplotypes in the complement factor H (CFH) and CFH-related (CFHR) family of genes protect against age-related macular degeneration: characterization, ethnic distribution and evolutionary implications. Ann Med 38(8):592–604

Hageman GS, Luthert PJ, Victor Chong NH, Johnson LV, Anderson DH, Mullins RF (2001) An integrated hypothesis that considers drusen as biomarkers of immune-mediated processes at the RPE-Bruch's membrane interface in aging and age-related macular degeneration. Prog Retin Eye Res 20(6):705–732

Haines JL, Hauser MA, Schmidt S, Scott WK, Olson LM, Gallins P, Spencer KL, Kwan SY, Noureddine M, Gilbert JR, Schnetz-Boutaud N, Agarwal A, Postel EA, Pericak-Vance MA (2005) Complement factor H variant increases the risk of age-related macular degeneration. Science 308(5720):419–421

Hammond CJ, Webster AR, Snieder H, Bird AC, Gilbert CE, Spector TD (2002) Genetic influence on early age-related maculopathy: a twin study. Ophthalmology 109(4):730–736

Heinen S, Hartmann A, Lauer N, Wiehl U, Dahse HM, Schirmer S, Gropp K, Enghardt T, Wallich R, Halbich S, Mihlan M, Schlotzer-Schrehardt U, Zipfel PF, Skerka C (2009) Factor H-related protein 1 (CFHR-1) inhibits complement C5 convertase activity and terminal complex formation. Blood 114(12):2439–2447

Hellwage J, Jokiranta TS, Koistinen V, Vaarala O, Meri S, Zipfel PF (1999) Functional properties of complement factor H-related proteins FHR-3 and FHR-4: binding to the C3d region of C3b and differential regulation by heparin. FEBS Lett 462(3):345–352

Herbert AP, Deakin JA, Schmidt CQ, Blaum BS, Egan C, Ferreira VP, Pangburn MK, Lyon M, Uhrin D, Barlow PN (2007) Structure shows that a glycosaminoglycan and protein recognition site in factor H is perturbed by age-related macular degeneration-linked single nucleotide polymorphism. J Biol Chem 282(26):18960–18968

Hocking HG, Herbert AP, Kavanagh D, Soares DC, Ferreira VP, Pangburn MK, Uhrin D, Barlow PN (2008) Structure of the N-terminal region of complement factor H and conformational implications of disease-linked sequence variations. J Biol Chem 283(14):9475–9487

Hughes AE, Orr N, Esfandiary H, Diaz-Torres M, Goodship T, Chakravarthy U (2006) A common CFH haplotype, with deletion of CFHR1 and CFHR3, is associated with lower risk of age-related macular degeneration. Nat Genet 38(10):1173–1177

Jakobsdottir J, Conley YP, Weeks DE, Mah TS, Ferrell RE, Gorin MB (2005) Susceptibility genes for age-related maculopathy on chromosome 10q26. Am J Hum Genet 77(3):389–407

Janssen BJ, Christodoulidou A, McCarthy A, Lambris JD, Gros P (2006) Structure of C3b reveals conformational changes that underlie complement activity. Nature 444(7116):213–216

Jarva H, Jokiranta TS, Hellwage J, Zipfel PF, Meri S (1999) Regulation of complement activation by C-reactive protein: targeting the complement inhibitory activity of factor H by an interaction with short consensus repeat domains 7 and 8-11. J Immunol 163(7):3957–3962

Ji SR, Wu Y, Potempa LA, Liang YH, Zhao J (2006a) Effect of modified C-reactive protein on complement activation: a possible complement regulatory role of modified or monomeric C-reactive protein in atherosclerotic lesions. Arterioscler Thromb Vasc Biol 26(4):935–941

Ji SR, Wu Y, Potempa LA, Qiu Q, Zhao J (2006b) Interactions of C-reactive protein with low-density lipoproteins: implications for an active role of modified C-reactive protein in atherosclerosis. Int J Biochem Cell Biol 38(4):648–661

Ji SR, Wu Y, Zhu L, Potempa LA, Sheng FL, Lu W, Zhao J (2007) Cell membranes and liposomes dissociate C-reactive protein (CRP) to form a new, biologically active structural intermediate: mCRP(m). FASEB J 21(1):284–294

Johnson LV, Leitner WP, Staples MK, Anderson DH (2001) Complement activation and inflammatory processes in Drusen formation and age related macular degeneration. Exp Eye Res 73(6):887–896

Johnson PT, Betts KE, Radeke MJ, Hageman GS, Anderson DH, Johnson LV (2006) Individuals homozygous for the age-related macular degeneration risk-conferring variant of complement factor H have elevated levels of CRP in the choroid. Proc Natl Acad Sci U S A 103(46):17456–17461

Jozsi M, Zipfel PF (2008) Factor H family proteins and human diseases. Trends Immunol 29(8):380–387

Kanda A, Chen W, Othman M, Branham KE, Brooks M, Khanna R, He S, Lyons R, Abecasis GR, Swaroop A (2007) A variant of mitochondrial protein LOC387715/ARMS2, not HTRA1, is strongly associated with age-related macular degeneration. Proc Natl Acad Sci U S A 104(41):16227–16232

Klein R, Peto T, Bird A, Vannewkirk MR (2004) The epidemiology of age-related macular degeneration. Am J Ophthalmol 137(3):486–495

Klein RJ, Zeiss C, Chew EY, Tsai JY, Sackler RS, Haynes C, Henning AK, SanGiovanni JP, Mane SM, Mayne ST, Bracken MB, Ferris FL, Ott J, Barnstable C, Hoh J (2005) Complement factor H polymorphism in age-related macular degeneration. Science 308(5720):385–389

Kuhn S, Zipfel PF (1996) Mapping of the domains required for decay acceleration activity of the human factor H-like protein 1 and factor H. Eur J Immunol 26(10):2383–2387

Laine M, Jarva H, Seitsonen S, Haapasalo K, Lehtinen MJ, Lindeman N, Anderson DH, Johnson PT, Jarvela I, Jokiranta TS, Hageman GS, Immonen I, Meri S (2007) Y402H polymorphism

of complement factor H affects binding affinity to C-reactive protein. J Immunol 178(6):3831–3836

Li CM, Clark ME, Chimento MF, Curcio CA (2006a) Apolipoprotein localization in isolated drusen and retinal apolipoprotein gene expression. Invest Ophthalmol Vis Sci 47(7):3119–3128

Li M, Atmaca-Sonmez P, Othman M, Branham KE, Khanna R, Wade MS, Li Y, Liang L, Zareparsi S, Swaroop A, Abecasis GR (2006b) CFH haplotypes without the Y402H coding variant show strong association with susceptibility to age-related macular degeneration. Nat Genet 38(9):1049–1054

Lokki ML, Koskimies SA (1991) Allelic differences in hemolytic activity and protein concentration of BF molecules are found in association with particular HLA haplotypes. Immunogenetics 34(4):242–246

Magnusson KP, Duan S, Sigurdsson H, Petursson H, Yang Z, Zhao Y, Bernstein PS, Ge J, Jonasson F, Stefansson E, Helgadottir G, Zabriskie NA, Jonsson T, Bjornsson A, Thorlacius T, Jonsson PV, Thorleifsson G, Kong A, Stefansson H, Zhang K, Stefansson K, Gulcher JR (2006) CFH Y402H confers similar risk of soft drusen and both forms of advanced AMD. PLoS Med 3(1):e5

Mihlan M, Stippa S, Jozsi M, Zipfel PF (2009) Monomeric CRP contributes to complement control in fluid phase and on cellular surfaces and increases phagocytosis by recruiting factor H. Cell Death Differ 16:1630–1640

Misasi R, Huemer HP, Schwaeble W, Solder E, Larcher C, Dierich MP (1989) Human complement factor H: an additional gene product of 43 kDa isolated from human plasma shows cofactor activity for the cleavage of the third component of complement. Eur J Immunol 19(9):1765–1768

Nozaki M, Raisler BJ, Sakurai E, Sarma JV, Barnum SR, Lambris JD, Chen Y, Zhang K, Ambati BK, Baffi JZ, Ambati J (2006) Drusen complement components C3a and C5a promote choroidal neovascularization. Proc Natl Acad Sci U S A 103(7):2328–2333

Oka C, Tsujimoto R, Kajikawa M, Koshiba-Takeuchi K, Ina J, Yano M, Tsuchiya A, Ueta Y, Soma A, Kanda H, Matsumoto M, Kawaichi M (2004) HtrA1 serine protease inhibits signaling mediated by Tgfbeta family proteins. Development 131(5):1041–1053

Okamoto H, Umeda S, Obazawa M, Minami M, Noda T, Mizota A, Honda M, Tanaka M, Koyama R, Takagi I, Sakamoto Y, Saito Y, Miyake Y, Iwata T (2006) Complement factor H polymorphisms in Japanese population with age-related macular degeneration. Mol Vis 12:156–158

Ormsby RJ, Ranganathan S, Tong JC, Griggs KM, Dimasi DP, Hewitt AW, Burdon KP, Craig JE, Hoh J, Gordon DL (2008) Functional and structural implications of the complement factor H Y402H polymorphism associated with age-related macular degeneration. Invest Ophthalmol Vis Sci 49(5):1763–1770

Pangburn MK, Schreiber RD, Muller-Eberhard HJ (1977) Human complement C3b inactivator: isolation, characterization, and demonstration of an absolute requirement for the serum protein beta1H for cleavage of C3b and C4b in solution. J Exp Med 146(1):257–270

Pascolini D, Mariotti SP, Pokharel GP, Pararajasegaram R, Etya'ale D, Negrel AD, Resnikoff S (2004) 2002 global update of available data on visual impairment: a compilation of population-based prevalence studies. Ophthalmic Epidemiol 11(2):67–115

Pepys MB, Hirschfield GM (2003) C-reactive protein: a critical update. J Clin Invest 111(12):1805–1812

Perez-Caballero D, Gonzalez-Rubio C, Gallardo ME, Vera M, Lopez-Trascasa M, Rodriguez de Cordoba S, Sanchez-Corral P (2001) Clustering of missense mutations in the C-terminal region of factor H in atypical hemolytic uremic syndrome. Am J Hum Genet 68(2):478–484

Potempa LA, Siegel JN, Fiedel BA, Potempa RT, Gewurz H (1987) Expression, detection and assay of a neoantigen (Neo-CRP) associated with a free, human C-reactive protein subunit. Mol Immunol 24(5):531–541

Prosser BE, Johnson S, Roversi P, Herbert AP, Blaum BS, Tyrrell J, Jowitt TA, Clark SJ, Tarelli E, Uhrin D, Barlow PN, Sim RB, Day AJ, Lea SM (2007) Structural basis for complement factor H linked age-related macular degeneration. J Exp Med 204(10):2277–2283

Rivera A, Fisher SA, Fritsche LG, Keilhauer CN, Lichtner P, Meitinger T, Weber BH (2005) Hypothetical LOC387715 is a second major susceptibility gene for age-related macular

degeneration, contributing independently of complement factor H to disease risk. Hum Mol Genet 14(21):3227–3236

Rodriguez de Cordoba S, Esparza-Gordillo J, Goicoechea de Jorge E, Lopez-Trascasa M, Sanchez-Corral P (2004) The human complement factor H: functional roles, genetic variations and disease associations. Mol Immunol 41(4):355–367

Ross RJ, Verma V, Rosenberg KI, Chan CC, Tuo J (2007) Genetic markers and biomarkers for age-related macular degeneration. Expert Rev Ophthalmol 2(3):443–457

Schmid-Kubista KE, Tosakulwong N, Wu Y, Ryu E, Hecker LA, Baratz KH, Brown WL, Edwards AO (2009) Contribution of copy number variation in the regulation of complement activation locus to development of age-related macular degeneration. Invest Ophthalmol Vis Sci 50(11):5070–5079

Scholl HP, Charbel Issa P, Walier M, Janzer S, Pollok-Kopp B, Borncke F, Fritsche LG, Chong NV, Fimmers R, Wienker T, Holz FG, Weber BH, Oppermann M (2008) Systemic complement activation in age-related macular degeneration. PLoS ONE 3(7):e2593

Seddon JM, Ajani UA, Mitchell BD (1997) Familial aggregation of age-related maculopathy. Am J Ophthalmol 123(2):199–206

Seddon JM, Cote J, Page WF, Aggen SH, Neale MC (2005) The US twin study of age-related macular degeneration: relative roles of genetic and environmental influences. Arch Ophthalmol 123(3):321–327

Seitsonen S, Lemmela S, Holopainen J, Tommila P, Ranta P, Kotamies A, Moilanen J, Palosaari T, Kaarniranta K, Meri S, Immonen I, Jarvela I (2006) Analysis of variants in the complement factor H, the elongation of very long chain fatty acids-like 4 and the hemicentin 1 genes of age-related macular degeneration in the Finnish population. Mol Vis 12:796–801

Simonelli F, Frisso G, Testa F, di Fiore R, Vitale DF, Manitto MP, Brancato R, Rinaldi E, Sacchetti L (2006) Polymorphism p. 402Y>H in the complement factor H protein is a risk factor for age related macular degeneration in an Italian population. Br J Ophthalmol 90(9):1142–1145

Sjoberg AP, Trouw LA, Clark SJ, Sjolander J, Heinegard D, Sim RB, Day AJ, Blom AM (2007) The factor H variant associated with age-related macular degeneration (His-384) and the non-disease-associated form bind differentially to C-reactive protein, fibromodulin, DNA, and necrotic cells. J Biol Chem 282(15):10894–10900

Skerka C, Horstmann RD, Zipfel PF (1991) Molecular cloning of a human serum protein structurally related to complement factor H. J Biol Chem 266(18):12015–12020

Skerka C, Lauer N, Weinberger AA, Keilhauer CN, Suhnel J, Smith R, Schlotzer-Schrehardt U, Fritsche L, Heinen S, Hartmann A, Weber BH, Zipfel PF (2007) Defective complement control of factor H (Y402H) and FHL-1 in age-related macular degeneration. Mol Immunol 44(13):3398–3406

Skerka C, Zipfel PF (2008) Complement factor H related proteins in immune diseases. Vaccine 26(Suppl 8):I9–14

Souied EH, Leveziel N, Richard F, Dragon-Durey MA, Coscas G, Soubrane G, Benlian P, Fremeaux-Bacchi V (2005) Y402H complement factor H polymorphism associated with exudative age-related macular degeneration in the French population. Mol Vis 11:1135–1140

Spencer KL, Hauser MA, Olson LM, Schmidt S, Scott WK, Gallins P, Agarwal A, Postel EA, Pericak-Vance MA, Haines JL (2008a) Deletion of CFHR3 and CFHR1 genes in age-related macular degeneration. Hum Mol Genet 17(7):971–977

Spencer KL, Olson LM, Anderson BM, Schnetz-Boutaud N, Scott WK, Gallins P, Agarwal A, Postel EA, Pericak-Vance MA, Haines JL (2008b) C3 R102G polymorphism increases risk of age-related macular degeneration. Hum Mol Genet 17(12):1821–1824

Thurman JM, Holers VM (2006) The central role of the alternative complement pathway in human disease. J Immunol 176(3):1305–1310

Tortajada A, Montes T, Martinez-Barricarte R, Morgan BP, Harris CL, de Cordoba SR (2009) The disease-protective complement factor H allotypic variant Ile62 shows increased binding affinity for C3b and enhanced cofactor activity. Hum Mol Genet 18(18):3452–3461

Vingerling JR, Klaver CC, Hofman A, de Jong PT (1995) Epidemiology of age-related maculopathy. Epidemiol Rev 17(2):347–360

Volanakis JE (2001) Human C-reactive protein: expression, structure, and function. Mol Immunol 38(2–3):189–197

Wang G, Spencer KL, Court BL, Olson LM, Scott WK, Haines JL, Pericak-Vance MA (2009) Localization of age-related macular degeneration-associated ARMS2 in cytosol, not mitochondria. Invest Ophthalmol Vis Sci 50(7):3084–3090

Wu J, Wu YQ, Ricklin D, Janssen BJ, Lambris JD, Gros P (2009) Structure of complement fragment C3b-factor H and implications for host protection by complement regulators. Nat Immunol 10(7):728–733

Wyatt RJ, Julian BA, Waldo FB, McLean RH (1987) Complement phenotypes (C3 and C4) in IgA nephropathy. Adv Exp Med Biol 216B:1569–1575

Yang Z, Camp NJ, Sun H, Tong Z, Gibbs D, Cameron DJ, Chen H, Zhao Y, Pearson E, Li X, Chien J, Dewan A, Harmon J, Bernstein PS, Shridhar V, Zabriskie NA, Hoh J, Howes K, Zhang K (2006) A variant of the HTRA1 gene increases susceptibility to age-related macular degeneration. Science 314(5801):992–993

Yates JR, Sepp T, Matharu BK, Khan JC, Thurlby DA, Shahid H, Clayton DG, Hayward C, Morgan J, Wright AF, Armbrecht AM, Dhillon B, Deary IJ, Redmond E, Bird AC, Moore AT (2007) Complement C3 variant and the risk of age-related macular degeneration. N Engl J Med 357(6):553–561

Yu J, Wiita P, Kawaguchi R, Honda J, Jorgensen A, Zhang K, Fischetti VA, Sun H (2007) Biochemical analysis of a common human polymorphism associated with age-related macular degeneration. Biochemistry 46(28):8451–8461

Zarbin MA (1998) Age-related macular degeneration: review of pathogenesis. Eur J Ophthalmol 8(4):199–206

Zipfel PF (2009) Complement and immune defense: from innate immunity to human diseases. Immunol Lett 126(1–2):1–7

Zipfel PF, Edey M, Heinen S, Jozsi M, Richter H, Misselwitz J, Hoppe B, Routledge D, Strain L, Hughes AE, Goodship JA, Licht C, Goodship TH, Skerka C (2007) Deletion of complement factor H-related genes CFHR1 and CFHR3 is associated with atypical hemolytic uremic syndrome. PLoS Genet 3(3):e41

Zipfel PF, Heinen S, Jozsi M, Skerka C (2006) Complement and diseases: defective alternative pathway control results in kidney and eye diseases. Mol Immunol 43(1–2):97–106

Zipfel PF, Skerka C (1999) FHL-1/reconectin: a human complement and immune regulator with cell-adhesive function. Immunol Today 20(3):135–140

Zipfel PF, Skerka C (2009) Complement regulators and inhibitory proteins. Nat Rev Immunol 9(10):729–740

2 The AMD-associated variation at position 62 does not, but the exchange at position 402 does affect surface regulation of the complement inhibitor FHL1.

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THE AMD-ASSOCIATED EXCHANGE AT POSITION 62 DOES NOT, BUT THAT AT POSITION 402 DOES AFFECT SURFACE REGULATION OF THE COMPLEMENT INHIBITOR FHL1*

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Running title: role of residues 62 (I vs. V) and 402 (Y vs. H) on FHL1 function

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Sequence variations in the human *Factor H gene*, that codes for the two alternative pathway regulators FHL1 and Factor H, are associated with age related macular degeneration. Genetic studies identify for two nonsynonymous SNPs (rs800292/I62V and rs1061170/Y402H), in the coding region of FHL1, a strong association with the disease. The FHL1 protein, which is expressed by retinal pigment epithelial cells, controls complement activation at the level of the C3 convertase. Here analyzed if the two SNPs, individually or in cooperation, affect FHL1-mediated surface attachment or complement control. The four FHL1 variants with either I vs. V at position 62, or Y vs. H at position 402 were recombinantly expressed, purified to homogeneity and assayed for C3b-, heparin- and cell binding, as well as for complement regulation on non-activator surfaces. The amino acid exchange at position 62 did not affect FHL1 binding to C3b, to heparin nor to cell surfaces. In contrast the exchange at position 402 affected heparin and cell surface binding. Consequently, the two FHL1 variants with the protective Y402 residue (i.e. FHL1_IY and FHL1_VY) protected sheep erythrocytes and nucleated CHO more efficiently from complement-mediated damage. Thus the variation in SCR7, but not that in SCR1, affects the regulatory activity of FHL1 at cellular surfaces.

The complement system is a central part of innate immunity and plays an essential role in the elimination of microbes, tissue homeostasis, clearance of modified self cells and in enhancing the adaptive immunity (1-3). Proper complement activity depends on the sensitive balance between activation and regulation. This allows targeting of foreign or modified surfaces with activation products such as C3b or TCC, and at the same time, allows protection of intact self surfaces and membranes.

Especially the spontaneously and continuously activated alternative pathway (AP) of complement, directs activation products to the surface of foreign cells or microbes. The initial steps of the AP occur by default, however cascade progression and C3b deposition is controlled by specific regulators. These regulators discriminate between self and foreign surfaces and allow almost exclusive complement activation on foreign or modified self surfaces. Inappropriate AP regulation results in C3b deposition and consequently in attack of bystander cells, which induces inflammation and damage of host tissues (4,5). A large panel of regulators and inhibitors control activation and amplification of complement, particularly of the AP. Such regulators represent either integral membrane proteins or soluble proteins that are distributed in plasma or body fluids and are able to bind surfaces. The soluble AP regulators include the plasma proteins FHL1 (Factor H-like protein 1), Factor H, CFHR3 (complement Factor H-related protein 3) as well as the terminal pathway regulators CFHR1 (complement Factor H-related protein 1), clusterin and vitronectin (6-8).

The human *Factor H gene* on chromosome 1 codes for two proteins: Factor H and FHL1, which is derived from an alternatively spliced transcript (9). FHL1 and Factor H, as major

inhibitors of the AP, are abundant plasma proteins, regulate the AP in fluid phase and can attach to the surface of host cells. Both proteins are composed of consecutive, individually folded protein domains, termed short consensus repeats (SCRs). The seven SCRs of FHL1 are identical to the first seven SCRs of Factor H, which itself is composed of 20 SCRs. The regulatory region of both proteins is located within the four N-terminal domains SCRs1-4. This region accelerates the decay of the central C3 convertase of the AP and acts as a cofactor for Factor I-mediated C3b cleavage (10,11). The C-termini of the two proteins, i.e. SCR7 of FHL1 and SCRs18-20 of Factor H, represent heparin and surface attachment regions. SCR7 and SCRs18-20 have similar binding characteristics but show low level of sequence identity. Both surface attachment regions bind heparin, the monomeric form of the inflammatory marker CRP (C-reactive protein) and contact host surfaces via surface components such as glycosaminoglycans (12,13). In addition a RGD motive in SCR4 is shared by FHL1 and by Factor H. This tripeptide mediates binding of FHL1 but not of Factor H to integrin receptors expressed on human fibroblast-like cells and on human melanoma cells (14). FHL1 has a specific *in-vivo* distribution and also a distinct binding profile that differs from that of Factor H (15). Due to its smaller size FHL1 is rapidly directed e.g. to sites of inflammation. Thus, FHL1 is a unique human complement regulator, which shares complement regulatory activity with Factor H, but has also specific functions in terms of surface attachment, distribution and adhesion.

Sequence variations in the human *Factor H gene* as well as copy number variations in the human *CFHR gene cluster*, are associated with various human diseases including the retinal disease age-related macular degeneration (AMD), the renal disorders membranoproliferative glomerulonephritis type II (MPGN II), atypical hemolytic uremic syndrome (aHUS) and also of the novel subform DEAP-HUS (16-20). Amino acid exchanges within these regulatory proteins modify their local regulatory functions, and consequently lead to enhanced generation of inflammatory molecules and toxic complement activation products (21). In AMD the two SNPs rs800292 and rs1061170 cause the nonsynonymous allelic exchanges I62V and Y402H that manifest in FHL1 and Factor H (22). Both SNPs are in strong linkage disequilibrium with each other (8). The amino acid isoleucin (I) at position 62 represents the protective residue and occurs with frequencies ranging from 17–25 % in AMD cases to 26–38 % in controls compared to valine (V) (23,24). For Factor H purified from plasma of genotyped individuals functional differences were reported for the I62 vs. V62 variants. In this case the protective I62 variant showed enhanced C3b binding which correlated with a stronger Factor I-mediated cleavage of fluid phase- and surface-bound C3b (25). The tyrosine (Y) to histidine (H) exchange at position 402 increases the risk for AMD about 2-4 fold for heterozygote and 5-7 fold for homozygote individuals (26). The protective Y402 variants of FHL1 and Factor H bind better to heparin-, to immobilized CRP and also with higher intensity to the surface of human retinal pigment epithelial cells (27-30). A combination of the two risk amino acids $V_{62}H_{402}$ within Factor H (and FHL1) appear in 57.4 % of AMD cases and 35.1 % of controls, while the protective combination $I_{62}Y_{402}$ is with 12 % less prominent in cases than in controls (22.4 %) (8).

Here we assayed, for the FHL1 protein, the functional consequences from SNP rs800292 and rs1061170. Therefore we compare –for the first time– how the AMD-associated amino acid exchanges at position 62 and 402, either separately or in combination, affect the regulatory activities of FHL1. By *in-vitro* mutagenesis the four FHL1 variants FHL1_$I_{62}Y_{402}$, FHL1_$V_{62}Y_{402}$, FHL1_$I_{62}H_{402}$ and FHL1_$V_{62}H_{402}$ were generated, expressed and purified. The exchange at position 62 (I to V) did neither affect C3b binding nor the regulatory activity of FHL1. However the exchange at position 402 (Y to H) affected heparin binding and cell surface attachment. These different binding characteristics affected the level of complement inhibition on non-activator surfaces and correlated with enhanced protection, when either sheep erythrocytes or nucleated CHO cells, were challenged by complement.

Experimental Procedures

Proteins and antibodies-
Purified human Factor H, C3b, Factor I as well as goat antiserum against human Factor H and goat anti-human C3 were obtained from Comptech (Tyler, Texas, USA). Horseradish

peroxidase (HRP)-conjugated swine anti-goat IgG (Dako, Hamburg, Germany) and AlexaFluor488 conjugated rabbit anti-goat, IgG (Invitrogen Karlsruhe, Germany) were used as secondary antibodies. Normal human serum (NHS) was obtained from healthy volunteers, Jena, Germany, upon informed consent. The generation of complement active FHL1- and Factor H reduced human serum (HSΔFHL1/CFH) was performed by immunadsorbance as described (6).

Expression of the four FHL1 variants and in-vitro mutagenesis-
The wild type FHL1 cDNA was amplified from the vector pBSV-FHL1 and subcloned into the vector Zero Blunt TOPO-TA (Invitrogen) (31). The nucleotide exchange in SCR1 at nucleotide position 257 from guanine (G) to adenine (A) was introduced by side-directed mutagenesis using the primers I62_fwd 5'-CTT GGA AAT **A**TA ATA ATG GTA TGC AGG AAG GG-3' and I62_rev 5'-CTT CCT GCA TAC CAT TAT TA**T** ATT TCC AAG AG-3' according to the manufactures introductions, (QuickChange, Stratagene, Netherlands) on already existing FHL1_Y402 as well as FHL1_H402 coding DNA templates. The single nucleotide exchange in SCR7 at nucleotide position 1277 from thymine to cytosine was described earlier (27). Finally four unique FHL1 sequence variants were generated. The constructs were excised by digestion with EcoR1 and Pst1 and cloned into the expression vector pBSV-8His. The vector was transfected together with baculogold DNA into *Spodoptera frugiperda (*Sf9) insect cells according to the manufactures recommendations and recombinant virus was isolated as described.

Sf9 insect cell culture and purification of the FHL1 variants-
Sf9 cells were grown in monolayers at 27 °C in Insect Express medium (Lonza, Viviers, Belgium) supplemented with FCS (4 %), penicillin (100 units / ml), streptomycin (100 mg / ml) and fungizone (250 ng / ml). For infection with the correspondent recombinant viruses of FHL1_$I_{62}Y_{402}$, FHL1_$V_{62}Y_{402}$, FHL1_$I_{62}H_{402}$ and FHL1_$V_{62}H_{402}$ approximately 3×10^6 cells were seeded in 25 ml serum-free Insect Express medium and infected using a multiplicity of infection of five. Recombinant proteins were isolated from the cell culture supernatant seven days after infection and purified by nickel affinity chromatography (31). Purified proteins were dialyzed against DPBS (Lonza) and concentrated using centrifugal filters (Vivaspin20, Sartorius, Germany). The protein concentration was measured by the UV spectrophotometer NanoDrop 1000 (ThermoFisher, Bonn, Germany).

SDS-Page and Western blotting-
Proteins were separated by SDS-PAGE and either visualized by silver staining or they were transferred onto a nitrocellulose membrane, and identified with an appropriate antiserum in combination with the corresponding HRP-coupled secondary goat antiserum. Visualization occurred with the Chemiluminescence Gel Documentation System MF-ChemBIS 3.2 (Biostep, Jahnsdorf, Germany) and image analyzing with the TotalLab100 software (Nonlinear, Biostep).

ELISA - binding of the FHL1 proteins to C3b and heparin-
MaxiSorp plastic plates (Nunc, Wiesbaden, Germany) were coated with C3b (10 µg / ml) over night at 4 °C. Nonspecific binding sites were blocked with BSA (2 %) in DPBS. The FHL1 variants (10 µg / ml) were added to each well and binding was detected with polyclonal Factor H antiserum and HRP-conjugated goat anti-swine IgG. Binding of the proteins to Heparin (Fluka, Seelze, Germany) was assayed using Heparin binding plates according to the manufacturer's instructions (BD Biosciences, Heidelberg, Germany).

Cofactor assay-
Fluid phase cofactor activity was measured by incubating C3b (0.8 µg / ml), Factor I (0.2 µg / ml) and each FHL1 variant (1 µg / ml). After 15 min incubation at 37° C, the samples were placed on ice, mixed with 10 µl of sample buffer containing beta 2-mercaptoethanol and boiled for 5 min at 95 °C. The mixture was separated by SDS-PAGE, blotted onto a nitrocellulose membrane and developed using goat anti-human C3 and HRP-conjugated rabbit anti-goat IgG.

Cultivation of CHO and ARPE-19 cells-.
The human retinal pigment epithelial cell line ARPE-19 (ATCC; CRL-2302) was cultivated as monolayers in a 1:1 mixture of DMEM and Ham's F12 medium supplemented with FCS

(10 %) at 37 °C in 5 % CO_2. Chinese hamster ovary cells (CHO, DSMZ ACC 110) were cultivated in DMEM medium under the same conditions than the ARPE-19 cells. Prior to binding studies cells were washed twice in DPBS and cultivated for additional 24 hrs in FCS-free culture medium. Detachment of the cells occurred by incubation with Accutase (PAA, Pasching, Austria) for 5 min at 37 °C.

Flow cytometry-
Binding of the FHL1 variants to intact ARPE-19 cells was examined by flow cytometry. Cells were washed twice in DPBS supplemented with BSA (1 %) then each FHL1 variant (10 µg / ml) was added. After extensive washing bound FHL1 proteins were detected with polyclonal Factor H antiserum together with an Alexa488 labeled goat Factor H antiserum. 10.000 cells were routinely counted in a BD LSRII flow cytometer and analyzed with the FACSDiva- (BD Biosciences) and FlowJo software (Tree Star, Ashland,OR, USA). Living, intact cells were identified as propidium iodide negative cells.

Hemolytic assay-
The complement regulatory activity of the FHL1 variants were analyzed in hemolytic assays in the presence of HEPES buffer: [HEPES (20 mM), NaCl (144 mM), $MgCl_2$ (7 mM), EGTA (10 mM), pH 7.5] on the surface of sheep erythrocytes (Biotrend, Köln, Germany). Sheep erythrocytes (sE) represent non-activator surfaces and remain intact when incubated in NHS. However, these cells are lysed, when incubated in complement active HSΔCFH. The replacement of the regulator Factor H has a dose-dependent protective effect (6). Therefore $1x10^7$ sE were incubated for 25 min at 37 °C with HSΔFHL1/CFH (30 %) and increasing amounts of each FHL1 protein. After centrifugation the fraction of lysed cells was determined by measuring the supernatant at 414 nm.

Viability assay-
The effect of the FHL1 variants on the metabolic capacity of cultivated CHO cells in the presence of HSΔFHL1/CFH was measured using the CellTiter-Blue® assay (Promega, Mannheim, Germany). Metabolic activity is characterized by enzymatic conversion of the non-fluorescent dye resazurin into the fluorescent dye resorufin. Therefore, 5×10^4 CHO cells were seeded into 96-well microplates (Nunc) and incubated for 2 days to allow adherence. The culture medium was replaced; cells were washed with HEPES buffer and then incubated for 1 h with each FHL1 variant (30 µg / ml) and Factor H (30 µg / ml) to allow surface attachment. After an additional washing procedure the cells were treated with NHS (5 %) or HSΔFHL1/CFH (5 %). Then, the CellTiter-Blue® was added to the cells, which were then further incubated for 4 hours. The light absorbance of the CellTiter-Blue® reagent is changed by intracellular reduction of resazurin into resorufin. The absorption maximum for resazurin is 605 nm and the absorption maximum for resorufin is 573 nm. Thus, the absorbance measurements at 570 nm and using 600 nm as a reference wavelength were used to monitor the results. Values are compared to blank well containing CellTiter-Blue® reagent without cells.

Statistical analysis-
PRISM GraphPad software was used for statistical analysis. After a significant one-way ANOVA, differences between groups were evaluated using Students t-test for independent samples. The results were considered statistically significant at a P value of ≤ 0.05 (*), ≤ 0.01 (**), or ≤ 0.001 (***).

RESULTS

FHL1 expression and purification-
To analyze whether and how the amino acid exchange at position 62 in SCR1 affects FHL1 function, and if this residue cooperates with the second AMD-associated variation at position 402, FHL1 protein variants, representing either the I62 or V62 isoform were generated by side-directed mutagenesis on the background of either FHL1_Y402 or FHL1_H402 coding cDNA templates (27). Thus four FHL1 isoforms (i.e. FHL1_IY, FHL1_VY, FHL1_IH and FHL1_VH) were recombinantly expressed using the baculovirus system and purified by nickel chelate chromatography. Following separation by SDS-PAGE the purified proteins were visualized by silver staining as single bands with a mobility of 43 kDa. No additional, contaminating bands were detectable (Figure 1A, lanes 1-4).

Neither residue 62 nor residue 402 of FHL1 influenced C3b binding or cofactor activity-

Residue 62 is positioned in SCR1, which is part of the N-terminal C3b binding and complement regulatory region of FHL1 and of Factor H (Figure 1B). Therefore the four FHL1 variants were first tested for C3b binding. C3b was immobilized and the FHL1 proteins were added in fluid phase. Each FHL1 variant bound to C3b and all four proteins bound with similar intensity (Figure 2A). Thus, neither the exchange at position 62, nor that at position 402 affected FHL1 binding to C3b.

Next cofactor activity of the four FHL1 variants for Factor I-mediated C3b cleavage was assayed in fluid phase. Therefore each FHL1 variant was added to Factor I and C3b. After 15 min incubation at 37° C, the reaction mixtures were separated by SDS-PAGE, transferred to a membrane and the C3b cleavage products were visualized by western blotting. All FHL1 variants displayed cofactor activity as revealed by cleavage of the C3b α'chain and the appearance of α'68 kDa and α'43 kDa cleavage products (Figure 2B, lane 2-5). Each FHL1 variant showed comparable cofactor activity as demonstrated by the almost similar intensities of the α'43 kDa band. This effect was confirmed when the intensity of the α'43 kDa was quantified by means of densitometry (Figure 2C). Thus neither the exchange in SCR1 (I62 to V62) nor that in SCR7 (Y402 to H402) influenced FHL1-mediated cofactor activity in fluid phase.

Residue 402, but not residue 62, affected heparin binding and cell surface attachment of FHL1-

Next we asked if the amino acid at position 62 influences heparin binding and cell surface attachment. First binding of the FHL1 variants to immobilized heparin was assayed by ELISA. Each FHL1 variant bound to heparin. The two FHL1 variants with the protective residue Y402 (i.e. FHL1_IY and FHL1_VY) bound stronger to heparin as the two risk variants with H402 (i.e. FHL1_IH and FHL1_VH) (Figure 3A). Heparin binding of the FHL1_IY variant was enhanced by 48 % ($p = 0.05$) and that of the FHL1_VY variant by 49 % ($p = 0.0062$). Thus, the amino acid exchange at position 62 did not, however that at position 402 did influence heparin binding.

In addition binding of the FHL1 variants to cultivated retinal pigment epithelial cells (ARPE-19) was assayed by flow cytometry. The two FHL1 variants with the protective Y402 residue bound to ARPE-19 cells with higher intensity, (i.e. FHL1_IY: MFI 4484; FHL1_VY: MFI 4256) as the variants with the risk residue H402 (i.e. FHL1_IH: MFI 3529 and FHL1_VH: MFI 3073). This difference was 22 % for FHL1_IY and 28 % for FHL1_VY, respectively. Again the exchange at position 402, but not that at position 62, affected FHL1 binding to ARPE-19 cells (Figure 3B). Thus residue 62 of FHL1 is not involved in cell surface binding.

The two 62-variants have comparable regulatory activity for controlling complement-mediated lysis of sheep erythrocytes-

Sheep erythrocytes (sE) represent non-activator surfaces and are protected from complement-mediated damage. Thus, when incubated in complement active NHS the cells remain intact and are not lysed. However sE are lysed when incubated in complement active human serum reduced for the regulators FHL1- and Factor H (HSΔFHL1/CFH). Greatly reduced FHL1 and Factor H concentration, to a level of about 20 %, in order to block AP activation, was confirmed by western blotting (Figure 4A). Addition of either FHL1 or Factor H to HSΔFHL1/CFH resulted in protection of sE from complement-mediated lysis (Figure 4B). This protective effect was dose-dependent and FHL1 and Factor H showed comparable activities.

The activity of the four FHL1 variants was assayed, in order to define, how exchanges at position 62 or 402 influence the protective effect on a non-activator surface. To this end sE were incubated with each FHL1 variant and subsequently challenged with complement active HSΔFHL1/CFH. First, the FHL1 variants that differ at residue 62 were compared. FHL1_IY and also FHL1_VY protected sE from complement-mediated lysis to the same extend (Figure 5A). Thus the exchange at position 62 did not influence the protective effect of the AP regulator FHL1.

In addition, the two FHL1 variants with the exchange at position 402 were compared. Again both FHL1 variants protected sE from complement-mediated lysis and the effects were dose-dependent (Figure 5B). At concentrations

of 20 and 30 µg / ml the FHL1_IY variant was significantly more effective as the FHL1_IH variant ($p = 0.0098$ and $p = 0.012$) The protective FHL1_IY and FHL1_VH, the second risk variant, showed a comparable difference (Figure 5C). At concentrations of 20 and 30 µg / ml, this difference was statistically significant ($p = 0.0061$ and $p = 0.0266$) and translated into a regulatory difference of about 25 %. Both variants with the risk residue H402 (i.e. FHL1_VH and FHL1_IH) showed comparable effects (Supplementary Figure 1A). This difference in regulatory activity reveals that the residue 402 is relevant for surface protection.

For either combination (FHL1_IY and FHL1_IH as well as FHL1_IY and FHL1_VH) the protective Y402 residue increased the regulatory activity at surfaces by almost 25 %. This effect correlates with the observed difference in binding to APRE-19 cells.

The two 62-variants protect nucleated cells from complement-mediated damage to a comparable extend-

Nucleated cells use surface attached as well as membrane bound regulators to control the continuous complement attack by the AP. Thus in the absence of surface attached regulators or when complement regulators are functionally inactivated, nucleated self cells are damaged by complement activation products (21,32). CHO cells which lack human membrane bound regulators were used as an additional cellular system, to evaluate the regulatory effects of the four FHL1 variants. As a read out for surface damage the cellular metabolic activity was assayed by monitoring the uptake of the non-fluorescent dye resazurin and intracellular conversion into the fluorescent dye resorufin. Metabolic activity correlated with a shift in absorbance over a period of 4 h. This sensitive test allowed assaying the protective effect of each FHL1 variant for CHO cells challenged with HSΔFHL1/CFH.

First, CHO cells were treated either with NHS or with HSΔFHL1/CFH. CHO cells exposed to NHS remained intact and were not damaged, as revealed by the potent metabolic activity. However the metabolic activity was reduced when CHO cells were challenged with complement active HSΔFHL1/CFH (Figure 6A). A significant difference in the metabolic activity for cells challenged with NHS vs. cells challenged with HSΔFHL1/CFH occurred at all four time points ($p=0.0012$ after 1 h; $p \leq 0.001$ for all time points > 1h). When FHL1 or Factor H were attached to the cell surface prior to the HSΔFHL1/CFH challenge, the metabolic activity increased, thus demonstrating that each complement regulator protects the cells from complement-mediated damage. In this set up, FHL1 and Factor H had comparable protective effects (Figure 6B).

This new metabolic assay was used to compare the activity of the four FHL1 variants. Each FHL1 protein was bound to the surface of CHO cells, then the cells were challenged with HSΔFHL1/CFH and the metabolic activity was followed over a period of 4 h. Cells treated with FHL1_IY and FHL1_VY, which differ only at residue 62, showed rather potent and comparable metabolic activity (Figure 7A). Thus both proteins have a similar protective effect. In contrast, the FHL1 variants with an exchange of residue 402 in SCR7 (i.e. FHL1_IY and FHL1_IH) had a different activity. Cells treated with the protective FHL1_IY variant showed higher metabolic activity as compared to cells treated with the FHL1_IH risk variant (Figure 7B). For all time points examined, CHO cells treated with the FHL1_IY variant, displayed a higher metabolic activity, as CHO cells to which the risk variant FHL1_IH was bound ($p=0.0299$ for 1 h; $p \leq 0.01$ for all time points > 1h). The protective activity of the FHL1_IY variant was also stronger as compared to the second risk variant FHL1_VH (Figure 7C). These effects were statistically significant after 3 h ($p = 0.017$ and 4 h ($p = 0.0076$). Both variants with the risk residue H402 (i.e. FHL1_VH and FHL1_IH) were less effective in complement control (Supplementary Figure 1B).

Thus, all four FHL1 variants protected cellular surfaces from complement-mediated damage, as demonstrated for nucleated CHO cells and sheep erythrocytes. In both scenarios residue 402 in SCR7, but not residue 62 in SCR1, affected the complement regulatory activity of FHL1 on the cellular surface. The difference among the protective vs. the FHL1 risk variants was in the range of 20 % to 25 %.

DISCUSSION

Sequence variations of the two human alternative pathway regulators FHL1 and Factor H are associated with AMD, a common form of blindness in western societies. Here we analyze the functional consequences of the two AMD-associated SNPs in the coding region of the FHL1 protein (rs800292/I62V and rs1061170/Y402H). We analyze how the amino acid exchanges that confer either a protective or a risk effect, affect complement regulatory function of FHL1. The amino acid exchange at position 62 in SCR1 did neither affect C3b-, heparin- nor cell surface binding. Also the exchange at position 402 in SCR7 did not influence C3b binding. However residue 402 affected cell surface binding. The two protective FHL1 variants (i.e. FHL1_IY and FHL1_VY) bound to heparin with higher intensity (48 %) and similarly also to the surface of ARPE-19 cells (approx 25 %). The increased heparin- and surface binding enhanced the regulatory activity of FHL1 on cellular surfaces and resulted in improved protection. Consequently, the two protective FHL1 variants enhanced protection from complement-mediated damage for sheep erythrocytes , by approx 25 % and also for nucleated CHO cells by approx 20 % (Table 1). In contrast the two H402 risk variants (FHL1_IH and FHL1_VH) were less efficient in surface binding and surface protection. Thus, the variation in SCR7, but not that in SCR1, affected the regulatory activity of FHL1 on cellular surfaces.

Genetic studies defined for SNP rs800292 (I62V) and SNP rs1061147 (Y402H) a significant contribution for AMD development (22,33). Here we identify, for the FHL1 variants, that the exchange of residue 62 has no or a rather minor effect on FHL1 protein function. In contrast the exchange at residue 402 does affect FHL1 ligand- and cell surface binding. This difference in surface binding enhanced the protective activity of FHL1 on surfaces. The effect is clear for the analyzed proteins when identical amounts of FHL1 were compared. However the difference between the protein- and the genetic data indicate that SNP rs800292 at nucleotide 257 (G to A) in the *Factor H gene* may affect gene transcription, mRNA stability, mRNA processing or translational efficiency. In addition other scenarios, like environmental factors or cigarette smoking may further modulate a protective effect.

Structural data show that residue 62 of SCR1 is not part of the interface and contact region that is formed between SCRs1-4 of FHL1/Factor H and C3b (34). In addition NMR studies suggest that residue 62 is not central for the architecture of SCR1. Residue 62 is partly buried in the SCR1 domain and thus not directly accessible for ligand interaction (35). In contrast, residue 402 of SCR7 is surface exposed and accessible for ligand interaction. In addition this residue is located close to a patch of positively charged heparin- and glycosaminoglycan binding amino acids (36). This can explain why the tyrosine to histidine exchange affects the binding characteristics of both FHL1 and Factor H for glycosaminoglycan- and also for surface binding (27,28,37,38). Thus this structural- and functional information demonstrates that residue 402 is relevant for heparin- or glycosaminoglycan binding and they further define no, or only a minor role, for residue 62.

For FHL1 the amino acid exchange in SCR1 did neither influence ligand-, nor cell surface binding. However for Factor H the same exchange affected Factor H ligand interaction and surface binding. In this case the protective Factor H variant (I62) showed increased affinity to C3b and also stronger cofactor activity both for fluid phase and on surface bound C3b (25). These functional differences between FHL1 and Factor H may reflect differences in the three dimensional conformation of the two related proteins. For Factor H a folded back structure as well as dimerization was proposed. Antibody blocking experiments indicate an omega type structure of native Factor H and in addition dimerization as well as oligomerizaiton was reported (13,39,40). FHL1 and Factor H differ in size (42 vs. 150 kDa), in the number of SCR domains (7 SCRs vs. 20 SCRs), in length, also in the number of C3b- as well as heparin binding sites and in serum concentration (50 µg/ml = 1.2µM vs. 500 µg/ml = 3.3µM). FHL1 has one binding site for C3b and one binding site for heparin. In contrast Factor H has three separate C3b- and also three heparin binding sites. Thus the two plasma proteins differ in the number of contact points for C3b, heparin and also cellular surfaces. Similarly FHL1 and Factor differ in integrin-mediated cell adhesion and binding to

integrin receptors (14). Expression of FHL1 and of Factor H is induced by proinflammatory cytokine IFN-γ, thus the local levels of the complement regulators is increased upon inflammation (41,42). FHL1 is expressed by RPE cells (27) and local FHL1 levels are relevant and beneficial for tissue protection. FHL1 mRNA expression is induced by dexamethasone and in addition the smaller size allows a better diffusion of the FHL1 protein (15). FHL1 and Factor H display overlapping complement regulatory activity. However the different regulation, various tissue distribution and expression levels show that FHL1 and Factor H have specific and unique roles in immune regulation (43).

The two AMD-associated SNPs rs800292 and rs1061147 in the *Factor H* gene that result in amino acid exchanges at positions 62 and 402 of both FHL1 and of the Factor H protein affect FHL1 protein function differently. The exchange at position 62 does not influence ligand binding and complement regulation. However the exchange at position 402 affects heparin and cell surface binding and also the regulatory activity at cellular surfaces. The protective Y402 variants of FHL1 bind more efficiently heparin and cellular surfaces (increased by 20 % - 25 %), which result in enhanced surface protection. Such initial differences are amplified during complement activation, chronic inflammation and over time. Increased and continuous complement activation can generate a pro-inflammatory scenario and the continuous complement challenge of cellular surfaces may ultimately lead to pathology.

A fine tuned activation and precise local levels of complement inhibitors is highly important to control the local activity of the short lived C3 convertase. Similarly the AMD-associated sequence exchange in the Factor B gene (R32Q) affects the stability and activity of the C3 convertase (44). In consequence defective regulation and an active C3 convertase increases local complement activity. This enhances inflammation and generates a pro-inflammatory environment. Over time this inflammatory scenario can cause cell damage e.g. in form of necrosis or apoptosis in RPE cells, deposition of complement activation fragments and release of inflammatory mediators. Such a scenario can lead to chronic inflammation at the Bruch's membrane resulting in drusen formation and pathology.

REFERENCES

1. Carroll, M. C. (2000) *Adv Immunol* **74**, 61-88
2. Ogden, C. A., and Elkon, K. B. (2006) *Curr Dir Autoimmun* **9**, 120-142
3. Walport, M. J. (2001) *N Engl J Med* **344**, 1058-1066
4. Pangburn, M. K., and Muller-Eberhard, H. J. (1983) *Ann N Y Acad Sci* **421**, 291-298
5. Zipfel, P. F., Mihlan, M., and Skerka, C. (2007) *Adv Exp Med Biol* **598**, 80-92
6. Heinen, S., Hartmann, A., Lauer, N., Wiehl, U., Dahse, H. M., Schirmer, S., Gropp, K., Enghardt, T., Wallich, R., Halbich, S., Mihlan, M., Schlotzer-Schrehardt, U., Zipfel, P. F., and Skerka, C. (2009) *Blood* **114**, 2439-2447
7. Preissner, K. T., and Seiffert, D. (1998) *Thromb Res* **89**, 1-21
8. Fritsche, L. G., Lauer, N., Hartmann, A., Stippa, S., Keilhauer, C. N., Oppermann, M., Pandey, M. K., Kohl, J., Zipfel, P. F., Weber, B. H., and Skerka, C. *Hum Mol Genet*
9. Misasi, R., Huemer, H. P., Schwaeble, W., Solder, E., Larcher, C., and Dierich, M. P. (1989) *Eur J Immunol* **19**, 1765-1768
10. Pangburn, M. K., Schreiber, R. D., and Muller-Eberhard, H. J. (1977) *J Exp Med* **146**, 257-270
11. Kuhn, S., and Zipfel, P. F. (1996) *Eur J Immunol* **26**, 2383-2387
12. Ferreira, V. P., Herbert, A. P., Hocking, H. G., Barlow, P. N., and Pangburn, M. K. (2006) *J Immunol* **177**, 6308-6316
13. Oppermann, M., Manuelian, T., Jozsi, M., Brandt, E., Jokiranta, T. S., Heinen, S., Meri, S., Skerka, C., Gotze, O., and Zipfel, P. F. (2006) *Clin Exp Immunol* **144**, 342-352
14. Hellwage, J., Kuhn, S., and Zipfel, P. F. (1997) *Biochem J* **326 (Pt 2)**, 321-327

15. Friese, M. A., Hellwage, J., Jokiranta, T. S., Meri, S., Muller-Quernheim, H. J., Peter, H. H., Eibel, H., and Zipfel, P. F. (2000) *Clin Exp Immunol* **121**, 406-415
16. Edwards, A. O., Ritter, R., 3rd, Abel, K. J., Manning, A., Panhuysen, C., and Farrer, L. A. (2005) *Science* **308**, 421-424
17. Licht, C., Heinen, S., Jozsi, M., Loschmann, I., Saunders, R. E., Perkins, S. J., Waldherr, R., Skerka, C., Kirschfink, M., Hoppe, B., and Zipfel, P. F. (2006) *Kidney Int* **70**, 42-50
18. Perez-Caballero, D., Gonzalez-Rubio, C., Gallardo, M. E., Vera, M., Lopez-Trascasa, M., Rodriguez de Cordoba, S., and Sanchez-Corral, P. (2001) *Am J Hum Genet* **68**, 478-484
19. Zipfel, P. F., Mache, C., Muller, D., Licht, C., Wigger, M., and Skerka, C. *Pediatr Nephrol*
20. Skerka, C., and Zipfel, P. F. (2008) *Vaccine* **26 Suppl 8**, I9-14
21. Thurman, J. M., and Holers, V. M. (2006) *J Immunol* **176**, 1305-1310
22. Hageman, G. S., Anderson, D. H., Johnson, L. V., Hancox, L. S., Taiber, A. J., Hardisty, L. I., Hageman, J. L., Stockman, H. A., Borchardt, J. D., Gehrs, K. M., Smith, R. J., Silvestri, G., Russell, S. R., Klaver, C. C., Barbazetto, I., Chang, S., Yannuzzi, L. A., Barile, G. R., Merriam, J. C., Smith, R. T., Olsh, A. K., Bergeron, J., Zernant, J., Merriam, J. E., Gold, B., Dean, M., and Allikmets, R. (2005) *Proc Natl Acad Sci U S A* **102**, 7227-7232
23. Bergeron-Sawitzke, J., Gold, B., Olsh, A., Schlotterbeck, S., Lemon, K., Visvanathan, K., Allikmets, R., and Dean, M. (2009) *Eur J Hum Genet* **17**, 1190-1199
24. Chen, L. J., Liu, D. T., Tam, P. O., Chan, W. M., Liu, K., Chong, K. K., Lam, D. S., and Pang, C. P. (2006) *Mol Vis* **12**, 1536-1542
25. Tortajada, A., Montes, T., Martinez-Barricarte, R., Morgan, B. P., Harris, C. L., and de Cordoba, S. R. (2009) *Hum Mol Genet* **18**, 3452-3461
26. Klein, R. J., Zeiss, C., Chew, E. Y., Tsai, J. Y., Sackler, R. S., Haynes, C., Henning, A. K., SanGiovanni, J. P., Mane, S. M., Mayne, S. T., Bracken, M. B., Ferris, F. L., Ott, J., Barnstable, C., and Hoh, J. (2005) *Science* **308**, 385-389
27. Skerka, C., Lauer, N., Weinberger, A. A., Keilhauer, C. N., Suhnel, J., Smith, R., Schlotzer-Schrehardt, U., Fritsche, L., Heinen, S., Hartmann, A., Weber, B. H., and Zipfel, P. F. (2007) *Mol Immunol* **44**, 3398-3406
28. Clark, S. J., Higman, V. A., Mulloy, B., Perkins, S. J., Lea, S. M., Sim, R. B., and Day, A. J. (2006) *J Biol Chem* **281**, 24713-24720
29. Laine, M., Jarva, H., Seitsonen, S., Haapasalo, K., Lehtinen, M. J., Lindeman, N., Anderson, D. H., Johnson, P. T., Jarvela, I., Jokiranta, T. S., Hageman, G. S., Immonen, I., and Meri, S. (2007) *J Immunol* **178**, 3831-3836
30. Sjoberg, A. P., Trouw, L. A., Clark, S. J., Sjolander, J., Heinegard, D., Sim, R. B., Day, A. J., and Blom, A. M. (2007) *J Biol Chem* **282**, 10894-10900
31. Kuhn, S., Skerka, C., and Zipfel, P. F. (1995) *J Immunol* **155**, 5663-5670
32. Zipfel, P. F., Edey, M., Heinen, S., Jozsi, M., Richter, H., Misselwitz, J., Hoppe, B., Routledge, D., Strain, L., Hughes, A. E., Goodship, J. A., Licht, C., Goodship, T. H., and Skerka, C. (2007) *PLoS Genet* **3**, e41
33. Pickering, M. C., de Jorge, E. G., Martinez-Barricarte, R., Recalde, S., Garcia-Layana, A., Rose, K. L., Moss, J., Walport, M. J., Cook, H. T., de Cordoba, S. R., and Botto, M. (2007) *J Exp Med* **204**, 1249-1256
34. Wu, J., Wu, Y. Q., Ricklin, D., Janssen, B. J., Lambris, J. D., and Gros, P. (2009) *Nat Immunol* **10**, 728-733
35. Hocking, H. G., Herbert, A. P., Kavanagh, D., Soares, D. C., Ferreira, V. P., Pangburn, M. K., Uhrin, D., and Barlow, P. N. (2008) *J Biol Chem* **283**, 9475-9487
36. Herbert, A. P., Deakin, J. A., Schmidt, C. Q., Blaum, B. S., Egan, C., Ferreira, V. P., Pangburn, M. K., Lyon, M., Uhrin, D., and Barlow, P. N. (2007) *J Biol Chem* **282**, 18960-18968
37. Prosser, B. E., Johnson, S., Roversi, P., Herbert, A. P., Blaum, B. S., Tyrrell, J., Jowitt, T. A., Clark, S. J., Tarelli, E., Uhrin, D., Barlow, P. N., Sim, R. B., Day, A. J., and Lea, S. M. (2007) *J Exp Med* **204**, 2277-2283

38. Jokiranta, T. S., Cheng, Z. Z., Seeberger, H., Jozsi, M., Heinen, S., Noris, M., Remuzzi, G., Ormsby, R., Gordon, D. L., Meri, S., Hellwage, J., and Zipfel, P. F. (2005) *Am J Pathol* **167**, 1173-1181
39. Pangburn, M. K., Rawal, N., Cortes, C., Alam, M. N., Ferreira, V. P., and Atkinson, M. A. (2009) *J Immunol* **182**, 1061-1068
40. Nan, R., Gor, J., and Perkins, S. J. (2008) *J Mol Biol* **375**, 891-900
41. Ripoche, J., Mitchell, J. A., Erdei, A., Madin, C., Moffatt, B., Mokoena, T., Gordon, S., and Sim, R. B. (1988) *J Exp Med* **168**, 1917-1922
42. Wu, Z., Lauer, T. W., Sick, A., Hackett, S. F., and Campochiaro, P. A. (2007) *J Biol Chem* **282**, 22414-22425
43. Zipfel, P. F., and Skerka, C. (1999) *Immunol Today* **20**, 135-140
44. Montes, T., Tortajada, A., Morgan, B. P., Rodriguez de Cordoba, S., and Harris, C. L. (2009) *Proc Natl Acad Sci U S A* **106**, 4366-4371

FOOTNOTES

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The abbreviations used are: aHUS, atypical hemolytic uremic syndrome; ARPE-19, retinal pigment epithelial cell line; AMD, age-related macular degeneration; AP, alternative pathway; C3b, complement component 3b; CFHR1, complement Factor H-related protein 1; CFHR3, complement Factor H-related protein 3; CHO, chinese hamster ovary cells; DEAP-HUS, deficient for CFHR proteins and Factor H autoantibody positive; I, isoleucin; H, histidine; HSΔFHL1/CFH, human serum depleted for FHL1 and Factor H; FHL1, Factor H-like protein; MPGN II, membranoproliferative glomerulonephritis type II; NHS, normal human serum; RGD, arginine-glycine-aspartate motive; SCR, short consensus repeat; sE, sheep erythrocytes; V, valine; Y, tyrosine;

FIGURE LEGENDS

Figure 1: Recombinant expression and purification of the four FHL1 variants. A) The four FHL1 variants with the indicated amino acid at position 62 in SCR1 and 402 in SCR7 were recombinantly expressed in Sf-9 insect cells and purified to homogeneity by gel chromatography. The four FHL1 variants together with Factor H were separated by SDS-PAGE and identified by silver staining as single bands. The mobility of the marker is indicated on the left. Data show one representative result out of five. **B**) Domain structure of FHL1. FHL1 is composed of 7 consecutive domains, termed short consensus repeat (SCRs) and Factor H of 20 domains. The N-terminal regions of both proteins, which include the four SCR domains (SCRs1-4) mediate the complement regulatory functions of FHL1 and Factor H, i.e. cofactor- and decay-accelerating activity. The C-termini of the both proteins, i.e. SCR7 of FHL1 and SCRs18-20 of Factor H, represent surface attachment regions that bind to heparin and to cell surfaces. The two AMD-associated amino acid exchanges at position 62 in SCR1 and 402 in SCR7 manifest in both regulators, FHL1 as well as Factor H. The protective residues I62 and Y402 are indicated green, the risk residues V62 and H402 red. **C)** Structural model of SCR1 and position of protective residue I62 (green). Residue 62 is largely buried in the SCR domain and is not surface exposed. **D)** Structural model of SCR7 and position of the protective residue Y402 (green). This residue is surface exposed and directly adjacent to several heparin binding residues that are shown in blue color.

Figure 2: Role of residues 62 and 402 on C3b binding and FHL1-mediated cofactor activity. A) Binding of the four recombinant FHL1 variants (FHL1_IY, FHL1_VY, FHL1_IH and FHL1_VH) to immobilized C3b was analyzed by ELISA. Each FHL1 variant bound with similar intensity to C3b. BSA did not bind to C3b (gray column) **B)** Cofactor activity of the FHL1 variants for Factor I-mediated cleavage of C3 in fluid phase. C3b and Factor I were incubated in the presence of the indicated FHL1 variant. Then the mixtures were separated by SDS-PAGE, proteins were transferred to a membrane and cleavage of C3b was followed. In the presence of the cofactor FHL1, Factor I-mediated cleavage of the C3b resulted in cleavage of the α'-chain (lane 2-5). The α'68 kDa and α'43 kDa cleavage products were of similar intensities, thus each of the four FHL1 variants showed comparable cofactor activity. **C)** Densitometric analysis of the α'43 fragment confirmed the similar intensity of the cleavage fragment demonstrating comparable cofactor activity of the four tested FHL1 variants. The columns represent the relative intensity of the α'-chain fragment normalized for the ß'-chain. The figure show mean values of one representative result and three independent experiments were performed. Error bars indicate SD.

Figure 3: Binding of the four FHL1 variants to heparin and to ARPE-19 cells. A) Binding of the recombinant FHL1 variants (FHL1_IY, FHL1_VY, FHL1_IH and FHL1_VH) to immobilized heparin was analyzed by ELISA. The two FHL1 variants with the protective Y402 (IY, black column; VY hatched column) bound significantly stronger to heparin, as compared to the H402 risk FHL1 variants (IH, dotted column; VH, white column). The variation of the amino acid at position 62 did not affect heparin binding. The data show mean values of a triplicate assays of one representative experiment. The experiment was repeated with similar results at least three times. Statistical analyses between two data points were done using students *t* test. Error bars indicate SD. *, $P < 0.05$; **, $P < 0.01$; ***, $P \leq 0.001$ **B-C)** Binding of the FHL1 proteins to intact ARPE-19 cells was analyzed by flow cytometry. Cell bound FHL1 protein was detected with a polyclonal antiserum in combination with an appropriate Alexa488 conjugated secondary antiserum. Unspecific antibody binding in the absence of protein is represented by the dotted line. The binding profiles show that the amino acid variation at position 402, but not that at position 62 affects FHL1 binding to retinal pigment cells. Binding of FHL1 variants with the protective tyrosine 402 are shown by the black (FHL1_IY) and gray (FHL1_VY) lines. The binding profiles of the two risk variants, i.e. FHL1_IH and FHL1_VH are shown by hatched lines. Representative results of one experiment are shown. A total of four independent experiments were performed which always showed the same profile.

Figure 4: FHL1 and Factor H protect sheep erythrocytes from complement-mediated lysis. The impact of FHL1 for surface protection of non-activator surfaces was assayed in a hemolytic assay using complement active FHL1- and Factor H depleted human serum (HS□FHL1/Factor H). **A)** Effect of FHL1 and Factor H depletion. Human serum was depleted from FHL1 and Factor H by incubation with specific antisera. Normal human serum (NHS) and HSΔFHL1/CFH were then separated by SDS-PAGE and transferred onto a nitrocellulose membrane. In NHS Factor H, FHL1 and both isoforms of CFHR1 were detected (lane 1). However in depleted serum the intensity of the Factor H band is reduced and FHL1- as well as CFHR1 are absent (lane 2). **B)** sE represent non activator surface and the cells are normally protected from complement-mediated lysis when incubated with complement active NHS (white diamond). However the same cells are lysed when they are incubated in complement active HSΔFHL1/CFH (black diamond). Addition of the alternative pathway regulators FHL1 (black squares) or of Factor H (crosses) protected erythrocytes from complement-mediated lysis. The effect was dose dependent. At the highest concentration of 40µg / ml FHL1 protected sE almost completely. FHL1 and Factor H showed comparable protective effects. The data represent mean values and standard deviation of one representative using triplicate samples. A total of three independent experiments were performed. Statistical analyses between two data points were done using students *t* test. Error bars indicate SD. *, $P < 0.05$; **, $P < 0.01$; ***, $P \leq 0.001$

Figure 5: Role of residues 62 or 402 of FHL1 on protection of complement-mediated lysis of sheep erythrocytes. The regulatory effect of the four FHL1 variants was assayed upon challenge of sheep erythrocytes (sE) with complement active human serum, which was depleted for FHL1- and

Factor H (HSΔFHL1/CFH). sE ($1x10^7$) were incubated in HSΔFHL1/CFH (30 %) at 37 °C for 30 min in the presence of the indicated amounts of each FHL1 variant. **A)** The role of the amino acid exchange at position 62 (i.e. V62 to I62) on complement-mediated lysis of sE was assayed in the presence of either FHL1_IY (black squares) or FHL1_VY (gray triangles). Both FHL1 variants protected sE dose-dependently from lysis with comparable effects. **B)** FHL1_IY (black squares) was more efficient in protecting sE as the FHL1_IH variant (white triangles). At concentrations of 20 µg / ml and 30 µg / ml the difference was statistically significant. **C)** Similarly FHL1_IY (black squares) had a stronger protective effect as compared to the FHL1_VH variant (white circles). At concentrations of 20 and 30 µg / ml this difference was statistically significant. The data represent mean values and the standard deviation of a representative experiment is shown. A total of three independent experiments were performed. A one-way ANOVA test was used to show that the tested groups are independent of each other. The values of F showed a range from 5.65 – 53.45 Statistical analyses between two data points were done using students t-test. Error bars indicate SD. *, $P < 0.05$; **, $P < 0.01$; ***, $P \leq 0.001$

Figure 6: FHL1 and Factor H protect nucleated CHO cells from complement challenge. The protective effect of the four FHL1 variants was also assayed using nucleated CHO cells that were challenged by complement activation. Therefore CHO cells were incubated with complement active HSΔFHL1/CFH or with NHS. Complement-mediated damage or membrane perturbation was assayed by following the metabolic activity via uptake and enzymatic conversion of the non fluorescent dye resazurin into the fluorescent dye resorufin. The shift in absorbance was recorded at 570 nm over a period of 4 h. The viability of CHO cells in the presence of NHS after 4h incubation was set to 100 %. **A)** CHO cells challenged with complement active HSΔFHL1/CFH (black diamonds and dotted line) showed lower metabolic activity as compared to cells which were incubated in complement active NHS in which both regulators FHL1 and Factor H were present (white diamonds and solid line). The effect was time-dependent and the differences were statistically significant for all time points analyzed. **B)** Preincubation of the CHO cells prior to complement challenge with FHL1_IY (black squares) and also Factor H (crosses) increased the metabolic activity of CHO. FHL1 and Factor H were used at 30 µg / ml and showed comparable protective effects. The data represent mean values of triplicates within one representative independent experiment. The experiment was repeated three times with similar results. A one-way ANOVA test was used to show that the tested groups are independent of each other. The values of F showed a range from18.13 – 64.89. Statistical analyses between two data points were done using students t-test. Error bars indicate SD. *, $P < 0.05$; **, $P < 0.01$; ***, $P \leq 0.001$

Figure 7: Role of residue 62 and 402 on FHL1-mediated protection of CHO cells upon complement challenge. The protective activity of the four FHL1 variants was compared for CHO cells which were challenged by complement active HSΔFHL1/CFH. Each FHL1 variant was first bound to CHO cells and then the cells were challenged with complement active HSΔFHL1/CFH. The metabolic activity of the cells was followed over a period of 4 h. The metabolic activity of CHO cells treated with NHS after 4h incubation was set to 100 %. **A)** FHL1_IY (black squares) and FHL1_VY (gray triangles), that differ only at position 62, had comparable protective effects for CHO cells exposed to HSΔFHL1/CFH. **B)** In contrast FHL1_IY (black squares) showed stronger protective effects as the FHL1_IH (white triangles) variant. This difference was statistically significant after over the whole period of 4 h. **C)** The risk variant FHL1_VH (white circles) displayed a lower protective effect for complement-mediated damage for CHO cells as compared to the protective FHL1_IY (black squares) variant. This effect was significant after 3 h and 4 h. The data show mean values of triplicates within one representative independent experiment. The experiment was repeated three times with similar results. A one-way ANOVA test was used to show that the tested groups are independent of each other. The values of F showed a range from 19.71 – 57.98. Statistical analyses between two data points were done using students t-test. Error bars indicate SD. *, $P < 0.05$; **, $P < 0.01$; ***, $P \leq 0.001$

SUPPLEMENTARY FIGURES LEGEND

Supplementary Figure 1: Regulatory effects of the two FHL1_IH and FHL1_VH risk variants A) The two FHL1 risk variants FHL1_VH (white circles) and FHL1_IH (white triangles) were assayed for their ability to protect complement mediated lysis of sE which were challenged with complement active HSΔFHL1/CFH. Both FHL1 variants protect sE dose-dependently from lysis and their effects were comparable. The data show mean values and standard deviation of one representative of three independent experiments. Error bars indicate SD. **B)** The two FHL1 risk variants FHL1_VH (white circles) and FHL1_IH (white triangles) showed comparable protective effects for complement mediated damage of nucleated CHO cells which were challenged with complement active HSΔFHL1/CFH. The metabolic conversion of the dye resazurin in CHO cells incubated with FHL1_VH (white circles) was comparable to cells incubated with FHL1_IH (white triangles). The data show mean values and standard deviation of one representative of three independent experiments. Error bars indicate SD. *, $P < 0.05$; **, $P < 0.01$; ***, $P \leq 0.001$

TABLES

Table 1: Binding and functional characteristics of the four FHL1 variants

FHL1 variant		cofactor	binding to:			protection of cells	
62	402	activity	C3b	Heparin	ARPE-19	sE	CHO
I	**Y**	++	++	++	++	++	++
V	**Y**	++	++	++	++	++	++
I	**H**	++	++	+	+	+	+
V	**H**	++	++	+	+	+	+

Table legend 1: The I62 to V62 exchange in SCR1 of FHL1 does neither influence Factor I-mediated cofactor activity, binding to C3b, to heparin nor to ARPE-19 cells. Similarly this exchange does not affect complement regulation on cellular surfaces, such as sheep red blood cells (sE) and nucleated chinese hamster ovary cells (CHO). In contrast the Y402 to H402 exchange in SCR7 of FHL1 influences heparin binding - as well as attachment to ARPE-19 cells. As a consequence the regulatory activity of FHL1_IY and FHL1_VY on cellular surfaces is enhanced (highlighted by a grey background).

Figure 1

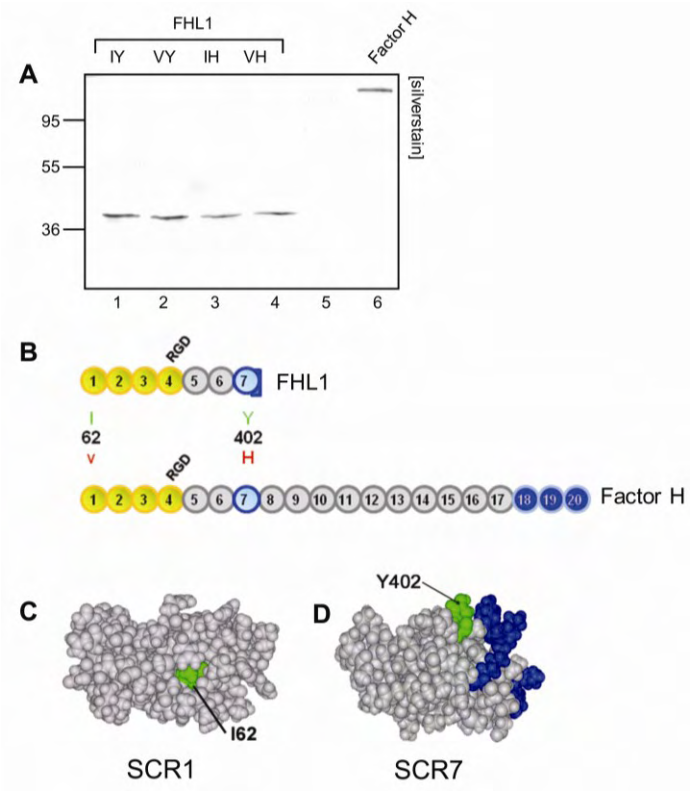


Figure 2

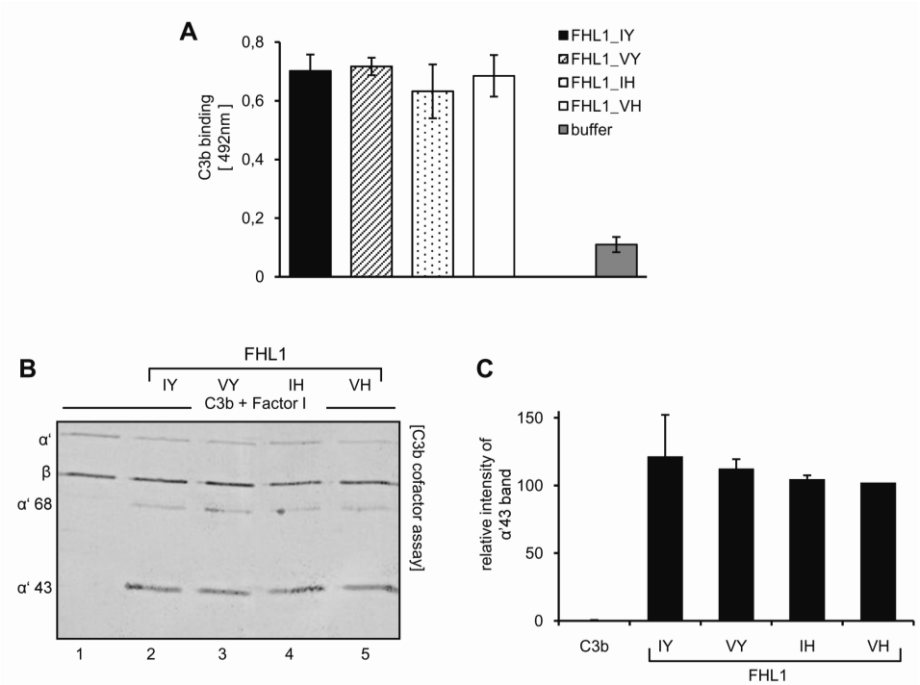


Figure 3

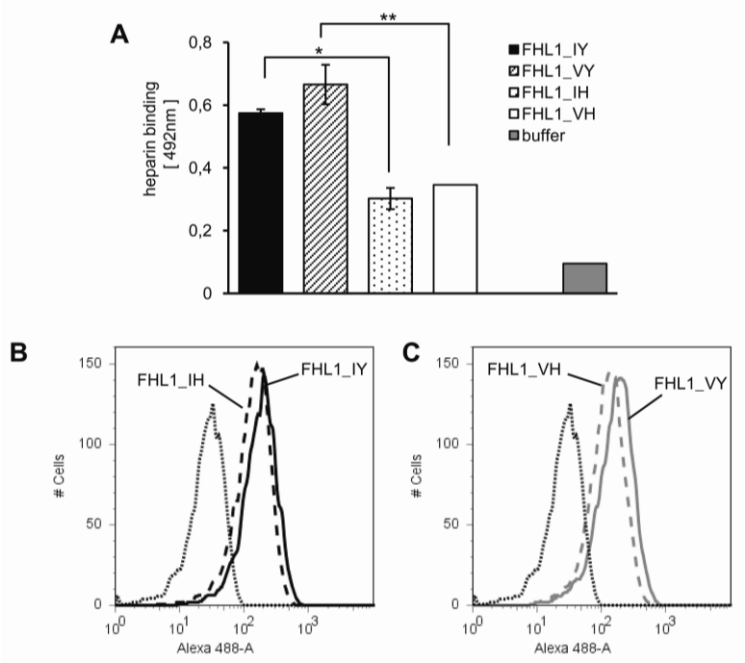


Figure 4

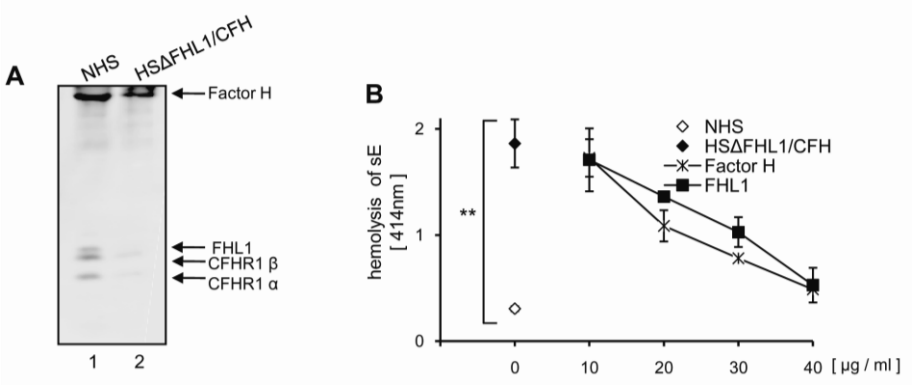


Figure 5

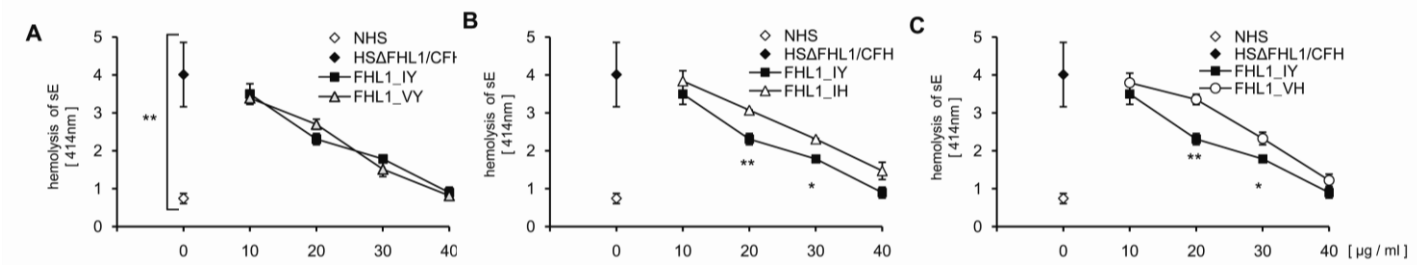


Figure 6

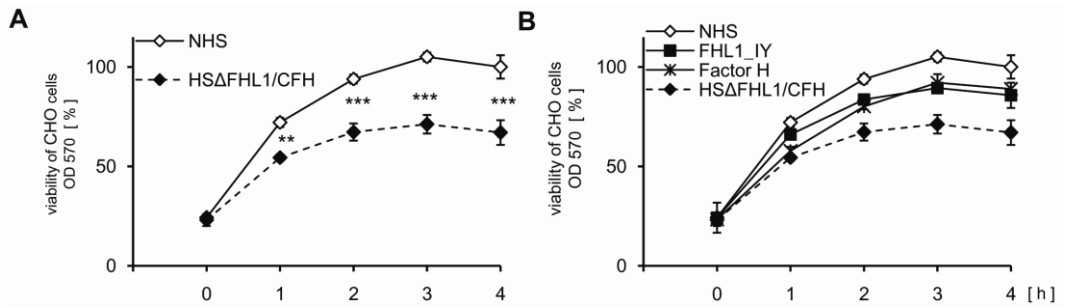
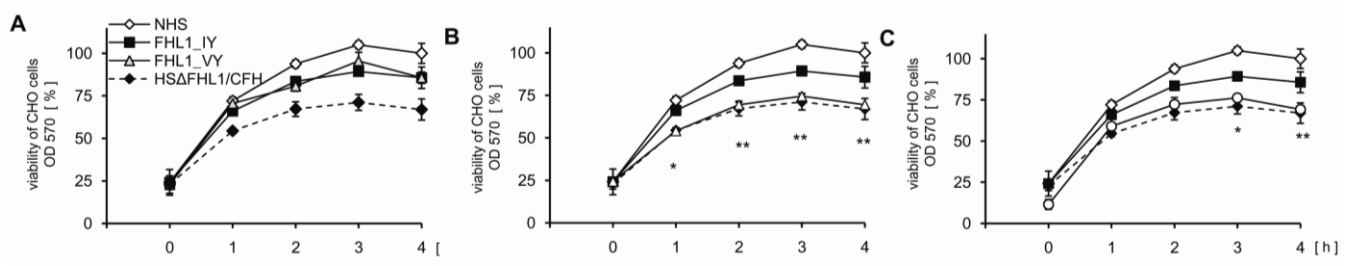
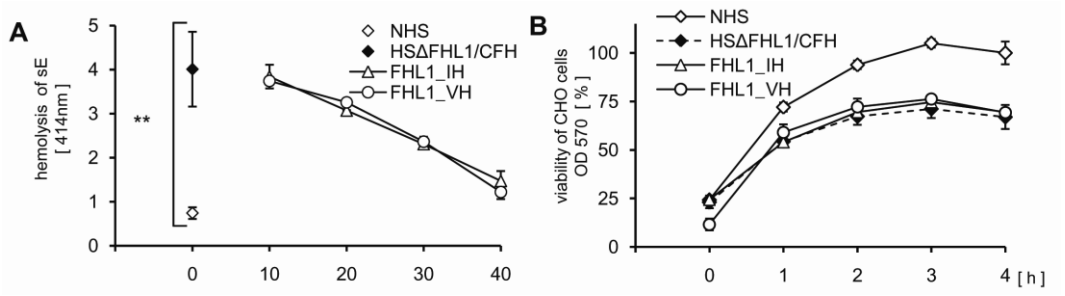


Figure 7



Supplementary Figure 1



3 Complement regulation at necrotic cell lesions is impaired by the AMD associated Factor H-H402 risk variant.

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COMPLEMENT REGULATION AT NECROTIC CELL LESIONS IS IMPAIRED BY THE AMD ASSOCIATED FACTOR H-H402 RISK VARIANT[1]

Running title: Y402H polymorphism affects complement regulation

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Age related macular degeneration a leading form of blindness in western countries is associated with a common SNP (rs 1061170/Y402H) in the Factor H gene, that encodes the two complement inhibitors Factor H and FHL1. However the functional consequences of this Y402H exchange in domain 7 are not precisely defined. Here we show that the Y402H sequence variation affects Factor H surface recruitment of Factor H by monomeric CRP (mCRP) to specific patches on the surface of necrotic retinal pigment epithelial cells. Enhanced attachment of the protective Y402 variants of both Factor H and FHL1 by mCRP results in more efficient complement control and further provides anti-inflammatory environment. In addition, we demonstrate that mCRP is generated on the surface of necrotic RPE cells and that this newly formed mCRP co-localizes with the cell damage marker annexin V. Bound to the cell surface Factor H – mCRP complexes allow complement inactivation and reduce the release of the pro-inflammatory cytokine TNF-α. This mCRP-mediated complement inhibitory- and anti-inflammatory activity at necrotic membrane lesions is affected by residue 402 of Factor H and defines a new role for mCRP, for Factor H and also for the mCRP-Factor H complex. The increased protective capacity of the Y402 Factor H variant allows better and more efficient clearance and removal of cellular debris, reduces inflammation and pathology.

Age related macular degeneration (AMD) is the leading cause of blindness in elderly population in Western societies. Approximately 20 million individuals in the United States and Europe suffer from this sight-threatening disease (1, 2). Late disease stages present in two severe forms that both result in central vision loss. Geographic atrophy is caused by atrophy of photoreceptors in the macular area that overlie degenerated retinal pigment epithelial cells (RPE). Choroidal neovascularisation develop due to the growth of new blood vessels into the retinal layer. A hallmark of AMD and usually one of the first clinical symptoms is the presence of ocular drusen (3). Proteomic and histochemical analyses show that these extracellular deposits contain complement components and inflammatory proteins (4-6).

During the last five years, mutations in several complement genes have been linked to AMD. Genetic polymorphisms in genes coding for Factor H (7-9), Factor B, C2 (10), C3 (11) as well as a 84kbp chromosomal deletion that results in the absence of the complement regulator CFHR1 and also of CFHR3 , significantly influence AMD pathogenesis (12-14). One prominent AMD-associated

polymorphism within the *Factor H gene* is a T → C substitution at nucleotide 1277 which results in a tyrosine (Y) to histidine (H) exchange at amino acid position 402. The Factor H risk variant H402 increases the risk for AMD about 2-4 fold for heterozygote and 5-7 fold for homozygote individuals (7-9).

The human *Factor H gene* encodes two proteins: Factor H itself and the Factor H-like protein (FHL1). Both plasma proteins are synthesized in the liver but are also expressed locally, e.g. in RPE cells (15). Factor H is composed of 20 consecutive protein domains, termed short consensus repeats (SCRs). FHL1, which is derived from an alternatively spliced transcript, represents the seven N-terminal SCRs of Factor H and has a unique C-terminal extension of four amino acids (16, 17). Both Factor H and FHL1 include the AMD relevant residue 402 in SCR 7 and are major regulators of the alternative complement pathway (18). The two proteins act as cofactors for Factor I mediated C3b inactivation and accelerate the decay of a preformed C3bBb convertase (19). Both regulators possess multiple binding sites for C3b, Heparin, C-reactive protein (CRP) and bind to cellular surfaces. In this context, SCR 7 binds to the ligands heparin, CRP and mediates cell surface binding (20, 21). The non-risk associated variant Y402 of Factor H and of FHL1 bind stronger to CRP as the H402 risk variants, suggesting a role of this AMD-associated polymorphism for ocular inflammation (15, 22-24).

CRP is an acute phase protein and the 125 kDa pentameric form (pCRP) is composed of five identical subunits, which are stabilized by calcium ions (25). pCRP is modified upon inflammation, surface attachment, oxidative stress, low pH, proteolytic cleavage, calcium depletion and *in-vitro* by heat or by urea treatment (26-28). Ultimately pCRP dissociates into units of 23 kDa, termed monomeric CRP (mCRP). We and others have recently shown that Factor H and FHL1 bind to mCRP, but not to pCRP and that mCRP recruits Factor H to the surface of apoptotic cells and apoptotic particles (29-31). Factor H attached to apoptotic surfaces controls complement progression, inhibits amplification of the complement cascade, enhances phagocytosis and has anti-inflammatory activity, e.g. by reducing release of the pro-inflammatory cytokine TNF-α (29).

The presence of CRP in drusen and ocular tissues, is indicative for chronic local inflammation within retinal layers (32). So far the two CRP isoforms were not discriminated in ocular tissues. However, individuals who are homozygote for the Factor H H402 risk variant show 2.5-fold higher CRP levels in the RPE-choroid layer, as compared to individuals homozygous for the non-risk associated Y402 variant (33). Persistent local inflammation near ocular drusen damages RPE cells and causes cell swelling, pigmentation and changes in organelle distribution (34, 35). Ultrastructural and histochemical analyses indicate, that during AMD RPE cells overlying drusen degenerate due to necrosis (6, 36). Rupture of the plasma membrane is a characteristic feature for necrotic cell death and is followed by release of cytoplasmatic contents in form of DNA, histones and other proteins which initiate and further amplify local inflammation within the retina and attract blood-derived macrophages (37, 38).

Here we localize pCRP and mCRP within retinal tissues and show that mCRP is generated from pCRP at distinct patches on necrotic cells and co-localizes with the cell damage marker annexin V. The non-risk associated Y402 variant of Factor H forms strong complexes with mCRP and is recruited by mCRP to these necrotic lesions. Recruited Factor H maintains complement regulatory activity, efficiently inactivates complement and blocks release of pro-inflammatory cytokine TNF-α. Reduced mCRP binding of the Factor H H402 risk variant results in complement activation, generation of inflammatory mediators, inflammation and finally in pathology. In conclusion, we define a physiological, anti-inflammatory function of mCRP on retinal cells and demonstrate that the reduced interaction of mCRP with the risk variant of Factor H (and also FHL1) has functional effects that can explain AMD pathology.

MATERIALS AND METHODS

Proteins and antibodies-

Serum-derived and recombinant human pCRP (Merck Biosciences, Schwalbach, Germany) was used to generate mCRP by urea treatment (29).

Goat and rabbit CRP antiserum and the monoclonal mCRP antibody (clone CRP-8) were purchased from Sigma–Aldrich (Taufkirchen, Germany), the monoclonal pCRP antibody (clone Mrz-12) from antibodies-online GmbH (Aachen, Germany). Purified human Factor H, Factor I, C3b and human Factor H antiserum were obtained from Comptech (Tyler, Texas, USA). AlexaFluor555, 488 or 647-conjugated anti-goat, anti-rabbit and anti-mouse IgG, were purchased from Invitrogen (Karlsruhe, Germany), the HRP-conjugated anti-goat IgG, anti-rabbit IgG, and anti-mouse IgG from Dako (Hamburg, Germany). All functional assays were performed in Tris-calcium buffer (TC buffer: 140 mM NaCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM Tris; pH 7.5) supplemented with 1 % BSA. Complement Factor H derived from genotyped AMD patients was purified from plasma, recombinant FHL1 was expressed in insect cells as described recently (15).

Patients-
Patients were of Caucasian origin and treated in the Departments of Ophthalmology, at the University of Bonn or the University Hospital of Würzburg. This study was approved by the ethical board of the Friedrich Schiller University Jena and the University Bonn. Genotyping of patients was performed as described by using genomic DNA isolated from blood leukocytes (15).

Cultivation of human retinal pigment epithelial cell lines
The immortalized human retinal pigment epithelial cell line ARPE-19 (ATCC number CRL-2302) was cultivated as monolayers in a 1:1 mixture of DMEM and Ham's F12 medium (ATCC) supplemented with 10 % FCS. Primary human retinal pigment epithelial cells isolated by ScienCell Research Laboratories from human retina were purchased from Innoprot (Derio, Spain, reference number P10873). Primary retinal cells were cultivated on poly-L-lysine coated flasks in complete epithelial cell medium according to the manufacturer's instructions (Innoprot). For detachment from their substratum both the immortalized and the primary cells were incubated with Accutase (PAA, Pasching, Austria) for 5 min at 37 °C. Necrosis of both cells was induced by incubating detached cells at 65 °C for 1 hr.

Enzyme-linked immunosorbent assay (ELISA)-
MaxiSorb microtiter plate wells (Nunc, Wiesbaden, Germany) were coated with the purified Factor H or FHL1 variants (5 µg / ml) over night at 4 °C, blocked and the binding of pCRP (10 µg / ml) and mCRP (0.1 – 10 µg / ml) was detected with polyclonal goat CRP antiserum and measured at 492 nm.

Flow Cytometry-
Necrotic ARPE-19 as well as necrotic primary RPE cells were incubated in 10 µg / ml of pCRP, mCRP, Factor H or FHL1 and binding of the different CRP isoforms was verified using a rabbit CRP antiserum followed by the appropriate Alexa488 labeled secondary antiserum. Binding of the Factor H or FHL1 variants in the presence or absence of either mCRP or pCRP was detected with Factor H antiserum and an Alexa488 labeled goat antiserum. In all experiments 10.000 cells were routinely counted in a BD LSRII flow cytometer and analyzed with the FACSDiva (BD Biosciences, Heidelberg, Germany) and FlowJo software (Tree Star, Ashland, Oregon, USA). Living intact cells were identified as propidium iodide negative cells and necrotic cells were identified as propidium iodide positive cells.

Confocal Microscopy-
Surface binding of 10 µg / ml pCRP, mCRP, Factor H or FHL1 to necrotic ARPE-19 as well as necrotic primary RPE cells was also visualized by confocal microscopy. Factor H and FHL1 were detected with polyclonal Factor H antiserum followed by an Alexa647 labeled goat antiserum. The CRP isoforms were localized with monoclonal antibodies to mCRP (CRP-8) or pCRP (Mrz-12) respectively, followed by Alexa488 coupled mouse antiserum. Co-localization of mCRP and Factor H was assayed by incubating the cells first with mCRP followed by Factor H. After washing monoclonal mCRP or polyclonal Factor H antiserum was added, followed by the corresponding Alexa488 or Alexa555 labeled antibodies. Cells were further stained with DAPI (Sigma-Aldrich) or annexin V labeled with allophycocyanin (APC, BD Biosciences) and examined with a laser scanning microscope LSM 510 META (Zeiss, Jena, Germany).

mCRP generation on the necrotic ARPE-19 surface-
Conversion of 15 µg / ml pCRP attached to the surface of necrotic ARPE-19 cells was followed over 18 hrs. At the indicated time points (0.5, 2, 3, 4, and 18 hrs) cells were washed and stained with the specific monoclonal antibody CRP-8. Addition of the Alexa488 conjugated mouse antiserum identified generated mCRP by flow cytometry and by confocal microscopy.

Cofactor assay-
mCRP was bound with different amounts (0.5 - 1.5 µg/ml) to necrotic retinal pigment epithelial cells, then either non-risk or risk Factor H or FHL1 variants were added, followed by a 15 min incubation of C3b and Factor I. The deposition of iC3b on the necrotic cell surface was assayed by flow cytometry using a monoclonal iC3b antibody (Quidel, San Diego, USA) and an Alexa488 labeled secondary mouse antiserum.

Immunohistochemistry-
Immunohistochemistry was performed on ocular tissues derived from two normal human donor eyes (79 and 85 years, 2 female) and two eyes with a history of clinically documented early AMD (81 and 87 years, 2 female). The donor eyes were obtained at autopsy and were processed less than 15 hours after death. Posterior eye poles were embedded in optimal cutting temperature compound and frozen in liquid nitrogen-cooled isopentane. Cryostat-cut sections (6 µm) were fixed in cold acetone, blocked and incubated with monoclonal antibodies to mCRP (CRP-8) or pCRP (Mrz-12) respectively, followed by Alexa488-conjugated secondary antiserum (Molecular Probes, Eugene OR). Nuclear counterstaining was performed with propidium iodide or with DAPI (Sigma-Aldrich). Immunolocalization of mCRP and pCRP to Bruch's membrane was confirmed by double labeling experiments with a polyclonal antibody against human aortic elastin (Elastin Products Company, Owensville, MO) followed by Alexa555-conjugated secondary antiserum. In negative control samples, the primary antibody was replaced by PBS or equimolar concentrations of nonimmune mouse IgG.

Cytokine Release-
THP1 monocytes were stimulated for 24 h with phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich) to induce a macrophage phenotype (29). Necrotic ARPE-19 cells incubated with the Factor H variants in the presence or absence of 1.5 µg/ml mCRP. After co-incubation for 20 h, the culture supernatant was collected and TNF-α content was determined using high sensitivity human ELISA Set (Immuno Tools, Friesoythe, Germany).

Peptide spot analysis-
Peptides that represent the SCR 7 region of complement Factor H / FHL1 were synthesized with a length of 12 and an overlap of ten amino acids and coupled to a cellulose membrane (MultiPep, Intavis Köln, Germany). After blocking, the membrane was incubated with 1 µg/ml mCRP for 2 hrs at room temperature. Bound mCRP was detected with monoclonal CRP-8 antibody followed by a HRP-labeled mouse antiserum. A second membrane treated in the same manner except that mCRP was absent was used as a negative control.

Structural modeling of SCR 7-
The published structure of SCR 7 (protein databank (PDB), www.rcsb.org, code 2jgx) was used to locate the mCRP binding amino acids. The molecular graphic was created using the UCSF Chimera software (http://www.cgl.ucsf.edu/chimera).

Statistical analysis-
PRISM GraphPad software was used for statistical analysis. Significant differences between two groups were analyzed by the unpaired Student's t-test. The results were considered statistically significant at a P value of ≤0.05 (*), ≤0.01 (**), or ≤0.001 (***).

RESULTS

mCRP binding of Factor H is affected by the Y402H variation-
To assay the effect of the Factor H Y402H variation on mCRP interaction, binding of pCRP or mCRP to the two immobilized Factor H variants was assayed by ELISA. Factor H, purified from plasma of genotyped AMD patients is free of contaminants as confirmed by SDS-PAGE and silver staining (Supplementary Figure 1A). The quality of mCRP-generated from pCRP was characterized by ELISA based on the reactivity with monoclonal antibodies

specific for either pCRP or for mCRP (Supplementary Figure 1B). mCRP, but not pCRP, bound dose-dependently to Factor H (Figure 1A). mCRP binding to the non-risk associated variant was about 35 - 45 % stronger as compared to the risk variant. This effect is statistically significant for mCRP concentrations of 5 and 10 µg / ml ($p<0.01$). Factor H – mCRP binding is specific as mCRP did not bind to immobilized BSA.

SCR 7 has two linear binding regions for mCRP-

To identify residues within SCR 7 that contact mCRP, linear peptides with a length of twelve and an overlap of ten residues were synthesized and spotted onto a membrane. This membrane was then incubated with mCRP and bound mCRP was detected (Figure 1B). mCRP bound to several peptides which form two motives: motive I is represented by 399-NQN**Y**GR**KFVQGK**SIDVAC-416 (overlapping residues of all peptides representing core motive I are underlined; the protective residue Y402 is highlighted). Motive II comprises 413-DVACHP**GYALPK**AQTTVT-430, (again core residues are underlined) (Figure 1C). Residues KFVQGK of motive I are directly adjacent to residue 402 and both core residues are surfaced exposed as revealed by molecular modeling (Figure 1D).

pCRP and mCRP are present in retinal tissues and located in drusen-

In order to analyze whether pCRP or mCRP are present *in-vivo*, immunohistochemistry was performed using retinal tissues from healthy individuals and from AMD patients. In tissues derived from healthy individuals, pCRP and mCRP reactivity was observed at low levels within the Bruch's membrane (Figure 2A, 2C, arrows). In tissues derived from AMD patients, pCRP and mCRP showed stronger staining in the Bruch's membrane and also in drusen (Figure 2B, 2D, arrows).

In order to localize pCRP reactivity at the Bruch's membrane co-staining with pCRP antibody and an elastin recognizing antiserum were performed. pCRP reactivity was identified at the Bruch's membrane (Figure 3A, image I: green fluorescence). Staining with the elastin specific antiserum (image II: red fluorescence) identified the layer of the Bruch's membrane. A merge of the two images showed costaining of the two markers as revealed by the yellow fluorescence (image III). To further confirm specificity of staining and to discriminate the specific fluorescence signal from autofluorescence sequential tissue sections were analyzed (Figure 3B). Sequential staining for pCRP with autofluorescence in sequential tissue sections revealed specific staining for pCRP in the Bruch's membrane and in drusen. Autofluorescence was identified in the Bruch's membrane. A merge of the two images allowed to discriminate specific pCRP reactivity from autofluorescence (Figure 3B, image III). The pCRP signal was also different from the autofluorescence of RPE cells (Figure 3A and 3B). In addition to provide additional proof that mCRP staining occurs in the Bruch's membrane sequential tissue sections were stained for mCRP and for elastin, as a component of the Bruch's membrane (Figure 3C, images I-IV).

Binding of Factor H, pCRP and mCRP to the surface of necrotic pigment epithelial cells-

To address whether the 402 sequence variation affects attachment of single proteins to damaged cellular surfaces, binding of both Factor H variants and also of the two CRP isoforms to necrotic ARPE-19 as well as primary RPE cells was assayed by flow cytometry. As single molecules each Factor H variant and also the two CRP isoforms bound to the surface of necrotic ARPE-19 (Figure 4A, 4B) and necrotic primary RPE cells with similar intensities (Figure 4F, 4G).

Confocal microscopy was used to localize bound proteins at the surface of necrotic cells (Figure 4C-E, Figure 4H-J). Factor H and pCRP were equally distributed over the surface of necrotic ARPE-19 (Figure 4C, 4D) and primary RPE cells (Figure 4H, 4I) and the risk and non-risk Factor H variants bound with equal intensities (data not shown). However, mCRP bound to specific sites of the necrotic retinal cells (Figure 4E, Figure 4J). The distribution of pCRP over the entire cell surface and the specific surface binding of mCRP was confirmed by three-dimensional imaging (Supplementary Figure 2).

Dissociation of pCRP into mCRP on the surface of necrotic ARPE-19 cells-
Prominent binding of mCRP to necrotic lesions suggested that mCRP is generated at sites of severe membrane damage. Therefore, pCRP was bound to the surface of necrotic ARPE-19 cells and mCRP generation was followed over time by flow cytometry using the mCRP specific mAb CRP-8. After binding pCRP to the surface of necrotic ARPE-19 cells, mCRP was already detectable after 0.5 hrs (Figure 5A). mCRP reactivity increased with time and after 18 hrs mCRP levels were significantly increased from background ($p \leq 0.007$). On the surface of necrotic cells gradual increase of mCRP reactivity over time was also confirmed by confocal microscopy (Figure 5B-G). With this method mCRP was detected after 2 hrs (Figure 4D) and again staining increased with time (Figure 5E-G).

mCRP-mediated surface recruitment of Factor H is affected by the Y402H variation-
As both Factor H variants bound equally to necrotic surfaces, but mCRP preferentially binds the non-risk variant of Factor H, we asked if the Y402H variation may affect mCRP - mediated surface recruitment of Factor H to retinal necrotic cells. mCRP enhanced surface recruitment of both Factor H variants (Figure 6A, compare last column of each triplet with first column) to ARPE-19 cells. However, binding of the non-risk associated Factor H variant was enhanced by about 115 % ($p \leq 0.0009$) and binding of the risk variant was increased by approximately 50 %. pCRP did not affect Factor H surface binding (Figure 6A, compare second with first column of each group).

mCRP - assisted recruitment of Factor H to the necrotic surface was visualized by confocal microscopy. To this end mCRP was bound to the surface of necrotic ARPE-19 cells and binding of the non-risk associated variant was analyzed (Figure 6B). mCRP recruited Factor H and formed a prominent patchy pattern at the surface of necrotic cells. This 'spot-like' staining in the presence of mCRP is clearly distinct from the even surface distribution observed for Factor H in the absence of mCRP (compare Figure 6B with Figure 4C). The staining pattern in the presence of mCRP suggests that mCRP recruits Factor H to specific sites on a necrotic cell. A very similar but not as pronounced effect was observed for necrotic primary RPE cells (Figure 6C and 6D). In this case mCRP enhanced surface recruitment of the protective Factor H variant by about 45 % ($p \leq 0.0057$) and the binding of the risk variant by 14 %. Again pCRP had no significant effect on Factor H surface recruitment (Figure 6 C). mCRP - mediated Factor H recruitment occurred also on surface of necrotic primary cells to a distinct spot of the cell (Figure 6D).

mCRP recruits Factor H to sites of severe membrane damage-
The binding of Factor H – mCRP complexes to specific surface spots, suggested that these patches represent sites of severe membrane damage. Therefore co-localization with annexin V, a marker for membrane damage, was assayed. Annexin V was distributed over the surface of necrotic ARPE-19 cells, and showed a more intense staining at specific patches (Figure 6E, red). Factor H – mCRP complexes bound preferentially to these specific patches (Figure 6F, mCRP green and Figure 6G, Factor H turquoise) and co-localized with annexin V (Figure 6H). Colocalization of mCRP and Factor H with annexin V and a similar surface distribution was also observed for necrotic primary RPE cells (data not shown). Formation of such prominent necrotic surface patches that bind annexin V and also Factor H - mCRP complexes is a new observation and has -to our knowledge- not been reported before. We hypothesize that these sites of severe membrane alterations represent damaged, possibly ruptured membranes that release cytoplasmic content. Based on the specific binding properties and the selective recruitment of markers for cell damage and of the Factor H– mCRP complexes we propose to term these sites: **u**rgent **r**egulation of **d**amage (URD) sites.

mCRP - assisted complement regulatory effects are influenced by the Y402H variation-
Necrotic ARPE-19 cells lack membrane bound complement regulators (Supplementary Figure 3). To allow efficient opsonisation of the surface by iC3b, these altered self surfaces attach fluid phase regulators such as Factor H. As mCRP enhances C3b cofactor activity of Factor H (29), we asked if local complement regulation on such necrotic cells is mediated by Factor H – mCRP complexes and if this activity is affected by the

Y402H polymorphism. mCRP enhanced Factor H - mediated iC3b deposition in a dose-dependent manner (Figure 7A). This effect was stronger for the non-risk variant. The functional difference of 35 % (at 1.5 µg / ml mCRP) between the non-risk and risk variants was statistically significant ($p \leq 0.026$). The same difference in iC3b inactivation was observed for Factor H – mCRP complexes in fluid phase (Supplementary Figure 4A, 4B). Similar results were observed in the presence of primary RPE cells. Again, mCRP enhanced Factor H cofactor activity and the two Factor H variants showed a functional difference of 17 % at the highest mCRP concentrations. However, the effect was not as prominent as for the ARPE-19 cell line (data not shown).

mCRP - mediated reduction of TNF-α release is affected by the Y402H variation-

Factor H displays anti-inflammatory activity on apoptotic particles. Consequently we asked: (i) if this effect also applies for necrotic retinal cells, (ii) if mCRP enhances this anti-inflammatory effect and (iii) if the effect is influenced by the Y402H variation. Therefore necrotic ARPE-19 cells were coated with one of the two Factor H variants and mCRP. These coated cells were then added to PMA - activated human macrophages to allow phagocytosis. After 20 h incubation release of the proinflammatory marker TNF-α was assayed in the supernatant. Necrotic ARPE-19 cells induced TNF-α release (Figure 7B column 4). Coating the cell surface with mCRP alone did not affect TNF-α secretion (column 5). However when the cells were coated with the Factor H – mCRP complexes TNF-α release was reduced. The protective Y402 Factor H variant reduced TNF-α release more efficiently (35 %, column 7) as compared to the H402 risk variant (1 %, column 9).

DISCUSSION

A tyrosine to histidine exchange at residue 402 of the complement regulator Factor H and of FHL1 increases the risk for AMD (7-9). Here we show how this polymorphism affects local complement control as well as anti-inflammatory processes on necrotic retinal cells. Reduced binding and recruitment of the risk variants by mCRP to specific necrotic cell lesions leads to functional differences that can develop into AMD. Necrotic cells lack membrane bound regulators and therefore require surface attached soluble inhibitors to allow opsonisation with iC3b, to inhibit progression of complement beyond the C3 convertase step and to allow efficient and non-inflammatory clearance. The consequences of the Y402H variation were proven here for the established immortalized cell line ARPE-19 as well as on the surface of primary RPE cells. On primary cells mCRP showed indeed the same characteristics than on ARPE-19 cells but the effects were not as pronounced. Most likely because of their origin primary cells are more sensitive to the applied necrotic procedures.

The role of the Y402H variation was also confirmed for FHL1, the second product of the *Factor H gene*. The non-risk and risk associated variants of FHL1 showed similar differences in mCRP - directed effects (Supplementary Figure 5A), mCRP - assisted recruitment to the surface of necrotic cells (Supplementary Figure 5B-D); mCRP - assisted binding to URD sites (Supplementary Figure 5E-I) and modified mCRP - mediated complement regulatory activities (Supplementary Figure 5J and 5K). Thus a second regulatory protein derived from the Factor H gene exists, whose function is impaired due to the Y402H variation. Factor H and FHL1 may act individually or in concert. However the 402 sequence variation influence mCRP binding, resulting in inappropriate complement control on the necrotic surface and mediates local inflammatory processes.

Currently, a specific physiological role for mCRP is emerging (29, 39). Here we identify both CRP isoforms in ocular tissues of AMD patients or healthy individuals (Figure 2, Figure 3). Enhanced mCRP staining in AMD tissues at the Bruch's membrane and also in drusen (Figure 2D) supports the relevance of mCRP *in-vivo* and particularly in AMD pathogenesis. mCRP is generated from pCRP on the surface of necrotic ARPE-19 cells (Figure 5). Similarly, mCRP generation on the surface of activated platelets was recently reported (28).

A mCRP binding region in SCR 7 which contains relevant residue 402 is described for Factor H and also for FHL1 (29). Residue 402 is surface exposed and affects the intensity of

mCRP interaction (Figure 1A-D) (15). Here we identify within SCR 7 two linear mCRP binding motives, that are located either in direct (motive I) or in close vicinity (motive II) to residue 402 (Figure 1D). When compared to the risk variants, the non-risk associated variants of Factor H and FHL1 bind mCRP with 40 -50 % higher intensity (Figure 1A and Supplementary Figure 5A). This difference influences mCRP - assisted surface recruitment to necrotic retinal pigment epithelial cells (Figure 6A, 6C and Supplementary Figure 5D) and ultimately affects local complement regulation and release of the pro-inflammatory cytokine TNF-α (Figure 7A, 7B, Supplementary Figure 5J).

AMD is an inflammatory process (5) and is associated with necrosis of RPE cells that overlie drusen (6, 36). Progression of cellular necrosis results in morphological changes, loss of membrane integrity, membrane rupture and release of cytoplasmic content(38). When not properly controlled and removed in a silent non-inflammatory manner, necrotic material released from damaged cells, activates complement and initiates local inflammation (34). It is of interested that histones, that are released during necrosis were also identified in drusen (4). Necrotic ARPE-19 cells lack membrane bound complement regulators, as demonstrated for CD46, CD55 and the terminal complement complex regulator CD59 (Supplementary Figure 3). Consequently, a necrotic surface will activate complement. Complement activation to the level of the C3 convertase allows opsonization of the surface with C3b/iC3b. This enhances phagocytosis and efficient removal of cellular debris. However progression of the cascade beyond the C3 convertase, generates the potent inflammatory marker C5a, allows terminal complement complex formation, causes inflammation and is therefore unfavorable. This scenario explains the need for surface acquired regulators such as Factor H and FHL1 to block complement activation and to allow silent, non-inflammatory removal of cellular debris (40, 41).

Factor H, FHL1, pCRP and mCRP bind to the surface of necrotic retinal pigment epithelial cells. Factor H (Figure 4C, 4H), FHL1 (Supplementary Figure 5C) and also pCRP (Figure 4D, 4I) are evenly distributed over the surface of necrotic cells. However, mCRP locates to specific patched (Figure 4E, 4J). Necrotic retinal pigment epithelial cells expose altered self epitopes, such as phosphatidyl serine (42), that bind annexin V and provide specific binding sites for mCRP (Figure 6E, Supplementary Figure 5F). Based on the unique staining, requirement for complement control and due to the unique binding features we propose to term these patches **u**rgent **r**egulation of **d**amage (URD) sites. mCRP - assisted recruitment of Factor H and of FHL1 to URDs (Figure 6A, 6C, Supplementary Figure 5D) is affected by the tyrosine to histidine exchange at position 402. The reduced binding of the risk variant results in lower complement regulation and accumulation of debris. Thus AMD patients with the risk allele at position 402 have a more "pro-inflammatory" intraocular environment than those who carry non-risk allele. Over time functional differences as outline here, extrapolate into more complement activation, increased damage and thus into pathology.

In summary, the Y402H polymorphisms of Factor H and FHL1 impair the mCRP - assisted complement control at necrotic surfaces (Supplementary Figure 6). The crucial differences between the risk and non-risk associated variants affect: (i) binding to the anti-inflammatory marker mCRP, (ii) mCRP - assisted recruitment to necrotic membrane lesions called URD sites, (iii) local complement inhibition at the level of the C3 convertase, as well as (iv) the release of the inflammatory cytokine TNF-α by human macrophages. Necrotic retinal epithelial cells bind the inflammatory marker pCRP and -at URD sites- the attached pCRP is dissociated into mCRP. Restriction of complement inactivation and anti inflammatory processes cause accumulation of damaged material or debris, and consequently translates into pathology of retinal tissue. Interfering and controlling these processes by complement based anti-inflammatory intervention will ultimately provide a promising approach for AMD therapy.

REFERENCES

1. Pascolini, D., S. P. Mariotti, G. P. Pokharel, R. Pararajasegaram, D. Etya'ale, A. D. Negrel, and S. Resnikoff. 2004. 2002 global update of available data on visual impairment: a compilation of population-based prevalence studies. *Ophthalmic Epidemiol* 11:67-115.
2. Congdon, N., B. O'Colmain, C. C. Klaver, R. Klein, B. Munoz, D. S. Friedman, J. Kempen, H. R. Taylor, and P. Mitchell. 2004. Causes and prevalence of visual impairment among adults in the United States. *Arch Ophthalmol* 122:477-485.
3. Jager, R. D., W. F. Mieler, and J. W. Miller. 2008. Age-related macular degeneration. *N Engl J Med* 358:2606-2617.
4. Crabb, J. W., M. Miyagi, X. Gu, K. Shadrach, K. A. West, H. Sakaguchi, M. Kamei, A. Hasan, L. Yan, M. E. Rayborn, R. G. Salomon, and J. G. Hollyfield. 2002. Drusen proteome analysis: an approach to the etiology of age-related macular degeneration. *Proc Natl Acad Sci U S A* 99:14682-14687.
5. Johnson, L. V., W. P. Leitner, M. K. Staples, and D. H. Anderson. 2001. Complement activation and inflammatory processes in Drusen formation and age related macular degeneration. *Exp Eye Res* 73:887-896.
6. Hageman, G. S., P. J. Luthert, N. H. Victor Chong, L. V. Johnson, D. H. Anderson, and R. F. Mullins. 2001. An integrated hypothesis that considers drusen as biomarkers of immune-mediated processes at the RPE-Bruch's membrane interface in aging and age-related macular degeneration. *Prog Retin Eye Res* 20:705-732.
7. Edwards, A. O., R. Ritter, 3rd, K. J. Abel, A. Manning, C. Panhuysen, and L. A. Farrer. 2005. Complement factor H polymorphism and age-related macular degeneration. *Science* 308:421-424.
8. Haines, J. L., M. A. Hauser, S. Schmidt, W. K. Scott, L. M. Olson, P. Gallins, K. L. Spencer, S. Y. Kwan, M. Noureddine, J. R. Gilbert, N. Schnetz-Boutaud, A. Agarwal, E. A. Postel, and M. A. Pericak-Vance. 2005. Complement factor H variant increases the risk of age-related macular degeneration. *Science* 308:419-421.
9. Klein, R. J., C. Zeiss, E. Y. Chew, J. Y. Tsai, R. S. Sackler, C. Haynes, A. K. Henning, J. P. SanGiovanni, S. M. Mane, S. T. Mayne, M. B. Bracken, F. L. Ferris, J. Ott, C. Barnstable, and J. Hoh. 2005. Complement factor H polymorphism in age-related macular degeneration. *Science* 308:385-389.
10. Gold, B., J. E. Merriam, J. Zernant, L. S. Hancox, A. J. Taiber, K. Gehrs, K. Cramer, J. Neel, J. Bergeron, G. R. Barile, R. T. Smith, G. S. Hageman, M. Dean, and R. Allikmets. 2006. Variation in factor B (BF) and complement component 2 (C2) genes is associated with age-related macular degeneration. *Nat Genet* 38:458-462.
11. Yates, J. R., T. Sepp, B. K. Matharu, J. C. Khan, D. A. Thurlby, H. Shahid, D. G. Clayton, C. Hayward, J. Morgan, A. F. Wright, A. M. Armbrecht, B. Dhillon, I. J. Deary, E. Redmond, A. C. Bird, and A. T. Moore. 2007. Complement C3 variant and the risk of age-related macular degeneration. *N Engl J Med* 357:553-561.
12. Hughes, A. E., N. Orr, H. Esfandiary, M. Diaz-Torres, T. Goodship, and U. Chakravarthy. 2006. A common CFH haplotype, with deletion of CFHR1 and CFHR3, is associated with lower risk of age-related macular degeneration. *Nat Genet* 38:1173-1177.
13. Heinen, S., A. Hartmann, N. Lauer, U. Wiehl, H. M. Dahse, S. Schirmer, K. Gropp, T. Enghardt, R. Wallich, S. Halbich, M. Mihlan, U. Schlotzer-Schrehardt, P. F. Zipfel, and C. Skerka. 2009. Factor H-related protein 1 (CFHR-1) inhibits complement C5 convertase activity and terminal complex formation. *Blood* 114:2439-2447.
14. Fritsche, L. G., N. Lauer, A. Hartmann, S. Stippa, C. N. Keilhauer, M. Oppermann, M. K. Pandey, J. Kohl, P. F. Zipfel, B. H. Weber, and C. Skerka. An imbalance of human complement regulatory proteins CFHR1, CFHR3 and factor H influences risk for age-related macular degeneration (AMD). *Hum Mol Genet*.
15. Skerka, C., N. Lauer, A. A. Weinberger, C. N. Keilhauer, J. Suhnel, R. Smith, U. Schlotzer-Schrehardt, L. Fritsche, S. Heinen, A. Hartmann, B. H. Weber, and P. F. Zipfel. 2007.

Defective complement control of factor H (Y402H) and FHL-1 in age-related macular degeneration. *Mol Immunol* 44:3398-3406.

16. Misasi, R., H. P. Huemer, W. Schwaeble, E. Solder, C. Larcher, and M. P. Dierich. 1989. Human complement factor H: an additional gene product of 43 kDa isolated from human plasma shows cofactor activity for the cleavage of the third component of complement. *Eur J Immunol* 19:1765-1768.
17. Hellwage, J., S. Kuhn, and P. F. Zipfel. 1997. The human complement regulatory factor-H-like protein 1, which represents a truncated form of factor H, displays cell-attachment activity. *Biochem J* 326 (Pt 2):321-327.
18. Kuhn, S., C. Skerka, and P. F. Zipfel. 1995. Mapping of the complement regulatory domains in the human factor H-like protein 1 and in factor H1. *J Immunol* 155:5663-5670.
19. Kuhn, S., and P. F. Zipfel. 1996. Mapping of the domains required for decay acceleration activity of the human factor H-like protein 1 and factor H. *Eur J Immunol* 26:2383-2387.
20. Giannakis, E., T. S. Jokiranta, D. A. Male, S. Ranganathan, R. J. Ormsby, V. A. Fischetti, C. Mold, and D. L. Gordon. 2003. A common site within factor H SCR 7 responsible for binding heparin, C-reactive protein and streptococcal M protein. *Eur J Immunol* 33:962-969.
21. Okemefuna, A. I., R. Nan, A. Miller, J. Gor, and S. J. Perkins. Complement factor H binds at two independent sites to C-reactive protein in acute phase concentrations. *J Biol Chem* 285:1053-1065.
22. Laine, M., H. Jarva, S. Seitsonen, K. Haapasalo, M. J. Lehtinen, N. Lindeman, D. H. Anderson, P. T. Johnson, I. Jarvela, T. S. Jokiranta, G. S. Hageman, I. Immonen, and S. Meri. 2007. Y402H polymorphism of complement factor H affects binding affinity to C-reactive protein. *J Immunol* 178:3831-3836.
23. Ormsby, R. J., S. Ranganathan, J. C. Tong, K. M. Griggs, D. P. Dimasi, A. W. Hewitt, K. P. Burdon, J. E. Craig, J. Hoh, and D. L. Gordon. 2008. Functional and structural implications of the complement factor H Y402H polymorphism associated with age-related macular degeneration. *Invest Ophthalmol Vis Sci* 49:1763-1770.
24. Sjoberg, A. P., L. A. Trouw, S. J. Clark, J. Sjolander, D. Heinegard, R. B. Sim, A. J. Day, and A. M. Blom. 2007. The factor H variant associated with age-related macular degeneration (His-384) and the non-disease-associated form bind differentially to C-reactive protein, fibromodulin, DNA, and necrotic cells. *J Biol Chem* 282:10894-10900.
25. Volanakis, J. E. 2001. Human C-reactive protein: expression, structure, and function. *Mol Immunol* 38:189-197.
26. Potempa, L. A., B. A. Maldonado, P. Laurent, E. S. Zemel, and H. Gewurz. 1983. Antigenic, electrophoretic and binding alterations of human C-reactive protein modified selectively in the absence of calcium. *Mol Immunol* 20:1165-1175.
27. Ji, S. R., Y. Wu, L. Zhu, L. A. Potempa, F. L. Sheng, W. Lu, and J. Zhao. 2007. Cell membranes and liposomes dissociate C-reactive protein (CRP) to form a new, biologically active structural intermediate: mCRP(m). *Faseb J* 21:284-294.
28. Eisenhardt, S. U., J. Habersberger, A. Murphy, Y. C. Chen, K. J. Woollard, N. Bassler, H. Qian, C. von Zur Muhlen, C. E. Hagemeyer, I. Ahrens, J. Chin-Dusting, A. Bobik, and K. Peter. 2009. Dissociation of pentameric to monomeric C-reactive protein on activated platelets localizes inflammation to atherosclerotic plaques. *Circ Res* 105:128-137.
29. Mihlan, M., S. Stippa, M. Jozsi, and P. F. Zipfel. 2009. Monomeric CRP contributes to complement control in fluid phase and on cellular surfaces and increases phagocytosis by recruiting factor H. *Cell Death Differ* 16:1630-1640.
30. Biro, A., Z. Rovo, D. Papp, L. Cervenak, L. Varga, G. Fust, N. M. Thielens, G. J. Arlaud, and Z. Prohaszka. 2007. Studies on the interactions between C-reactive protein and complement proteins. *Immunology* 121:40-50.
31. Hakobyan, S., C. L. Harris, C. W. van den Berg, M. C. Fernandez-Alonso, E. G. de Jorge, S. R. de Cordoba, G. Rivas, P. Mangione, M. B. Pepys, and B. P. Morgan. 2008. Complement factor H binds to denatured rather than to native pentameric C-reactive protein. *J Biol Chem* 283:30451-30460.

10

32. Mullins, R. F., S. R. Russell, D. H. Anderson, and G. S. Hageman. 2000. Drusen associated with aging and age-related macular degeneration contain proteins common to extracellular deposits associated with atherosclerosis, elastosis, amyloidosis, and dense deposit disease. *Faseb J* 14:835-846.
33. Johnson, P. T., K. E. Betts, M. J. Radeke, G. S. Hageman, D. H. Anderson, and L. V. Johnson. 2006. Individuals homozygous for the age-related macular degeneration risk-conferring variant of complement factor H have elevated levels of CRP in the choroid. *Proc Natl Acad Sci U S A* 103:17456-17461.
34. Anderson, D. H., R. F. Mullins, G. S. Hageman, and L. V. Johnson. 2002. A role for local inflammation in the formation of drusen in the aging eye. *Am J Ophthalmol* 134:411-431.
35. Thurman, J. M., B. Renner, K. Kunchithapautham, V. P. Ferreira, M. K. Pangburn, Z. Ablonczy, S. Tomlinson, V. M. Holers, and B. Rohrer. 2009. Oxidative stress renders retinal pigment epithelial cells susceptible to complement-mediated injury. *J Biol Chem* 284:16939-16947.
36. Farkas, T. G., V. Sylvester, and D. Archer. 1971. The ultrastructure of drusen. *Am J Ophthalmol* 71:1196-1205.
37. Caicedo, A., D. G. Espinosa-Heidmann, Y. Pina, E. P. Hernandez, and S. W. Cousins. 2005. Blood-derived macrophages infiltrate the retina and activate Muller glial cells under experimental choroidal neovascularization. *Exp Eye Res* 81:38-47.
38. Hotchkiss, R. S., A. Strasser, J. E. McDunn, and P. E. Swanson. 2009. Cell death. *N Engl J Med* 361:1570-1583.
39. Ji, S. R., Y. Wu, L. A. Potempa, Y. H. Liang, and J. Zhao. 2006. Effect of modified C-reactive protein on complement activation: a possible complement regulatory role of modified or monomeric C-reactive protein in atherosclerotic lesions. *Arterioscler Thromb Vasc Biol* 26:935-941.
40. Sohn, J. H., H. J. Kaplan, H. J. Suk, P. S. Bora, and N. S. Bora. 2000. Chronic low level complement activation within the eye is controlled by intraocular complement regulatory proteins. *Invest Ophthalmol Vis Sci* 41:3492-3502.
41. Zipfel, P. F., and C. Skerka. 2009. Complement regulators and inhibitory proteins. *Nat Rev Immunol* 9:729-740.
42. Krysko, O., L. De Ridder, and M. Cornelissen. 2004. Phosphatidylserine exposure during early primary necrosis (oncosis) in JB6 cells as evidenced by immunogold labeling technique. *Apoptosis* 9:495-500.

FOOTNOTES

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[3]Abbreviations
AMD, age-related macular degeneration; APC, allophycocyanin; ARPE-19, immortalized retinal pigment epithelial cell line; CRP, C-reactive protein; FHL1, Factor H-like protein; mCRP, monomeric CRP; pCRP, pentameric CRP; RPE, retinal pigment epithelial cells; SCR, short consensus repeat; URD, urgent regulation of damage

FIGURE LEGENDS

Figure 1: mCRP, but not pCRP, binds to Factor H by interaction with two linear mCRP binding motives within SCR 7.
A) Factor H variants purified from genotyped AMD patients were immobilized on a microtiter plate and pCRP and mCRP were applied in fluid phase and detected with a polyclonal CRP antiserum. mCRP, but not pCRP, bound both Factor H variants in a dose-dependent manner. mCRP bound up to 35 % - 45 % stronger to the non-risk associated Factor H variant (filled squares, solid line) as compared to the risk variant (open squares, dashed line). mCRP did not bind to BSA which was used a control (dotted line). The figure show mean values of a triplicate assay of one representative experiment. The experiment was repeated three times with similar results. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. The Greek letter Δ denotes the percent difference between the corresponding OD values for mCRP binding the presence of either Factor H Y402 or Factor H H402. **B)** Amino acids within SCR 7 that mediate linear binding motives for mCRP were identified by peptide spot analysis. The cellulose membrane was incubated with purified mCRP and bound mCRP was detected with the monoclonal CRP-8 antibody. mCRP bound to two regions each represented by four consecutive spots and are indicated by black squares. One representative membrane of two independent experiments each is shown. **C)** Each binding region represents a linear sequence of 18 residues that are highlighted by a grey background in the selected sequence of SCR 7. Overlapping residues that are present in each peptide are shown in blue. The AMD associated tyrosine at position 402 is colored green. **D)** The identified core sequences of the two identified linear mCRP binding motives (blue) are surface exposed and close to the relevant 402 residue (green) as revealed by molecular modeling.

Figure 2: pCRP and mCRP are present in retinal tissues of healthy individuals and AMD patients.
Tissues derived from two human donor eyes with no and with clinically documented AMD were fixed in cold acetone, blocked and incubated in a monoclonal mouse antibody to pCRP (MRz-12) or mCRP (CRP-8) respectively overnight at 4 °C. Bound antibodies were visualized using an Alexa488-conjugated secondary antiserum (green). Nuclear counterstaining was performed with propidium iodide (red) **A)** In the tissue derived from a healthy individual pCRP is detected at the Bruch's membrane (arrows) **B)** In AMD-patients pCRP staining is more pronounced and appears in the choroidal capillaries, the Bruch's membrane and within drusen (arrows). **C-D)** mCRP showed similar reactivity as pCRP: weak staining in ocular tissue derived from healthy individuals (**C**) and stronger reactivity for material derived from AMD patients (**D**) predominantly in the Bruch's membrane and within drusen (arrows). Autofluorescence of lipofuscin containing cells appears yellow. The images are representative of two independent experiments. D = Drusen, BM = Bruch´s membrane, Ch = Choroid, RPE = retinal pigment epithelium. Bar = 200µm

Figure 3: Immunohistological localization of pCRP and mCRP in retinal tissue of AMD patients.
Tissues derived from two patients with clinical AMD were fixed in cold acetone, blocked and incubated in a monoclonal mouse antibody specific for pCRP (MRz-12), mCRP (CRP-8) and a polyclonal anti-elastin antiserum, respectively, overnight at 4°C. Bound antibodies were visualized using an Alexa488-conjugated anti-mouse (green) or an Alexa555-conjugated anti-rabbit (red) secondary antibody. Nuclear counterstaining was performed with DAPI (blue). **A)** Double labeling for pCRP (green, image I) and elastin (red, image II) revealed co-localization (yellow, image III) along Bruch's membrane (arrows). **B)** Differentiation between specific pCRP fluorescence (green, image I) and autofluorescence (red, image II) of RPE cells in AMD tissue revealed specific pCRP immunoreactivity within drusen and Bruch's membrane (green, image III, arrows). **C)** Differentiation between specific mCRP fluorescence (green, image I) and autofluorescence (red, image II) revealed specific staining for mCRP within Bruch's membrane and drusen (green, image III, arrows).

Figure 4: Factor H, pCRP and mCRP bind to necrotic retinal pigment epithelial cells.
Binding of the non-risk and risk variant of Factor H as well as of the two CRP isoforms to necrotic

ARPE-19 as well as primary RPE cells was analyzed by flow cytometry. Bound Factor H was detected with polyclonal Factor H antiserum. pCRP and mCRP binding was analyzed with a polyclonal CRP antiserum which detects both forms. Unspecific antibody binding in the absence of proteins is shown by the dotted line. **A/F**) Factor H Y402 (black line) and Factor H H402 (grey line) bind to the surface of necrotic ARPE-19 (A) as well as primary RPE cells (F) with similar intensity. **B/G)** Also the two CRP isoforms, pCRP (black dashed line) and mCRP (grey dashed line) bound with similar intensity to necrotic ARPE-19 (B) and primary cells (G). Representative histogram profiles from five independent experiments are shown. **C-J)** Binding of Factor H, pCRP or mCRP was assayed by confocal microscopy. The protective variant of Factor H was detected with a polyclonal Factor H antiserum together with an Alexa647 conjugated secondary antiserum (red). Bound CRP forms were identified with monoclonal antibodies (Mrz-12 for pCRP and CRP-8 for mCRP) together with an Alexa488 labeled secondary anti-mouse serum (green). Cell nuclei were stained with DAPI (blue). Factor H (**C/H**) and pCRP (**D/I**) are evenly distributed over the surface of necrotic retinal pigment epithelial cells. In contrast, mCRP binding (**E/J**) is restricted to a specific site. The images C – E and H – J are representative of five independent experiments. Bar = 20µm

Figure 5: Formation of mCRP on the surface of necrotic ARPE-19 cells.
A) Generation of mCRP derived from the pentameric isoform on the necrotic cell surface was assayed by flow cytometry. Therefore pCRP was bound to the surface of necrotic ARPE-19 cells and formation of mCRP was followed using the mCRP specific CRP-8 antibody. mCRP formation was followed over time for 18 hrs. Background MFI at time 0 was subtracted and the increase in fluorescence is shown. .mCRP was already detectable after 0.5 hrs and specific mCRP reactivity increased over an 18 h period gradually. The data represent the mean ± SD for three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. **B-G)** Formation of mCRP on the surface of necrotic ARPE cells was also followed by confocal microscopy using the monoclonal CRP-8 antibody and the Alexa488 labeled secondary mouse antiserum (green). Cell nuclei were stained with DAPI (blue). mCRP generation was detectable after 2 hrs (**D**) and signal is more prominent after 3 hrs, 4hrs and 18 hrs respectively (**E-G**). The images B - G are representative of two independent experiments. Bar = 20µm

Figure 6: mCRP, but not pCRP, recruits Factor H to the surface of necrotic RPE cells.
Recruitment of Factor H to necrotic retinal pigment epithelial cells by the two CRP isoforms was assayed by flow cytometry using a polyclonal Factor H antiserum and an Alexa488 labeled goat antiserum. **A)** Both, the non-risk (black columns) and risk variant (white columns) of Factor H bind to the surface of necrotic ARPE-19 cells (compare first column of each triplet with control). mCRP enhanced surface recruitment of the non-risk Factor H variant by ca. 115 % and of the risk variant by ca. 50 % (compare last column of each triplet with the first column). pCRP did not affect surface binding of the Factor H variants (compare second column of each group with the first column). **C)** On the surface of primary RPE cells enhanced mCRP surface recruitment of the non-risk Factor H variant by 45 % and of the risk variant by 14 %. Again, pCRP did not affect surface binding of the Factor H variants. Binding of both Factor H variants without preincubation of mCRP or pCRP was set to 100 %. The percent enhancement (Δ) in the presence of mCRP was calculated by dividing the value obtained in the presence of the corresponding Factor H - mCRP complexes by the value of the Factor H binding alone. The data represent the mean ± SD for three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. **B/D)** The distribution of mCRP - recruited protective Factor H to necrotic ARPE-19 (B) and necrotic primary RPE cells (D) was analyzed by confocal microscopy using a polyclonal Factor H antiserum together with an Alexa555 conjugated secondary antiserum (red). Cell nuclei were stained with DAPI (blue). Recruited Factor H binds specifically one distinct site of the necrotic membrane. The image is representative of five independent experiments. **E-H)** Co-localization with annexin V, coupled to the fluorescent dye APC was assayed. Annexin V (**E,** red) binds to the surface of necrotic cells and apparently binds with higher intensity to the specific site of the necrotic cell, that co-localizes with mCRP (**F**, green) and Factor H (**G,** pseudocolor turquoise) as

revealed by the yellow color (**H**). The images E - H are representative of two independent experiments. Bar = 20µm

Figure 7: The Y402H variation affects mCRP - assisted cofactor activity and release of TNF-α. **A)** The complement proteins C3b and Factor I were added to Factor H - mCRP complexes, which were preincubated on the surface of necrotic ARPE-19 cells. The deposition of iC3b, on the retinal surface was followed by flow cytometry, using a monoclonal iC3b antibody and a corresponding Alexa488 coupled mouse antiserum. mCRP increased the iC3b deposition for both Factor H variants dose-dependently. The enhancement was more pronounced for the non-risk Factor H variant (solid line, filled squares) as compared to the risk variant (dashed line, open squares). At the highest used mCRP concentration this difference reaches 35 %. The antibody controls (grey bars) show background signals in the absence of all proteins and the specific detection of iC3b in contrast to C3b by the used iC3b antibody. The iC3b deposition in the absence of mCRP was set to 100 %. The effect of the Factor H – mCRP interaction was calculated by dividing the value obtained in the presence of mCRP by the value in the absence of mCRP. The Greek letter Δ denotes the percent difference between the values for iC3b deposition in the presence of 1.5 µg / ml mCRP of either Factor H Y402 or Factor H H402. The data represent the mean ± SD for three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. **B)** Necrotic ARPE-19 cells were added to activated human THP-1 macrophages for 20 h to induce the secretion of TNF-α (column 4) Factor H coating on necrotic ARPE-19 cells did not reduce TNF-α release (column 6, first black column and column 8, first white column). In the presence of the non-risk Factor H variant TNF-α secretion was reduced upon mCRP recruitment (column 7) by 35 % and for the risk variant by 1 % (column 9). Attachment of mCRP to the necrotic cells did not influence TNF-α protection (column 5) neither did necrotic ARPE-19 cells alone (column 2). THP-1 macrophages in the absence of necrotic cells showed back ground levels for TNF-α (column 1). The TNF-α release of the macrophages in the presence of necrotic particles but in the absence of mCRP and Factor H was set to 100 %. The percent inhibition (Δ) of the corresponding Factor H – mCRP complexes was calculated by dividing the value obtained in the presence of each Factor H - mCRP complex by the value of the corresponding Factor H effect alone. The data represent the mean ± SD for six independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Figure 1

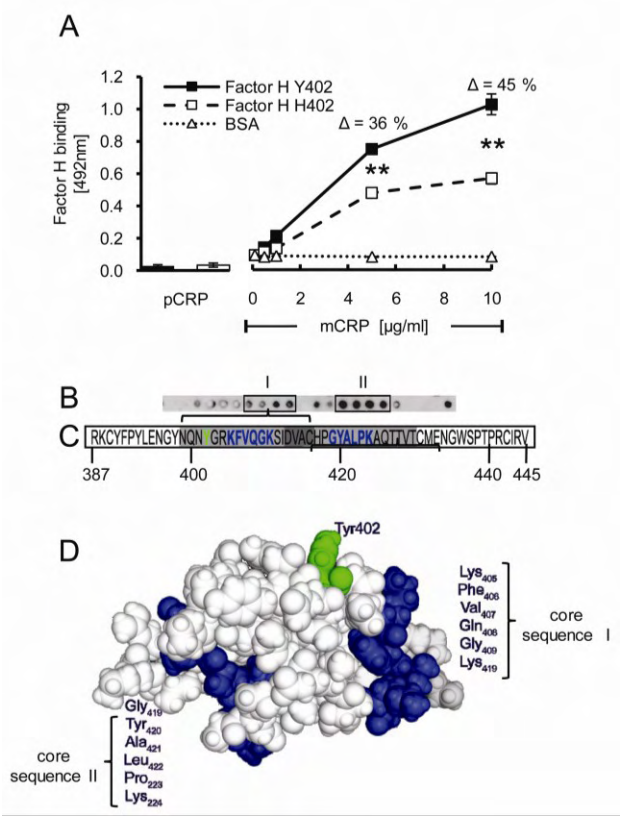


Figure 2

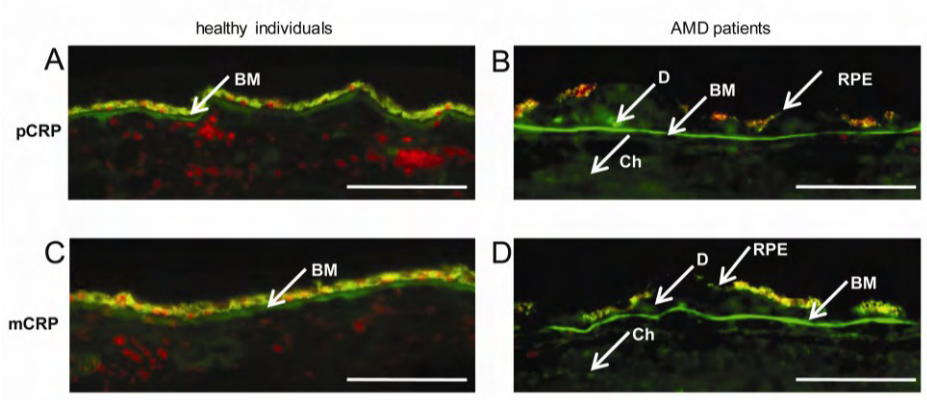


Figure 3

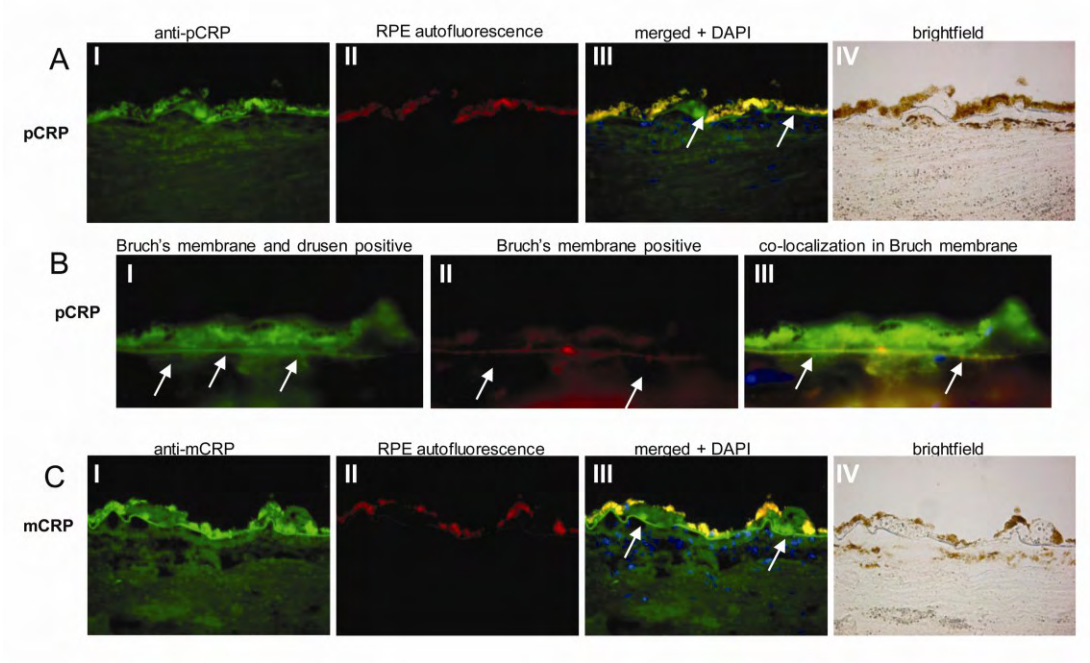


Figure 4

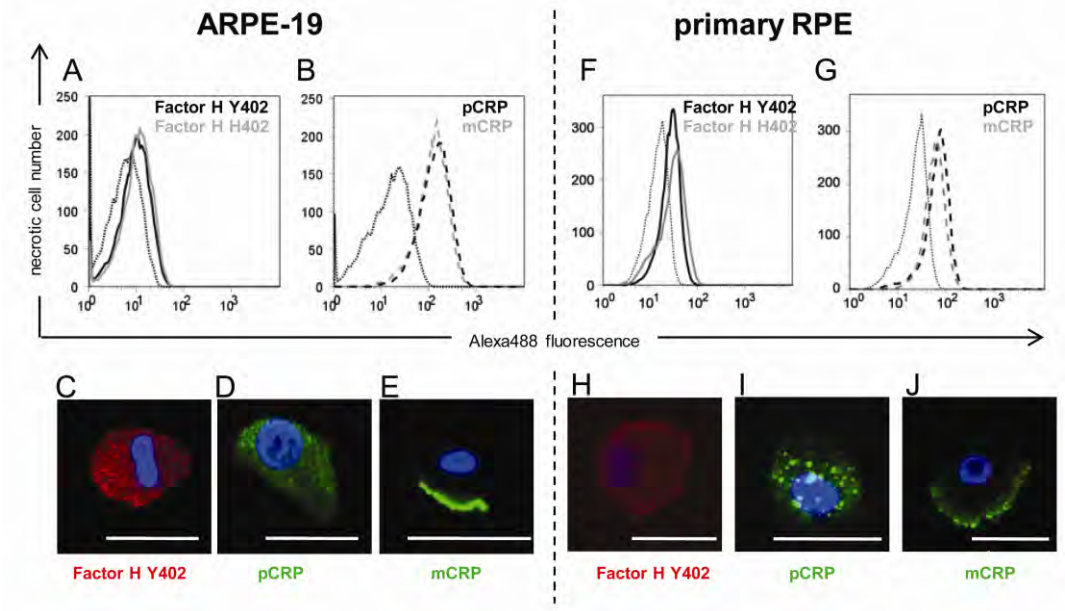


Figure 5

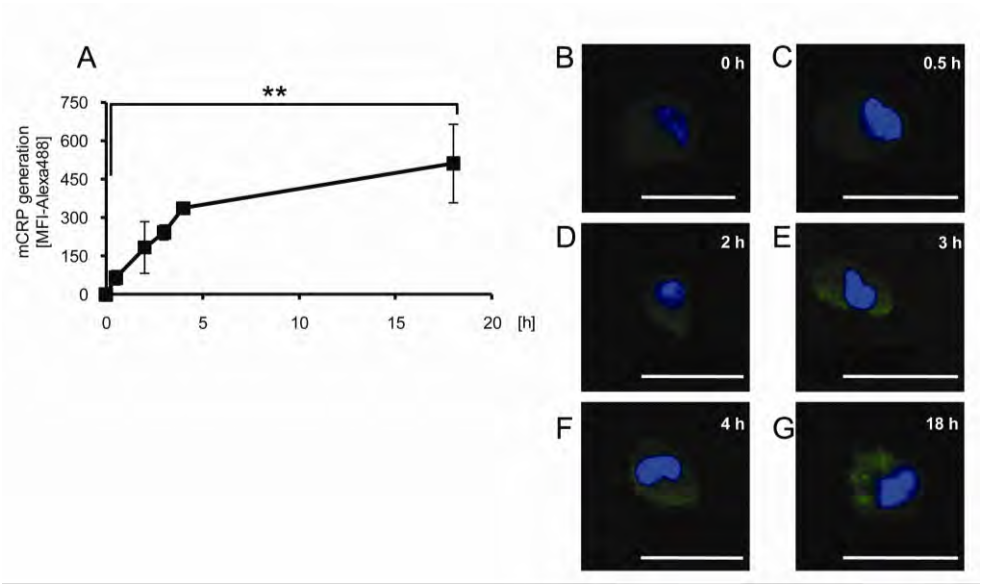


Figure 6

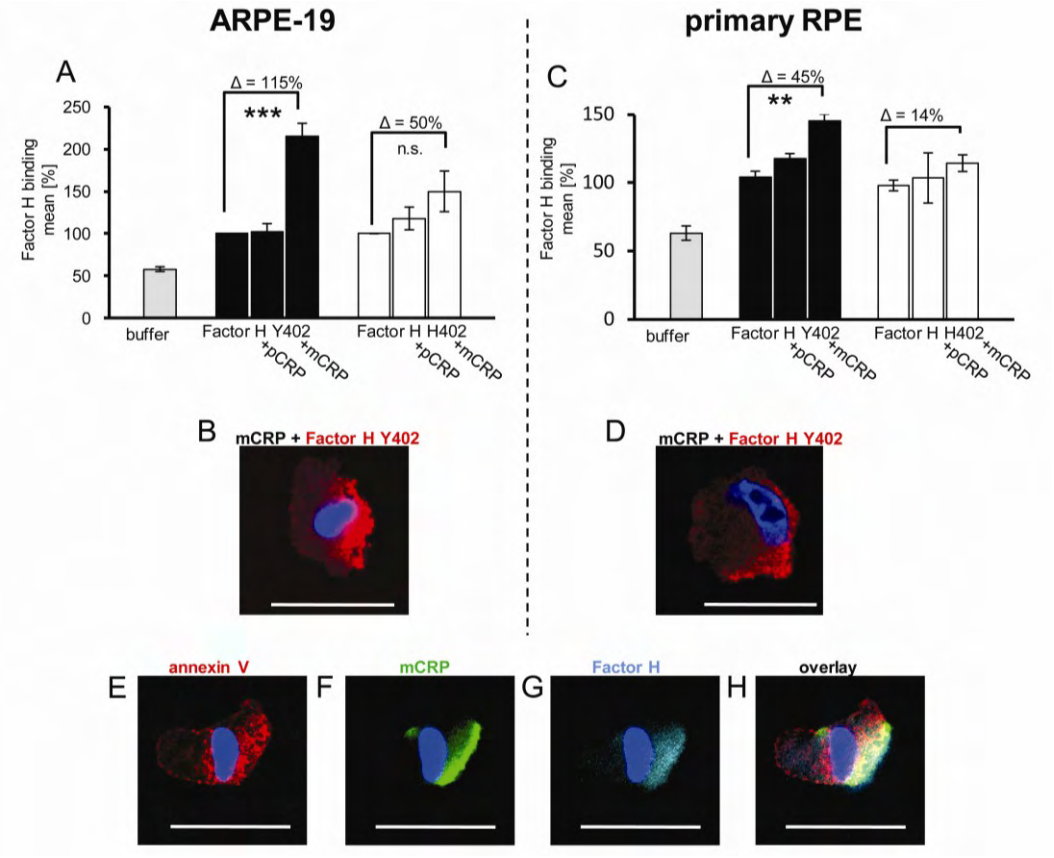
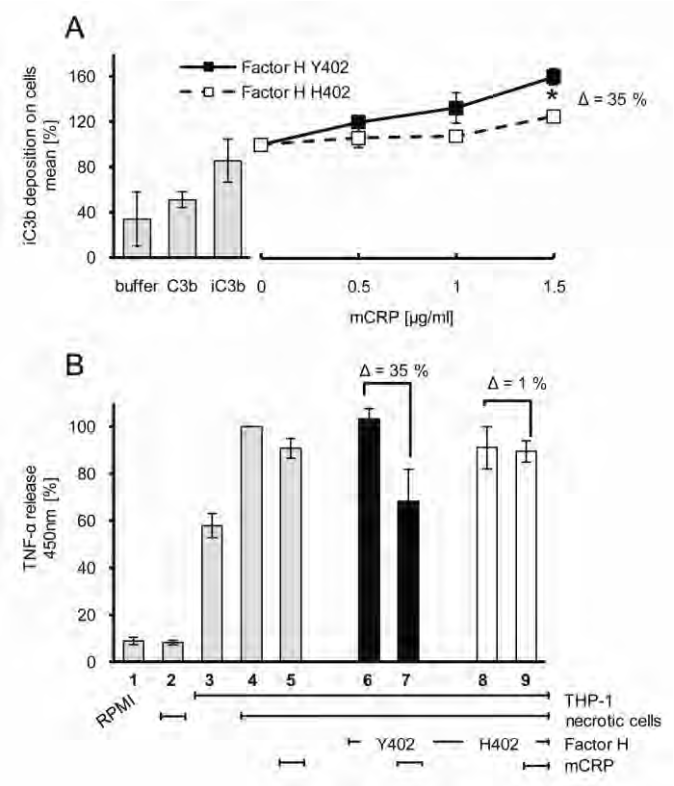


Figure 7


A
iC3b deposition on cells
mean [%]
Factor H Y402
Factor H H402
Δ = 35 %
buffer C3b iC3b 0 0.5 1 1.5
mCRP [µg/ml]
B
TNF-α release
450nm [%]
Δ = 35 %
Δ = 1 %
1 2 3 4 5 6 7 8 9
RPMI
THP-1
necrotic cells
Y402 H402 Factor H
mCRP

4 Factor H related protein 1 (CFHR-1) inhibits complement C5 convertase activity and terminal complex formation.

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IMMUNOBIOLOGY

Factor H–related protein 1 (CFHR-1) inhibits complement C5 convertase activity and terminal complex formation

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Homozygous deletion of a 84-kb genomic fragment in human chromosome 1 that encompasses the *CFHR1* and *CFHR3* genes represents a risk factor for hemolytic uremic syndrome (HUS) but has a protective effect in age-related macular degeneration (AMD). Here we identify CFHR1 as a novel inhibitor of the complement pathway that blocks C5 convertase activity and interferes with C5b surface deposition and MAC formation. This activity is distinct from complement factor H, and apparently factor H and CFHR1 control complement activation in a sequential manner. As both proteins bind to the same or similar sites at the cellular surfaces, the gain of CFHR1 activity presumably is at the expense of CFH-mediated function (inhibition of the C3 convertase). In HUS, the absence of CFHR1 may result in reduced inhibition of terminal complex formation and in reduced protection of endothelial cells upon complement attack. These findings provide new insights into complement regulation on the cell surface and biosurfaces and likely define the role of CFHR1 in human diseases. (Blood. 2009;114:2439-2447)

Introduction

The complement system is important for host innate and adaptive immunity and mounts a protective immune response to invading microbes.[1] The alternative complement pathway is spontaneously activated, and generates C3 convertases (C3bBb) that cleave the central component C3 to the anaphylactic peptide C3a and C3b.[2,3] C3b attached to a foreign surface binds factor B and generates the C3 convertase (C3bBb), which enhances further complement activation resulting in opsonization and phagocytosis of particles. Binding of an additional C3b molecule to the C3 convertase forms the C5 convertase (C3bBbC3b) of the alternative pathway. This convertase cleaves C5 and generates the potent chemoattractant C5a as well as C5b, which initiates the terminal complement pathway assembly.[4] C5b immediately undergoes conformational changes and binds C6 and C7 in a nonenzymatic manner. The assembled C5b67 complex is released from the convertase and attaches to lipid bilayers. Upon binding of C8 and C9, the lytic membrane attack complex (MAC) is formed.[3,5]

Once activated, this powerful defense system is tightly controlled on host cell surfaces by both membrane-anchored and surface-attached soluble regulators. Proper and coordinated function of these regulators is essential for tissue integrity. Single gene mutations predispose to severe renal and retinal diseases, that is, hemolytic uremic syndrome (HUS; OMIM no. 235400), membranoproliferative glomerulonephritis type II (MPGN II; OMIM no. 609814), or age-related macular degeneration (AMD; OMIM no. 603075).[6,7]

HUS is caused by occlusion of arterioles and capillaries in the kidney, due to endothelial cell and platelet damage.[8] MPGN II is a rare renal disease, with formation of dense deposits at the glomerular basement membrane and thickening of the peripheral capillary walls.[9] Similarly, the retinal disease AMD, which causes visual impairment of elderly people, is caused by deposits (drusen) that form on the Bruch membrane and lead to atrophy of the retinal pigment epithelium and chorodial neovascularization under the macular area.[10]

These diverse diseases are caused by defective local complement regulation and are associated with gene variations and mutations coding for complement components and regulators, such as complement factor H (CFH).[8,11] In addition deletion of a 84-kb genomic fragment on human chromosome 1, which leads to the loss of the complement factor H–related genes 1 and 3 *(CFHR1* and *CFHR3),* is associated with both HUS[12] and AMD.[13] However, this chromosomal deletion has opposite effects, for HUS the deletion increases the risk for the disease and for AMD the deletion has a protective effect.

In atypical HUS (aHUS), the absence of CFHR1 and CFHR3 in plasma correlates with the presence of autoantibodies to CFH.[14] The autoantibodies bind to the C-terminal surface binding region of CFH[15] and inhibit CFH surface attachment resulting in damage of endothelial cells as well as platelets.[16] The CFHR1 plasma protein is composed of 5 short consensus repeats and is identified in 2 glycosylated forms. CFHR1β (42 kDa) has 2 and CFHR1α (37 kDa) has one attached carbohydrate side chain.[17-19] The high sequence identity of the 3 C-terminal SCRs of CFHR1 and CFH (100%, 100%, and 98%) suggests related functions. CFHR1 lacks

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cofactor and decay-accelerating activity,[20] and the function is currently unknown. Given the opposing effects of CFHR1/CFHR3 deficiency in human diseases, we were interested to identify the function of CFHR1 protein. Here we identify CFHR1 as a regulator of the complement pathway that inhibits C5 convertase activity and MAC assembly.

Methods

Proteins and antibodies

Recombinant CFHR1 and deletion mutants CFHR1 SCR1-2 (CFHR1/1-2) and CFHR1 SCR3-5 (CFHR1/3-5) were expressed as described.[21] CFHR1/1-2 cDNA was cloned into vector pPICZαB using specific primers $CFHR1_{1\text{-}2}$ forward, 5′TTTCTGCAGCCGAAGCAACATTTTGTGATTTTCAA3′ and $CFHR1_{1\text{-}2}$ reverse, 5′TTTTCTAGAGCAGTGGACCTGCATTTGGGAGGGGT3′. CFH/SCR11-15 was expressed in insect cells, as previously described.[14,15] All proteins were expressed in *Picha pastoris* and purified by nickel chelate affinity chromatography.[14] Vitronectin was purchased from BD Biosciences; C3b, C5, C5b6, factor H, and factor I, from Merck Biosciences; C7, C8, C9, from Comptech. CFHR1 was purified from human plasma by HiTrap Heparin HP column (GE Healthcare) affinity chromatography. Pooled elution fractions were concentrated (Superdex 200; GE Healthcare) and separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE); CFHR1 was dissected from the gel, concentrated, and dialyzed (1× PBS, pH 4.7). Mouse monoclonal antibody (mAb) C18[22] (Alexis) was used to detect the C-terminus of CFH and CFHR1. A novel CFHR1 mAb JHD10 was generated by immunizing mice with purified CFHR1/SCR1-2 fragments.

Serum probes

Normal human plasma (HP) was obtained from healthy volunteers (Jena, Germany) upon informed consent obtained in accordance with the Declaration of Helsinki. CFH depletion was performed by immunadsorbance as described.[12] For C8 depletion, goat polyclonal anti-C8 (Comptech) was used as described for CFH depletion. The study is approved by the ethics board of the Friedrich Schiller University of Jena. The board approved study of patient material (DNA and blood) for genetic and molecular characterization of complement factor H and complement factor H family members in HUS, MPGN2, and AMD.

Cell culture and confocal microscopy

Human umbilical vein endothelial cells (HUVECs; ATCC CRL-1730) were cultivated as described.[23] For confocal microscopy (LSM 510 META; Zeiss) HUVECs were grown on chamber slides (Nunc) and incubated for 60 minutes with CFHR1 or CFH (100 μg/mL), and binding was visualized using mAb JHD10 (CFHR1) or polyclonal anti–SCR1-4 (CFH).[24] C5 deposition on erythrocytes incubated in $HP_{\Delta CFH\Delta C8}$ was detected with C5 mAb (Comptech).

Immunohistochemistry

Immunohistochemistry was performed on tissues derived from 2 human donor eyes (2 female patients, 79 and 81 years of age) with history neither of clinically documented early AMD nor of morphologic evidence of ocular disease. The donor eyes were obtained at autopsy and were processed fewer than 15 hours after death. In addition, normal kidney tissue was obtained from 2 human adult donor kidneys that were not used for transplantation. Posterior eye poles and portions of decapsulated kidneys were embedded in optimal cutting temperature compound and frozen in isopentane-cooled liquid nitrogen. Cryostat-cut sections (6 μm) were fixed in cold acetone, blocked with 10% normal goat serum, and incubated in a monoclonal mouse antibody to CFHR1 (JHD10) diluted 1:100 in phosphate-buffered saline (PBS) overnight at 4°C. Antibody binding was detected by Alexa 488–conjugated secondary antibodies (Molecular Probes). Nuclear counterstaining was performed with propidium iodide. For preabsorption experiments, the primary antibody was treated for 1 hour with either CFHR1 or CFH.

Binding of CFHR1 to heparin, C3b, C5, and C5b6

MaxiSorp plastic plates (Nunc) were coated with heparin (500 units/well) or C3b (10 μg/mL), C5, or C5bC6 (5 μg/mL) and incubated with CFHR1 (50 μg/mL) or CFH (75 μg/mL) dissolved in binding buffer B (10 nM Na_2HPO_4, 27 mM KCl, 1.4 M NaCl, 2% BSA, pH 7.4). Bound CFHR1 was detected with mAb C18 and bound CFH with SCR1-4 antiserum. In control experiments, buffer B was added without proteins.

For identification of C5 binding site in CFHR1, mAb JHD10 or mAb C18 (15 μg/mL) was immobilized to a microtiter plate and used to catch CFHR1 (30 μg/mL). After washing, C5 or C5b6 (5 μg/mL) was added in gelatin veronal buffer (Sigma) and bound proteins were identified using a C5 mAb. CFHR1-specific antiserum was used to confirm binding of CFHR1 to the immobilized mAbs. C5 binding to CFH or CFHR1 was measured by immobilizing equimolar concentrations of CFH or CFHR1 to a microtiter plate. C5 binding to immobilized CFHR1 and CFH was assayed by incubation of increasing concentrations of C5 (1.5-50 μg/mL) for 1 hour, and bound C5 was identified with mouse monoclonal antibodies (1:2000; Quidel). In addition, binding of C5 (50, 150, 200, and 400 nM) to immobilized CFHR1 or CFH/SCR10-13 (carboxylated dextran chip CM5; Biacore AB) was assayed by surface plasmon resonance in 75 mM PBS at a flow rate of 5 μL/minute at 25°C. Controls (binding of the fluid phase ligand to the uncoated surface) were subtracted.

Cofactor assay

Cofactor activity of heparin-bound CFH was analyzed by measuring factor-I–mediated degradation of C3b after SDS-PAGE and Western blot analysis. For competition CFH immobilized to heparin-coated microtiter plates (EpranEx; Plasso) was incubated with CFHR1 (0.13 μg-13.3 μg). Then C3b (2 μg) and CFI (0.28 μg) were added and cofactor activity was determined.[24] Degradation products (β chain band and α'43 band) were analyzed by densitometry.

ELISA

To investigate CFHR1 regulation of the C3 convertase, the C3 convertase was generated by incubation of C3b (2 μg/mL) and C3 (80 μg/mL) with factor D (4 μg/mL) and factor B (40 μg/mL) in activation buffer C (20 mM Hepes, 144 mM NaCl, 7 mM $MgCl_2$, 10 mM EGTA, pH 7.4). Activity of C3 convertase was determined after incubation of constant amounts of C3 (80 μg/mL) and increasing amounts of CFHR1 (25 and 50 μg/mL), CFH (50 g/mL), or 25 μg/mL human serum albumin (HSA) by C3a generation. C3a concentrations were determined by enzyme-linked immunosorbent assay (ELISA; Quidel). Sheep erythrocytes (10^9) were incubated with C3b (10 μg/mL) in veronal buffer overnight at 4°C. C3 and C5 convertase was generated by incubation of erythrocytes for 40 minutes at 30°C with C3 (10 μg/mL), factor D (5 μg/mL), and factor B (10 μg/mL) in veronal buffer supplemented with Ni^{2+} and properdin (5 μg/mL). C5 convertase–loaded erythrocytes were preincubated with recombinant or plasma-derived CFHR1 or CFH (50 μg/mL) or with BSA (50 μg/mL) before the addition of C5 (50 μg/mL). C5a generation was analyzed after 15 minutes by ELISA (DRG Diagnostics).

Erythrocyte lysis assay

Increasing concentrations of CFHR1 (5-160 μg/mL) or CFH, vitronectin, or HSA were added to CFH- and CFHR1-depleted plasma (30%) and incubated at 37°C for 15 minutes with approximately 2×10^7 sheep erythrocytes in activation buffer C (see above). Supernatants were recorded at 415 nm. In similar experiments, generation of complement activation products C3a and C5a was followed by ELISA (Quidel; DRG Diagnostics).

Hemolysis of chicken erythrocytes, which are more sensitive for MAC-induced hemolysis, was investigated in complement-inactivated (20 mM HEPES, 144 mM NaCl, 10 mM EDTA, pH 7.4) defHP with constant concentrations of C5b6 (5 ng/mL), increasing amounts of CFHR1 (25-100 μg/mL), CFH, or BSA. In addition C5b6 complexes (5 ng/mL)

were preincubated with either recombinant CFHR1 (50 μg/mL), purified CFHR1 (12.5 μg/mL), vitronectin (12.5 μg/mL), or BSA (12.5 μg/mL) in 20 mM HEPES, 144 mM NaCl, 10 mM EDTA, pH 7.4, for 5 minutes at 20°C. Lysis of sheep erythrocytes (2×10^7) was followed after addition of C7 (final concentration, 1 μg/mL) C8 (0.2 μg/mL), and C9 (0.2 μg/mL) by absorbance at 415 nm. To compare similar molar amounts of the plasma-purified CFHR1 with vitronectin, C5b6 complexes (5 ng/mL) were preincubated with increasing concentrations (1-300 nM) of either plasma-purified CFHR1, vitronectin, or BSA for 5 minutes at 20°C. After addition of C7 (final concentration, 1 μg/mL), C8 (0.2 μg/mL), and C9 (0.2 μg/mL) the mixture was added to sheep erythrocytes (2×10^7) and incubated for 30 minutes at 37°C. Percentage of cell lysis (absorbance at 415 nm) was calculated by the formula: [(sample − background)/(100% lysis − background)] × 100, where background hemoglobin release was obtained from sheep erythrocytes incubated in buffer only, and 100% lysis was achieved using distilled water.

Flow cytometry

CFHR1/CFHR3-deficient plasma was depleted of CFH and C8 ($HP_{\Delta CFH\Delta C8}$) by immunoaffinity chromatography and added to sheep erythrocytes in the presence or absence of 50 μg/mL CFHR1 in 20 mM HEPES, 250 mM mannitol, 8 mM $MgCl_2$, 10 mM EGTA, pH 7.4. At each time point, the sample was transferred to ice-cold buffer with 1% wt/vol BSA with protease inhibitor mix (Complete Inhibitor Mix; Roche). C5 was detected using C5 mAb (Comptech).

Binding of serum-derived CFHR1 to HUVECs was investigated by incubating HUVECs, which have been grown serum free for 3 days, in 25% normal human serum with mAb JHD10.

Immunoprecipitation

Monoclonal C5 antibodies (Comptech) were immobilized to protein A sepharose beads (GE Healthcare) by incubation overnight at 4°C. Antibody-loaded beads were washed 3 times in PBS (1×) and incubated for 2 hours at 4°C with 50% NHP. The beads were washed 5 times in PBS (1×) and bound antibodies and proteins were eluted with 0.1 M glycine/0.5 M NaCl, pH 2.7. Eluates were separated by SDS-PAGE and transferred to a membrane, and CFHR1 was detected with a polyclonal antiserum and C5, with a rabbit polyclonal antiserum in combination with a secondary horseradish peroxidase–conjugated antiserum.

Statistical analysis

Statistics were analyzed with the Student *t* test; *P* values less than .05 were considered significant. Statistical analysis of defined groups was performed with the Jonckheere-Terpstra trend test[25]; *P* values were calculated for the 2-sided test of no trend.

Results

CFHR1 binds to C3b, C3d, heparin, and human cells

The 3 C-terminal SCRs of CFHR1 display almost sequence identity to the central C3b and surface binding region of CFH (ie, SCR18-20; Figure 1A). This homology suggests related functions. Therefore binding of CFHR1 to the ligand C3b was investigated. CFHR1 binds to C3b, and CFH, which harbors 3 interaction sites for C3b, showed more pronounced binding (Figure 1B).

CFHR1 uses the C-terminus for both C3b and heparin binding: Surface plasmon resonance showed that deletion mutant CFHR1/3-5 (supplemental Figure 2A blue line, available on the *Blood* website; see the Supplemental Materials link at the top of the online article), but not CFHR1/1-2 (black line), bound to immobilized C3b and to heparin (supplemental Figure 2A-B). The binding affinity of the C-termini of CFHR1 and of CFH was determined by surface plasmon resonance. The affinity of CFHR1/3-5 to C3b is $K_D = 6.4 \times 10^{-6}$ M and that of CFH/18-20 is $K_D = 2.6 \times 10^{-6}$ M. Thus, the C-terminus of CFHR1 binds C3b with lower affinity than that of CFH (supplemental Figure 2C-D).

Binding of CFHR1 to cell surfaces

Binding of CFHR1 to human cells was investigated by flow cytometry. HUVECs were incubated in normal human plasma (HP) and binding of native, plasma-derived CFHR1 was detected with the novel CFHR1-specific mAb JHD10 (Figure 1C). Binding of recombinant CFHR1 to HUVECs, as well as to epithelial cells (ARPE-19), was confirmed by confocal microscopy. On the cell surface, CFHR1 showed a patchy distribution (supplemental Figure 3AI-II). In addition CFHR1 bound to C3b-treated rabbit erythrocytes (supplemental Figure 3AIII). The secondary antibodies alone showed no binding (supplemental Figure 3AIa-IIIa). The C-terminus of CFHR1 mediates cell binding, as the deletion mutant CFHR1/3-5, but not CFHR1/1-2, bound to ARPE-19 cells (supplemental Figure 3B). Thus, CFHR1 binds via the C-terminal binding region to C3b and to human cells.

CFHR-1 binding to kidney and retinal tissue

Having demonstrated that CFHR1 binds cell surfaces, we analyzed in vivo CFHR1 expression in human retinal and ocular tissues by immunohistochemistry. In renal tissue, CFHR1 was detected in the lining of blood vessels (green fluorescence), particularly of large arteries and of afferent and efferent arterioles at the vascular poles of glomeruli (Figure 1Di). In the posterior segment of a retina, CFHR1 staining was specifically localized to the Bruch membrane and the choriocapillaries of the choroids (Figure 1Div). Preincubation of the antibodies with CFH did not affect reactivity (Figure 1Diii,vi), but preincubation with CFHR1 did (Figure 1Dii,v). Thus, CFHR1 is present at the surface of endothelial cells and at the Bruch membrane.

CFHR1 and CFH recognize overlapping binding sites at the cell surface

The almost identical C-termini and the conserved binding characteristics of CFHR1 and CFH suggested coordinated as well as competitive binding. Therefore simultaneous binding to cell surfaces was investigated. HUVECs were incubated with CFHR1 and CFH. Surface-bound CFHR1 was visualized by red—and bound CFH by green—fluorescence (Figure 1Ei-ii). A merge of the 2 images revealed overlapping binding as indicated by the yellow fluorescence (Figure 1Eiii).

To investigate whether CFHR1 replaces CFH and reduces local CFH-mediated activity, heparin-bound CFH was competed with increasing concentrations of CFHR1, and cofactor activity was assayed by analyzing the generation of the C3b degradation products α'68 and α'41/43. CFHR1, by replacing CFH, reduced the local regulatory activity as demonstrated by the lower amount of C3b degradation fragments (Figure 1F top panel). Densitometric analyses showed reduction of the α'43 band (as demonstrated by the α'43/β75 ratio) when equal molar amounts of CFHR1 and CFH were used (Figure 1F bottom panel, column 3). This effect demonstrates that CFHR1 may reduce local CFH-mediated complement control.

CFHR1 regulates complement pathway activation

Binding of CFHR1 to C3b suggested a unique regulatory function(s) on the level of C3 convertase. To identify such an activity, a hemolytic assay[12,26,27] was used. Sheep erythrocytes representing nonactivator surfaces remain intact when incubated in human plasma (HP). However, these cells are lysed when incubated in

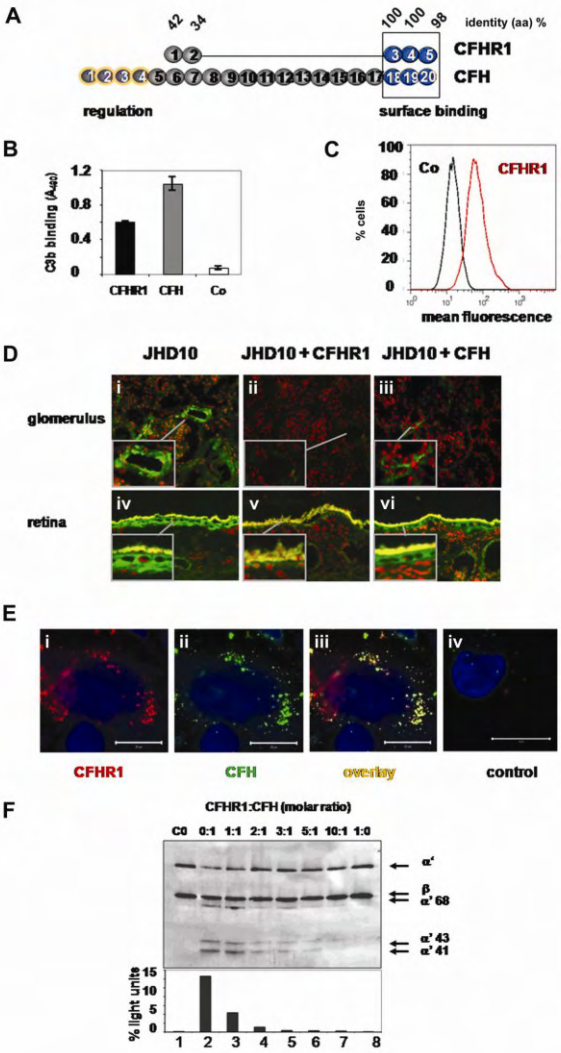

Figure 1. CFHR1 binds to C3b and cells and competes with CFH. (A) CFHR1 is composed of 5 SCRs domains. SCR1 and SCR2 show 42% and 34% sequence identity to SCR6 and SCR7 of factor H (CFH), respectively. The 3 C-terminal SCRs show 100%, 100%, and 98% sequence identity to SCR18, SCR19, and SCR20 of CFH, respectively, which comprise the C-terminal surface binding region. (B) Equimolar concentrations of CFHR1 (25 μg/mL) and CFH (75 μg/mL) bind to immobilized C3b. Data represent mean values ± SDs from 3 separate experiments. Control represents binding of antibodies to immobilized BSA. (C) Plasma-derived CFHR1 (red curve) binds to HUVECs. Cells were incubated in human plasma, and bound CFHR1 was visualized with specific mAb (JHD10) by flow cytometry. Control: cells were treated with the secondary antibody alone (black curve). (D) Immunofluorescence staining of CFHR1 (green fluorescence) in renal and retinal human tissue. CFHR1 is detected at the lining of renal (i) and ocular (iv) blood vessels including large arteries, afferent and efferent arterioles associated with glomeruli (i), or the choriocapillaries (iv) as well as the Bruch membrane (nuclear counterstain: propidium iodide; original magnification, ×100). Preabsorption of mAb JHD10 with CFHR1 blocks reactivity (ii,v). In contrast, preabsorption with CFH (iii,vi) does not affect reactivity and demonstrates specificity of signals in panels i and iv. Thus, CFHR1 is present at the surface of endothelial cells and at the Bruch membrane. Inlays represent magnifications. Autofluorescence of lipofuscin-containing cells appears yellow. (E) HUVECs were incubated with a combination of CFHR1 (100 μg/mL) and CFH (100 μg/mL). After addition of the appropriate secondary antibodies, bound proteins were identified by confocal laser scanning microscopy. CFHR1 binding was detected with the CFHR1-specific mAb JHD10 in combination with a secondary anti–mouse antibody labeled with Alexa 647 (red fluorescence; i) and binding of CFH with a polyclonal antiserum specific for the N-terminal domains of CFH (anti–SCR1-4) together with a secondary goat anti–rabbit antibody labeled with Alexa 488 (ii; green fluorescence). An overlay of subpanels i and ii (iii) reveals colocalization of the 2 regulators as indicated by the yellow signal. Binding of primary (JHD10) and secondary antibodies showed no signal (control; iv). All cells were stained with DAPI to visualize the cell nucleus (bar represents 20 μm). (F) CFHR1 competes with factor H for heparin binding and thus affects the regulatory activity at surfaces. Constant amounts of factor H (10 μg/mL) were bound to immobilized heparin, and CFHR1 was used at increasing concentrations (0.1-20 μg/mL) as competitor. After competition, C3b and factor I were added. After incubation for 30 minutes the supernatant was removed, separated by SDS-PAGE, and transferred to a membrane, and C3b and degradation fragments were visualized with C3 antiserum (top panel). The mobility of the α' and β chain as well as the degradation fragments are indicated. A densitometric analysis as determined by the ratio of the α' 43 band and the β chain is shown in the bottom panel. A representative experiment of 3 separate experiments is shown.

complement active HP depleted of CFH and CFHR1 (HPΔCFH). In this system, the absence of CFH results in erythrocyte lysis, and addition of CFH has a dose-dependent protective effect (Figure 2A gray squares). The role of CFHR1 on complement activation was analyzed in this serum. Addition of CFHR1 to $HP_{\Delta CFH}$ reduced lysis of sheep erythrocytes dose dependently (Figure 2A black triangles) and HSA showed no effect (black squares). The protective effect of CFHR1, used at 80 μg/mL, was 13% and that of CFH was 75%. The inhibitory activity of CFHR1 was comparable with the known terminal pathway inhibitor vitronectin (Figure 2A compare black triangles and gray diamonds).

The regulatory role of CFHR1 on the complement pathway was investigated for the C3 convertase. C3 convertases were formed in vitro by incubating C3 with factor B and factor D, and, after addition of C3, convertase activity was monitored by C3a generation. CFHR1 (25 and 50 μg/mL) did not significantly affect C3a levels (Figure 2B columns 5-6), demonstrating that CFHR1 does not affect the C3 convertase. In contrast, CFH (50 μg/mL) strongly inhibited C3a generation (Figure 2B column 7). Thus CFHR1 does not affect the C3 convertase and likely acts downstream of this convertase.

CFHR1 regulates the C5 convertase of the alternative pathway

CFHR1 binds C3b and inhibits alternative complement activation before MAC assembly and insertion. Therefore, we hypothesized that CFHR1 inhibits the C5 convertase of the alternative pathway.

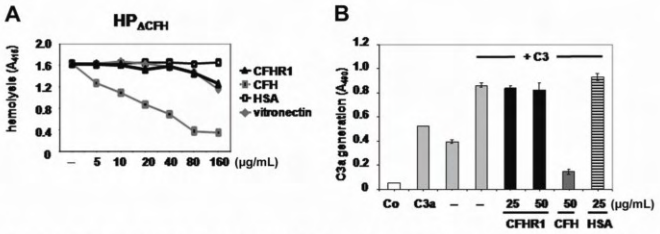

Figure 2. CFHR1 is a regulator of the alternative pathway of complement. (A) Hemolysis of sheep erythrocytes in the presence of CFHR1- and CFH-depleted normal human plasma ($HP_{\Delta CFH}$). Sheep erythrocytes represent nonactivator surfaces when incubated in complement active human plasma. However, when the same cells are incubated in $HP_{\Delta CFH}$, these cells represent activator surfaces and are lysed. Factor H acts as a surface protector and reverts the effect (▯). Addition of CFHR1 (5-160 μg/mL) results in a reduction of erythrocytes lysis, which indicates a regulatory effect of this protein in complement control (▲). Similar inhibition of hemolysis is observed with vitronectin (◈). HSA has no effect on hemolysis (■). Data show 1 representative of 3 experiments. A_{415} indicates absorbance at 415 nm. (B) CFHR1 does not affect C3a generation of an in vitro–assembled C3 convertase. C3 was incubated with factor D and factor B, and C3 convertase activity was analyzed by comparing C3a before (column 3) and after (column 4) addition of C3. Addition of CFHR1 (25 or 50 μg/mL) did not significantly effect C3a generation (columns 5-6). CFH (50 μg/mL) strongly effected C3 convertase activity (column 7). Addition of human serum albumin (HSA) did not affect C3a generation (column 8). C3 mAb, which reacts with C3a (1 μg/mL, standard; column 2) did not react with an empty well (co). A representative result of 3 independent experiments is shown and SDs are given. A_{490} indicates absorbance at 490 nm.

To this end, sheep erythrocytes were incubated in complement active $HP_{\Delta CFH}$ and C3a, and C5a generation was determined. CFHR1 showed a dose-dependent protective effect on erythrocyte lysis, did not affect C3a generation, but reduced C5a generation (Figure 3A). Thus CFHR1 inhibits the C5 convertase of the alternative pathway.

Having demonstrated that CFHR1 inhibits the C5 convertase, we next aimed to characterize this inhibitory effect in more detail. To this end, sheep erythrocytes were incubated in CFH-depleted, complement active human plasma, which, in this case, was depleted for CFH and C8 ($HP_{\Delta CFH\Delta C8}$). Surface deposition of C3b and C5b was analyzed by flow cytometry. CFHR1 (10, 20, and 40 μg/mL) did not, but CFH (10, 20, and 40 μg/mL) strongly reduced C3b deposition (compare Figure 3B solid red and blue lines). However, CFHR1 (10, 20, and 40 μg/mL) reduced C5b deposition by 40%, 50%, and 60%, respectively (Figure 3B red

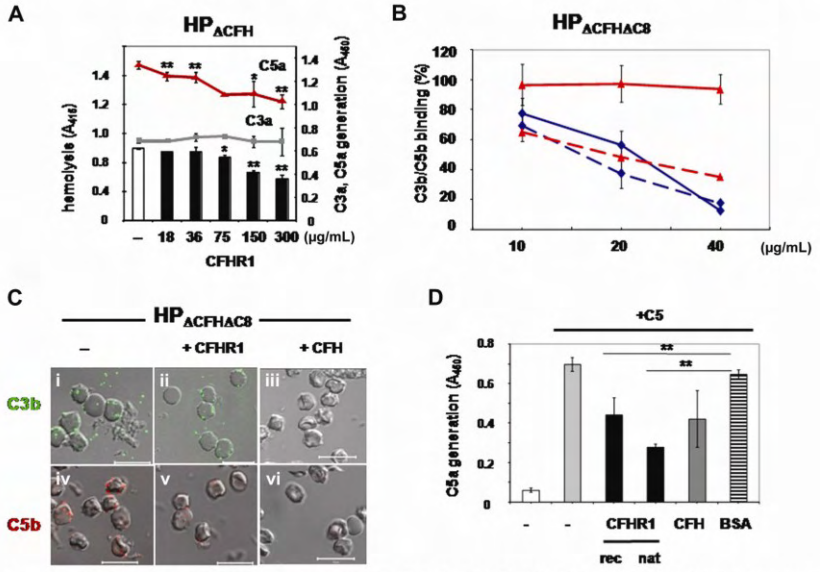

Figure 3. CFHR1 regulates C5 convertase activity, binds to C5 and C5b6, and inhibits binding of C5b6 to the cell surfaces. (A) Sheep erythrocytes were incubated in complement active CFH/CFHR1-depleted HP ($HP_{\Delta CFH}$) in the presence or absence of CFHR1 (18-300 μg/mL). Lysis was recorded after 30 minutes. In addition, the concentration of C3a (gray squares) and C5a (red triangles) was assayed in the supernatant. Data represent mean values of 3 separate experiments and SDs are indicated. $^*P < .05$, $^{**}P < .005$ versus control. (B) Effect of CFHR1 on C3b (solid line) and C5/C5b (stippled line) deposition on the surface of sheep erythrocytes. Sheep erythrocytes were incubated in $HP_{\Delta CFH\text{-}\Delta C8}$ plasma, and deposition of C3b and C5b was assayed in the presence of increasing concentrations of CFHR1 (10, 20, and 40 μg/mL, red solid and stippled lines) and CFH (10, 20, and 40 μg/mL, blue solid and stippled lines) by flow cytometry. Data represent mean values ± SDs of 3 separate experiments. (C) CFHR1 inhibits C5/C5b deposition on sheep erythrocytes (v) that were incubated in human CFH- and C8-depleted plasma ($HP_{\Delta CFH\text{-}\Delta C8}$). CFHR1 does not effect C3b deposition (ii). Factor H (CFH) inhibits both C3b and C5/C5b deposition (iii and vi). Bars represent 10 μm. (D) CFHR1 inhibits C5 convertase activity. Recombinant CFHR1 (column 3) as well as plasma-purified CFHR1 (column 4) inhibit the C5 convertase and cleavage of C5. The C5 convertase was generated on the surface of sheep erythrocytes using purified C3, factor B, and factor D in the presence of Ni^{2+} and properdin. Cleavage and C5a generation was determined by ELISA. CFH also affected C5a generation (column 5) in contrast to BSA (column 6). Data represent mean values of 3 separate experiments and SDs are indicated. $^*P < .05$, $^{**}P < .005$ versus BSA.

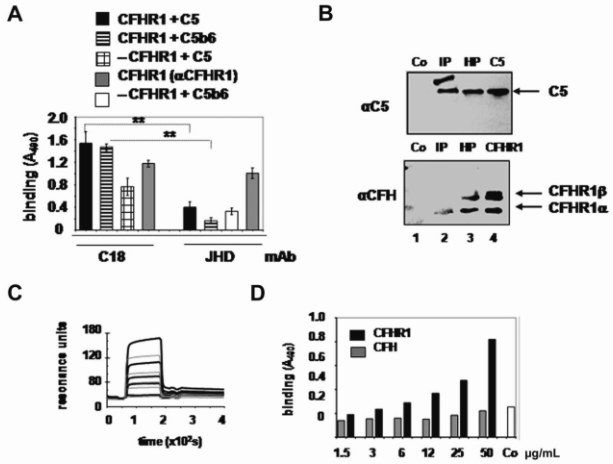

Figure 4. CFHR1 but not CFH binds to C5. (A) CFHR1 was captured to the surface of a microtiter plate using the C-terminal (C18) or the N-terminal (JHD10) binding mAbs. C5 or C5b6 was applied with the fluid phase, and bound proteins were detected with mAb to C5. Attachment of CFHR1 to C18 or JHD10 was verified with polyclonal CFHR1 antiserum (columns 4 and 8). In addition, binding of C5b6 to the mAb JHD10 in the absence of CFHR1 was assayed and was at background levels (column 7). Data represent mean values ± SD of 3 separate experiments. $^{**}P < .001$ versus binding of C5 or C5b6 to C18-mediated CFHR1 immobilization. (B) Formation of native CFHR1/C5 complexes in plasma was identified by immunoprecipitation. C5 antibodies were used to capture C5 complexed with CFHR1 from human plasma as shown by Western blot using polyclonal CFH antibodies (bottom panel, lane 2, arrows) and monoclonal C5 antibodies (top panel, lane 2, arrow). CFHR1 in human plasma (HP, bottom panel, lane 3) or plasma-purified CFHR1 (bottom panel, lane 4) shows similar mobilities. In addition, C5 in human plasma (top panel, lane 3) or purified C5 (top panel, lane 4) shows similar mobilities compared with the immunoprecipitated C5 protein (top panel, lane 2). Eluates derived from noncoated columns incubated with HP contain neither CFHR1 (bottom panel, lane 1) nor C5 (top panel, lane 1). The extra band in the top panel, lane 1, is considered unspecific. (C) Binding of C5 to immobilized CFHR1. CFHR1 (black line) or CFH/SCR10-13 (gray line) was immobilized to the surface of a sensor chip and C5 (50, 150, 200, 400 nM) was added in the fluid phase. (D) Binding of C5 to immobilized CFHR1 and CFH. CFHR1 (10 μg/mL) and CFH (30 μg/mL) were immobilized to a microtiter plate and incubated with increasing concentrations of C5 (1.5-50 μg/mL). Binding of C5 was detected with monoclonal C5 antibodies. Co represents reactivity of the mAb C5 to immobilized CFHR1 in the absence of C5. Representative data from 2 independent experiments are shown.

stippled line). In the presence of CFH, which acts on the C3 convertase, no further progression to the level of C5 convertase occurs (Figure 3B stippled blue line). These results show that CFHR1 controls complement activation at the level of C5.

The inhibitory effect of CFHR1 on C5b but not C3b generation was further investigated by analyzing surface deposition of C5/C5b and C3b on sheep erythrocytes by microscopy. Again, CFHR1 inhibited C5/C5b but not C3b surface deposition (Figure 3Cii,iv). The C3 convertase inhibitor CFH affected both C3b and C5/C5b deposition (Figure 3Ciii,vi). These results confirm that CFHR1 inhibits the C5 convertase of the alternative pathway and prevents C5 cleavage, as demonstrated by reduction of C5/C5b deposition and C5a generation.

To confirm the CFHR1 inhibitory effects on the C5 convertase, the C5 convertase was generated in vitro using purified complement components. Activity of the convertase was demonstrated by an increase of C5a levels upon incubation of convertase-loaded sheep erythrocytes with C5 (Figure 3D column 2). Both recombinant and plasma-purified CFHR1 (supplemental Figure 1C) reduced C5a generation (Figure 3D columns 3-4). Similarly, C5 cleavage was reduced by CFH (column 5) but not by BSA (Figure 3D column 6). These results confirm that CFHR1 inhibits the C5 convertase and reduces C5 cleavage.

CFHR1 binds C5 and C5b6

CFHR1 inhibits the alternative pathway C5 convertase, therefore direct binding of CFHR1 to C5 and to the activation product C5b6 was studied. CFHR1 was captured with mAbs that bind to either the C-terminus (C18) or the N-terminus (JHD10). C5 and C5b6 bound to CFHR1, which was immobilized via the C-terminus and that has the N-terminal SCRs accessible (Figure 4A columns 1-2). Reduced binding of C5 or C5b6 to CFHR1 was observed when CFHR1 was immobilized via JHD10, which binds the N-terminal epitope and has the C-terminus accessible (Figure 4A columns 5-6). These results indicate that the N-terminal region of CFHR1 binds C5 and C5b6.

To analyze interaction of CFHR1 with C5 in plasma, CFHR1/C5 complex formation in plasma was analyzed by immunoprecipitation. Normal human plasma was incubated with a C5 monoclonal antibody that was bound to a protein A matrix. Bound complexes were eluted from the matrix, separated by SDS-PAGE, and transferred to a membrane, and C5 and CFHR1 were identified by Western blotting. CFHR1 forms complexes with C5 as demonstrated by the presence of both proteins CFHR1 and C5 in the immunoabsorbed sample (Figure 4B lane 2; compare top panel for C5 and bottom panel for CFHR1). An eluate derived from a CFHR1 or C5 lacking sample showed the absence of either protein (Figure 4B lane 1, Co).

Interaction between CFHR1 and C5 was further confirmed by surface plasmon resonance and by ELISA. CFHR1 showed binding to C5 as indicated by dose-dependent association and reduced dissociation of CFHR1 to C5 (black lines). In contrast to CFHR1, the CFH fragment including SCR10-13 (gray lines) did not interact with C5 (Figure 4C). C5 binding to CFHR1 or CFH was also measured by ELISA and demonstrated dose-dependent binding to CFHR1 but no interaction with CFH (Figure 4D). Thus the interaction with C5 represents a distinct characteristic of CFHR1.

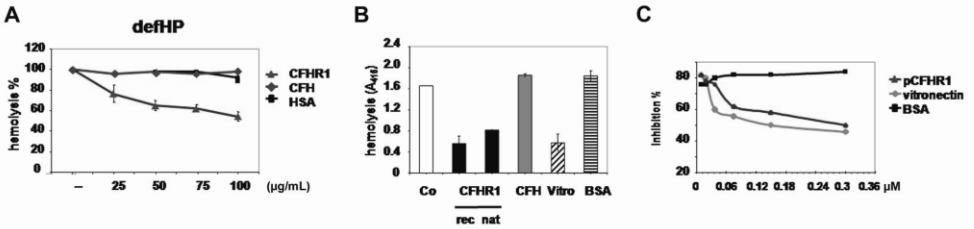

Figure 5. CFHR1 inhibits nonenzymatic steps of the terminal complement pathway. (A) Chicken erythrocytes were incubated with C5b6 complexes (5 ng/mL) and increasing concentrations of CFHR1, and nonlytic complement inactive defHP was added as a source for terminal complement components. MAC formation was assayed by lyses of erythrocytes. Hemolysis was recorded after 30 minutes by measuring the absorbance at 415 nm. Increasing concentrations of CFHR1 (25-100 μg/mL) affected MAC activity, and CFH or human serum albumin (HSA) showed no effect. Data represent mean values in percentage ± SD derived from 3 independent assays. (B) MAC formation on sheep erythrocytes was induced by incubation with C5b6, C7, C8, and C9 components and detected by hemolysis of cells (column 1). Preincubation of C5b6 with recombinant CFHR1 (50 μg/mL) or plasma-purified CFHR1 (12.5 μg/mL) inhibited hemolysis (columns 2 and 3, respectively). CFH (12.5 μg/mL) showed no effect on MAC formation (column 4), but vitronectin did (12.5 μg/mL; column 5). BSA (12.5 μg/mL) did not induce hemolysis (column 6). Data represent mean values ± SD of 3 separate experiments (except for plasma-purified CFHR1). (C) Inhibitory role of CFHR1 on MAC formation. CFHR1 purified from human plasma (0.1-0.3 μM) inhibited MAC formation on the surface of sheep erythrocytes (red triangles, red line). In addition, the established MAC inhibitor vitronectin was assayed (blue diamond, blue line). CFHR1 and vitronectin had similar activity and BSA did not affect MAC formation (black squares, black line). A representative experiment of 2 is shown.

CFHR1 prevents nonenzymatic assembly of MAC

Cleavage of C5 is the last enzymatic reaction of the complement cascade, and MAC complexes are formed by conformational changes and protein assembly. Therefore we asked whether CFHR1 might inhibit MAC assembly and deposition on the lipid bilayer. To this end, chicken erythrocytes were incubated with increasing amounts of CFHR1 together with C5b6 and added to nonlytic, complement inactive HP deficient of CFHR1 and CFHR3, which was used as a source for C7 to C9. MAC formation was followed by assaying lysis of erythrocytes. CFHR1 inhibited lysis in a dose-dependent manner; at 70 μg/mL FHR1 reduced lysis by 38% (Figure 5A red triangles). This inhibitory effect was specific for CFHR1 and was observed neither for CFH nor for BSA (Figure 5A blue diamonds and black squares, respectively). These data identify a second regulatory function of CFHR1: inhibition of MAC assembly or formation.

This complement regulatory activity was confirmed for native plasma-derived CFHR1 (supplemental Figure 1B lanes 3 and 6). Sheep erythrocytes, which are incubated with the terminal complement components C5b6, C7, C8, and C9, are lysed (Figure 5B column 1). Preincubation of C5b6 complexes with recombinant (50 μg/mL) or native (12.5 μg/mL) CFHR1 proteins inhibited hemolysis (Figure 5B columns 2-3). Similarly, preincubation of C5b6 complexes with the MAC inhibitor vitronectin (12.5 μg/mL) inhibited hemolysis (Figure 5B column 5). In this setup, CFH (12.5 μg/mL; column 4) or BSA (12.5 μg/mL; Figure 5D column 6) had no effect. The inhibitory activity of plasma-purified CFHR1 and vitronectin on MAC assembly was compared. Both proteins were added to C5b6 and the terminal components C7, C8, and C9 and then to erythrocytes. Plasma-derived CFHR1 and vitronectin showed similar inhibitory effects on sheep erythrocyte lysis (Figure 5C). These data demonstrate that both recombinant and plasma-derived CFHR1 block MAC formation.

CFHR1- and CFHR3-deficient serum reduces viability of human nucleated cells

As CFHR1 and CFHR3 deficiency increases the risk for aHUS,[12] we asked whether the lack of CFHR1 in plasma may result in enhanced complement activation on the surface of nucleated human cells. To this end the metabolic activity of human endothelial cells (HUVECs) upon complement challenge was monitored after uptake of the nonfluorescent substrate resazurin by following the intracellular conversion to the fluorescent dye resorufin. Cellular vitality results in a typical metabolic response that is followed by fluorescence generation (supplemental Figure 4, HP). However, when cells were incubated in complement active, CFHR1/CFHR3-deficient HP (defHP), derived from 3 healthy individuals, cell viability was reduced, as shown by the slower turnover of the substrate (supplemental Figure 4, defHP). When plasma from 3 CFHR1/CFHR3-deficient HUS patients was assayed, cell vitality and metabolic activity were significantly reduced (supplemental Figure 4, defHP, -aAb, HUS). These data indicate that the absence of CFHR1 and CFHR3 results in reduced cell vitality, likely due to inappropriate control of complement activation at the surface of nucleated human endothelial cells.

Discussion

Here we identify CFHR1 as a novel human complement regulator. CFHR1 is a human plasma protein, composed of 5 SCR domains, with 2 functional regions. The N-terminus (ie, SCR1-2) binds C5 and C5b6, and the C-terminus (ie, SCR3-5) binds C3b/C3d and heparin and to host cells. CFHR1 is a complement regulator that controls the activity of the C5 convertase and also assembly and membrane insertion of MAC. This is—to our knowledge—the first description of a regulator of the C5 convertase that does not affect the C3 convertase. CFHR1 and CFH have almost identical C-terminal surface binding regions and the 2 proteins bind to the same ligands (ie, C3b and heparin) and colocalize at the surface of endothelial cells (Figure 1C-D). This simultaneous binding suggests a sequential and coordinated action at the cell surface. As CFHR1 mutations and absence of the protein in plasma are linked to renal and retinal diseases, such as HUS and AMD, the characterization of CFHR1 as a complement regulator deepens our understanding on the molecular mechanisms leading to pathology.

The CFHR1 cDNA identified in 1991 represented the first member of the group of CFH-related plasma proteins.[17,18,28] Each of the known 5 CFHR proteins is encoded by a unique gene, which is located adjacent to the *Factor H* gene on human chromosome 1.[29] The 5 CFHR proteins show immune cross-reactivity, and individual domains have significant sequence identity to domains of CTH. The 2 N-terminal SCRs of CFHR1 (ie, SCR1 and SCR2) show 36% and 45% sequence identity to

SCR6 and SCR7 of CFH, respectively. The 3 C-terminal SCRs are almost identical to the C-terminus of CFH, except for the residues L290 and A296 in SCR5 of CFHR1 that correspond to S1191 and V1197 in SCR20 of CFH, respectively. The CFHR1 plasma concentration is approximately 70 to 100 μg/mL (1.6 to 2.4 μM; data not shown) and thus comparable with the concentrations of the terminal complement components C5 to C9. Due to its association with high-density lipoproteins,[19] the concentration of CFHR1 in the circulation might actually be higher.

CFHR1 is a complement regulator acting in the late alternative and early terminal complement pathway: (1) CFHR1 is—to our knowledge—the first human regulator of the C5 convertase of the alternative pathway that does not inhibit the C3 convertase. CFHR1 at physiologic concentrations inhibits C5 cleavage and prevents C5a generation (Figure 3). This effect is in agreement with C3b and C5 binding, suggesting that CFHR1 binds C3b and C5 simultaneously and thus may contact the C5 convertase (C3bBbC3b) and the substrate C5 at the same time.[4,30] CFHR1 regulates C5 convertase activity and inhibits further complement activation. The physiologic effects are inhibition of C5a and C5b generation. Thus, formation of the potent anaphylactic peptide C5a is blocked and also MAC complex formation and cytolysis.[30-32]

(2) CFHR1, but not CFH, inhibits assembly of C5b6(7) complexes and prevents surface attachment (Figures 3-5). CFHR1 may act in concert with the soluble terminal pathway inhibitors clusterin[33] and vitronectin.[34] Binding and inhibitory activity on the C5b6 complexes is independent of convertase activity (Figure 5). These complement regulatory functions could also be the reason why pathogens such as *Candida albicans, Aspergillus fumigatus,* or *Pseudomonas aeruginosa* bind CFHR1 to their surfaces.[35-37]

(3) The C-terminus of CFHR1, similar to that of CFH, binds to C3b, heparin, and cell surfaces (Figure 1, supplemental Figure 2). Thus CFHR1 likely has the capacity to discriminate between self- and foreign surfaces. The 3 C-terminal SCRs of CFHR1 bind immobilized C3b with lower affinity compared with the 3 C-terminal SCRs of CFH (K_D 6.4×10^{-6} vs 2.6×10^{-6} M; supplemental Figure 2C-D). The lower affinity of CFHR1 to C3b confirms previous results that demonstrate reduced heparin and cell surface binding of a HUS-associated CFH mutant, which encompasses the CFHR1-specific residues at positions 1191 and 1197.[21,38] CFHR1 competes with CFH at a heparin surface and thus reduces local CFH-mediated regulatory activity (Figure 1F). CFHR1 may replace CFH at surfaces, and reduction of CFH-mediated C3 convertase inhibition is for the gain of C5 regulatory activity. This balance may explain the opposing effects of CFHR1 deficiency: risk in HUS versus protection in AMD.

CFHR1 and CFHR3 deficiency is a predisposing factor for aHUS,[12] correlates with the presence of autoantibodies to CFH,[14] and defines a new subgroup of aHUS in children termed DEAP HUS (*de*ficiency of CFHR proteins and CFH *a*utoantibody *p*ositive).[14] The risk effect of CFHR1 deficiency in aHUS suggests that the coordinated action of the 2 regulators CFHR1 and CFH is required for surface integrity in situations of complement stress. Both CFHR1 and CFH inhibit complement activation, prevent cell lysis of sheep erythrocytes, and protect human endothelial cells (Figure 2 and supplemental Figure 4). Thus the absence of CFHR1 may result in enhanced complement activation on host cell surfaces leading to endothelial cell or platelet damage and to pathology (supplemental Figure 5). This effect is in agreement with the recent detection of CFHR1 in dense deposits of patients with dense deposit disease (MPGNII), suggesting that CFHR1 plays a role in this renal disease.[39] However, additional investigations with more patient samples are necessary to define the pathomechanism.

Deficiency of CFHR1 and CFHR3 is a risk factor in HUS, yet has a protective effect in AMD. Currently the reasons for these opposing effects are poorly understood. Based on the presented data, we hypothesize that the unique functions of CFHR1 and CFH are responsible for this difference. CFH is a regulator of the C3 convertase and promotes degradation of C3b and opsonization of a particle with C3b that results in phagocytosis. CFHR1 controls the later steps of the complement activation, and regulates C5 convertase activity and early MAC assembly. Thus CFHR1 blocks C5a formation and consequently inhibits inflammation. Therefore, in the absence of CFHR1 and CFHR3, local CFH binding and activity are increased, resulting in enhanced iC3b deposition and likely phagocytosis of opsonized particles. In the retina, this scenario may be advantageous for the clearance of cellular debris. The prevalence, especially of AMD, is growing, in the background of increasing longevity of the population. Starting to resolve the functional role of the disease-associated protein CFHR1 and of CFHR3 is a further step to define the underlying biologic mechanism of the complement system for the development of disorders such as AMD, HUS, and MPGN.

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Authorship

Contribution: S. Heinen, A.H., N.L., U.W., H.-M.D., S.S., K.G., T.E., S. Hälbich, and M.M. performed experiments and discussed results; R.W. generated the monoclonal antibody JHD10; U.S.-S. performed immunohistology; and P.F.Z. and C.S. conceived and directed the study and wrote the paper.

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References

1. Janeway CA Jr, Medzhitov R. Innate immune recognition. *Annu Rev Immunol.* 2002;20:97-216.
2. Walport MJ. Complement: first of two parts. *N Engl J Med.* 2001(14);344:1058-1066.
3. Walport MJ. Complement: second of two parts. *N Engl J Med.* 2001(15);344:1140-1144.
4. Rawal N, Pangburn MK. Formation of high-affinity C5 convertases of the alternative pathway of complement. *J Immunol.* 2001(3);166:2635-2642.
5. Morgan BP, Harris CL. Complement Regulatory Proteins. San Diego, CA: Academic Press; 1999.
6. Zipfel PF, Heinen S, Józsi M, Skerka C. Complement and diseases: defective alternative pathway control results in kidney and eye diseases. *Mol Immunol.* 2006(1-2);43:97-106.
7. de Córdoba SR, de Jorge EG. Translational mini-review series on complement factor H: genetics and disease associations of human complement factor H. *Clin Exp Immunol.* 2008(1);151:1-13.

8. Atkinson JP, Goodship TH. Complement factor H and the hemolytic uremic syndrome. *J Exp Med.* 2007(6);204:1245-1248.
9. Appel GB, Cook HT, Hageman G, et al. Membranoproliferative glomerulonephritis type II (dense deposit disease): an update. *J Am Soc Nephrol.* 2005(5);16:1392-1403.
10. de Jong PT. Age-related macular degeneration. *N Engl J Med.* 2006(14);355:1474-1485.
11. Saunders RE, Abarrategui-Garrido C, Frémeaux-Bacchi V, et al. The interactive Factor H-atypical hemolytic uremic syndrome mutation database and website: update and integration of membrane cofactor protein and Factor I mutations with structural models. *Hum Mutat.* 2007(3);28:222-234.
12. Zipfel PF, Edey M, Heinen S, et al. Deletion of complement factor H-related genes CFHR1 and CFHR3 is associated with atypical hemolytic uremic syndrome. *PloS Genet.* 30041(2007);3:e41.
13. Hughes AE, Orr N, Esfandiary H, et al. A common CFH haplotype, with deletion of CFHR1 and CFHR3, is associated with lower risk of age-related macular degeneration. *Nat Genet.* 2006; 38(10):1173-1177.
14. Józsi M, Licht C, Strobel S, et al. Factor H autoantibodies in atypical hemolytic uremic syndrome correlate with CFHR1/CFHR3 deficiency. *Blood.* 2008;111(3):1512-1514.
15. Józsi M, Strobel S, Dahse HM, et al. Anti factor H autoantibodies block C-terminal recognition function of factor H in hemolytic uremic syndrome. *Blood.* 2007;110(5):1516-1518.
16. Ståhl AL, Vaziri-Sani F, Heinen S, et al. Factor H dysfunction in patients with atypical hemolytic uremic syndrome contributes to complement deposition on platelets and their activation. *Blood.* 2008;111(11):5307-5315.
17. Skerka C, Horstmann RD, Zipfel PF. Molecular cloning of a human serum protein structurally related to complement factor H. *J Biol Chem.* 1991; 266(18):12015-12020.
18. Schwaeble W, Feifel E, Estaller C, et al. Human complement factor H: molecular cloning and cDNA expression reveals variability in the factor H-related mRNA species of 1.4 kb. *Immunobiology.* 1991;182(3-4):307-322.
19. Park CT, Wright SD. Plasma lipopolysaccharide-binding protein is found associated with a particle containing apolipoprotein A-I, phospholipid, and factor H-related proteins. *J Biol Chem.* 1996; 271(30):18054-18060.
20. Timmann C, Leippe M, Horstmann RD. Two major serum components antigenically related to complement factor H are different glycosylation forms of a single protein with no factor H-like complement regulatory functions. *J Immunol.* 1991;146(4):1265-1270.
21. Heinen S, Sanchez-Corral P, Jackson MS, et al. De novo gene conversion in the RCA gene cluster (1q32) causes mutations in complement factor H associated with atypical hemolytic uremic syndrome. *Hum Mutat.* 2006;27(3):292-293.
22. Oppermann M, Manuelian T, Józsi M, et al. The C-terminus of complement regulator Factor H mediates target recognition: evidence for a compact conformation of the native protein. *Clin Exp Immunol.* 2006;144(2):342-352.
23. Manuelian T, Hellwage J, Meri S, et al. Mutations in factor H reduce binding affinity to C3b and heparin and surface attachment to endothelial cells in hemolytic uremic syndrome. *J Clin Invest.* 2003; 111(8):181-190.
24. Skerka C, Lauer N, Weinberger AA, et al. Defective complement control of factor H (Y402H) and FHL-1 in age-related macular degeneration. *Mol Immunol.* 2007;44(13):3398-3406.
25. Lehmann, EH. *Nonparametrics: Statistical Methods based on Ranks.* San Francisco, CA; Holden Day: 1975;233.
26. Pangburn MK. Cutting edge: localization of the host recognition functions of complement factor H at the carboxyl-terminal: implications for hemolytic uremic syndrome. *J Immunol.* 2002;169(9): 4702-4706.
27. Heinen S, Józsi M, Hartmann A, et al. Hemolytic uremic syndrome: a factor H mutation (E1172Stop) causes defective complement control at the surface of endothelial cells. *J Am Soc Nephrol.* 2007;18(2):506-514.
28. Zipfel PF, Skerka C. Complement factor H related proteins in immune diseases. *Vaccine.* 2008; 26(8):109-114.
29. Male DA, Ormsby RJ, Ranganathan S, Giannakis E, Gordon DL. Complement factor H: sequence analysis of 221 kb of human genomic DNA containing the entire fH, fHR-1 and fHR-3 genes. *Mol Immunol.* 2000(1-2);37:41-52.
30. Rawal N, Pangburn MK. C5 convertase of the alternative pathway of complement: kinetic analysis of the free and surface-bound forms of the enzyme. *J Biol Chem.* 1998;273(27):16828-16835.
31. Haas PJ, van Strijp J. Anaphylatoxins: their role in bacterial infection and inflammation. *Immunol Res.* 2007;37(3):161-175.
32. Guo RF, Ward PA. Role of C5a in inflammatory responses. *Annu Rev Immunol.* 2005;23:821-852.
33. Jenne DE, Tschopp J. Clusterin: the intriguing guises of a widely expressed glycoprotein. *Trends Biochem Sci.* 1992;4(4):154-159.
34. Podack ER, Preissner KT, Müller-Eberhard HJ. Inhibition of C9 polymerization within the SC5b-9 complex of complement by S-protein. *Acta Pathol Microbiol Immunol Scand Suppl.* 1984;284:89-96.
35. Poltermann S, Kunert A, von der Heide M, Eck R, Hartmann A, Zipfel PF. Gpm1p is a factor H-, FHL-1-, and plasminogenbinding surface protein of Candida albicans. *J Biol Chem.* 2007;282(52): 37537-37544.
36. Behnsen J, Hartmann A, Schmaler J, Gehrke A, Brakhage AA, Zipfel PF. The opportunistic human pathogenic fungus Aspergillus fumigatus evades the host complement system. *Infect Immun.* 2008;76(2):820-827.
37. Kunert A, Losse J, Gruszin C, et al. Immune evasion of the human pathogen Pseudomonas aeruginosa: elongation factor Tuf is a factor H and plasminogen binding protein. *J Immunol.* 2007;179(5):2979-2988.
38. Józsi M, Heinen S, Sanchez-Corral P, et al. Factor H and atypical hemolytic uremic syndrome: mutations in the C-terminus cause structural changes and defective recognition functions. *J Am Soc Nephrol.* 2006;17(1):170-177.
39. Sethi S, Garnez JD, Vrana JA, et al. Glomeruli of Dense Deposite Disease contain components of the alternative and terminal complement pathway. *Kidney Int.* 2009;75(9):952-960.

5 An imbalance of human complement regulatory proteins CFHR1, CFHR3 and factor H influences risk for age-related macular degeneration.

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An imbalance of human complement regulatory proteins CFHR1, CFHR3 and factor H influences risk for age-related macular degeneration (AMD)

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A frequent deletion of complement factor H (*CFH*)-related genes *CFHR3* and *CFHR1* (Δ*CFHR3/CFHR1*) is considered to have a protective effect against age-related macular degeneration (AMD), although the underlying mechanism remains elusive. The deletion seems to be linked to one of the two protective *CFH* haplotypes which are both tagged by the protective allele of single nucleotide polymorphism rs2274700 (CFH:A473A). In a German cohort of 530 AMD patients, we now show that protection against AMD conferred by Δ*CFHR3/CFHR1* is independent of the effects of rs2274700 and rs1061170 (CFH:Y402H). This suggests a functional role of CFHR1 and/or CFHR3 in disease pathogenesis. We therefore characterized the CFHR3 function and identified CFHR3 as a novel human complement regulator that inhibits C3 convertase activity. CFHR3 displays anti-inflammatory effects by blocking C5a generation and C5a-mediated chemoattraction of neutrophils. In addition, CFHR3 and CFHR1 compete with factor H for binding to the central complement component C3. Thus, deficiency of CFHR3 and CFHR1 results in a loss of complement control but enhances local regulation by factor H. Our findings allude to a critical balance between the complement regulators CFHR3, CFHR1 and factor H and further emphasize the central role of complement regulation in AMD pathology.

INTRODUCTION

Age-related macular degeneration (AMD, OMIM #603075) is a prevalent cause of visual impairment in older individuals of industrialized countries (1). Several studies have identified the complement factor H (*CFH*) gene on chromosome 1q31 as a major AMD susceptibility gene (2–5). At this locus, the common single nucleotide polymorphism (SNP) rs1061170:T>C (Y402H) was identified as the putative risk variant. A plethora of studies have replicated that these initial findings and initial reports on functional consequences of the Y402H amino acid change demonstrated reduced heparin and CRP binding of the risk variant (6–9). However, the potential risk modifying the role of the common protective haplotypes at the *CFH* locus is still unknown.

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A common copy number polymorphism (CNP) downstream of *CFH* encompasses an 84 kb region that includes the *CFH*-related genes *CFHR3* and *CFHR1*. This CNP (Δ*CFHR3/CFHR1*) is of particular interest as the corresponding deletion is reported to exclusively occur on one of the two common protective *CFH* haplotypes. This possibly highlights an independent mechanism of action unrelated to the CFH:His402 risk variant (10–12). It is unclear, however, whether the deletion carrying haplotype alters AMD risk independently of rs1061170 (10), or whether the deletion itself has a protective effect at least partially caused by the strong causative effect of the rs1061170:C allele (11). Interestingly, the Δ*CFHR3/CFHR1* also represents a risk factor for an autoimmune form of the severe kidney disease atypical hemolytic uremic syndrome, called DEAP-HUS (13,14). Most HUS patients with this chromosomal deletion have auto-antibodies to factor H. These auto-antibodies bind to the C-terminal region of factor H and consequently inhibit surface binding, resulting in inappropriate protection of endothelial cells as well as platelets (15,16).

CFHR3 and *CFHR1* are members of the factor H gene family and are located telomeric to *CFH* on human chromosome 1 (17,18). Each of the known five *CFHR* genes (*CFHR1–CFHR5*) encodes a unique plasma protein. CFHR3 and CFHR1 are both composed of five domains, termed short consensus repeat (SCR) (Supplementary Material, Fig. S1A) (19). The last two SCRs of CFHR3 show a high degree of amino acid identity to SCR6 and SCR7 of factor H (96%) and the three C-terminal SCRs of CFHR1 have almost the identical amino acid sequence as SCR18-20 of factor H (98%), indicating related functions of the CFHR proteins. CFHR3 is a human plasma (HP) protein, which is expressed in four glycosylated forms with molecular masses ranging from 45 to 56 kDa. Similarly, CFHR1 appears in two glycosylated forms of 41 and 43 kDa (Supplementary Material, Fig. S1B) (20,21). The plasma concentration of CFHR3 is 50–80 μg/ml (1–1.6 μM) (data not shown) and that of CFHR1 70–100 μg/ml (1.6–2.4 μM) (7). CFHR1 is associated with high-density lipoproteins and therefore the concentration of CFHR1 in the circulation might be even higher (22). CFHR1 represents a complement regulator that acts downstream of factor H by inhibiting the C5 convertase and the assembly and membrane insertion of the terminal complement complex (TCC) (7,23). CFHR1 lacks cofactor and decay accelerating activity on the C3 convertase. The specific function of CFHR3 is currently unknown.

By completing our previously established case–control data set of *CFH* haplotypes with the CNP genotypes, we specifically were interested to correlate the common *CFH* haplotypes with the Δ*CFHR3/CFHR1* data. To further determine the impact of the CNV and polymorphisms on AMD risk, we also included rs1061170 and rs2274700:G>A (CFH:A473A), two of the most pronounced factor H risk determinants in a logistic regression calculation (3,4,10,24,25). As Δ*CFHR3/CFHR1* represents an excellent candidate for a functional variant, we further aimed to define the function of the CFHR3 protein, identify its role in complement activation and compare the function of CFHR3 with that of CFHR1 and factor H.

We now confirm that Δ*CFHR3/CFHR1* confers a reduced risk for AMD. Furthermore, we show that this protective effect is independent of both risk variants rs1061170 and rs2274700, indicating a functional role of CFHR3 and/or CFHR1 in AMD. Subsequent investigations identified CFHR3 as a novel human complement regulator of the C3 convertase that acts in concert with CFHR1 and factor H. Imbalances between CFHR3, CFHR1 and factor H levels may have functional effects and shift the delicate balance of complement control and activation. Such an imbalance may explain the risk-modifying character of one of the protective haplotypes, namely Δ*CFHR3/CFHR1*.

RESULTS

Δ*CFHR3/CFHR1* is in strong linkage disequilibrium with *CFH* SNPs rs1061170, rs2274700 and rs412852

To assess linkage disequilibrium (LD) between *CFH* SNPs and Δ*CFHR3/CFHR1*, we resorted to *CFH* genotypes determined previously in 110 AMD patients and 67 controls (26) and additionally established the CNP status for Δ*CFHR3/CFHR1* by multiplex ligation-dependent probe amplification (Supplementary Material, Table S1). In this cohort, we observed LD between the three *CFH* SNPs such as rs1061170, rs2274700, rs412852 and Δ*CFHR3/CFHR1* but not between Δ*CFHR3/CFHR1* and SNPs such as rs800292, rs1048663 and rs11582939 (Fig. 1) (26).

Haplotype analysis revealed Δ*CFHR3/CFHR1* to occur predominantly on one of the two protective haplotypes (P2) (Supplementary Material, Table S2) confirming previous studies (11,12). To further dissect the dependencies between Δ*CFHR3/CFHR1* and the major risk variant rs1061170 as well as the major protective variant rs2274700 (3,10,24,25), we increased the sample size by genotyping an additional 420 AMD patients and 246 matched controls for Δ*CFHR3/CFHR1* and the two SNPs rs1061170 and rs2274700. The frequency of Δ*CFHR3/CFHR1* in the combined sample of 530 AMD patients and 313 control individuals was in perfect agreement with the data reported by Schmid-Kubista *et al.* (11) (Supplementary Material, Table S1). Rare findings of deletions of *CFHR3* only and of *CFHR1* only as well as duplications of *CFHR3/CFHR1* and *CFHR1* alone were identified and were consistent with previous reports (11,27) (Supplementary Material, Table S1). Individuals with gene duplications ($n = 3$) were excluded from further analyses.

Δ*CFHR3/CFHR1* is protective to AMD

Logistic regression analysis confirmed the reported protective effect of Δ*CFHR3/CFHR1* in the additive model [odds ratio (OR) = 0.36 (0.26–0.48), $P_{adj} = 3.6 \times 10^{-11}$]. When conditioned for rs1061170, the effect remained statistically significant and showed association with a reduced risk for AMD [OR = 0.50 (0.35–0.69), $P_{adj} = 3.5 \times 10^{-5}$], even after including rs2274700 [OR = 0.67 (0.45–0.99), $P_{adj} = 0.044$] (Table 1). Interestingly, when considering the *CFHR3* and *CFHR1* deletions separately, i.e. by adding individuals with Δ*CFHR3* only or Δ*CFHR1* only, we detected a stronger effect for Δ*CFHR1* compared with Δ*CFHR3* throughout the analyses (Table 1).

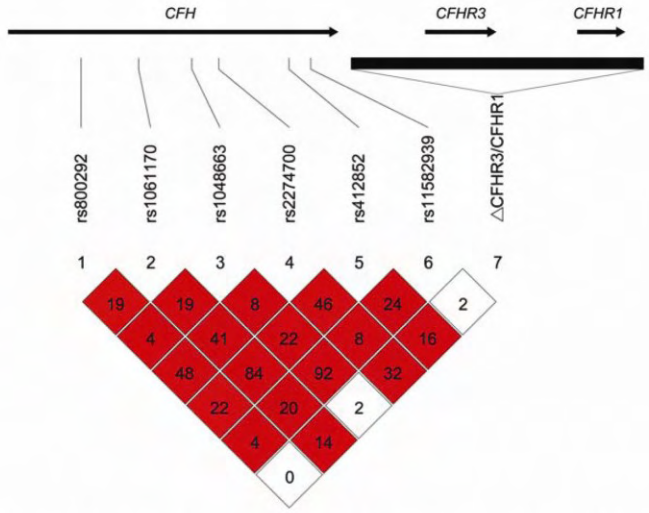

Figure 1. Pairwise LD between six *CFH* SNPs (rs800292, rs1061170, rs1048663, rs2274700, rs412852, rs11582939) and the *CFHR3/CFHR1* deletion (ΔCFHR3/CFHR1, black bar) in 110 AMD patients and 67 control individuals. Genes are depicted as black arrows above the plot. Values of r^2 are given within square shading, which indicates the strength of LD (white, low D'; red, high D').

The observed strong effects of the rs1061170:C allele [OR 2.19 (1.75–2.73); $P_{ADD} = 5.0 \times 10^{-12}$] and the rs2274700:A allele [OR 0.36 (0.29–0.47); $P_{ADD} = 6.1 \times 10^{-16}$] were in accordance with previous reports (Table 1) (10,25) which consistently show a stronger association between AMD and rs2274700 than with rs1061170. To estimate the unique effects of both major risk variants, we performed a conditional SNP analysis. Conditioning rs1061170 for rs2274700 revealed a marked risk reduction from an OR of 2.19 (1.75–2.73) to an OR of 1.34 (0.99–1.82) which then revealed only borderline association with AMD ($P_{ADD} = 0.057$). In contrast, rs2274700 conditioned for rs1061170 did not result in a similarly prominent risk alteration [OR 0.36 (0.29–0.47) to OR 0.45 (0.33–0.63)] and remained statistically significant ($P_{ADD} = 1.7 \times 10^{-6}$). This indicates that the protective effect of rs2274700 has a greater influence on AMD risk than rs1061170. Considering that the two haplotypes P1 and P2 are uniquely tagged by the rs2274700:A allele (Supplementary Material, Table S2), this suggests that the protective effect of rs2274700 represents a combined effect of the two common protective haplotypes.

In summary, our results show that Δ*CFHR3/CFHR1* is associated with a decreased risk for AMD, and is independent of the strong risk variant rs1061170. This indicates that the absence of CFHR1 and/or CFHR3 confers beneficial effects for an AMD risk. To clarify the role of CFHR3 and CFHR1 in complement regulation, we determined the function of the CFHR3 plasma protein and compared CFHR3 function with that of CFHR1 and factor H.

CFHR3 is a complement regulator

To define whether CFHR3 displays complement regulatory activities, hemolysis assays were performed with sheep erythrocytes and factor H depleted plasma (7). The effect of CFHR3 on complement-mediated hemolysis was analyzed by adding CFHR3 to factor H depleted, complement active plasma and sheep erythrocytes. CFHR3 reduced complement-mediated hemolysis of sheep erythrocytes by about 15% (Fig. 2A). This effect was comparable with that of CFHR1 or with the terminal complement pathway regulator vitronectin, which reduced lysis by 15 and 20%, respectively. Factor H inhibited hemolysis by 80% (Fig. 2A). The inhibitory effect of CFHR3 was dose dependent (Supplemental Material, Fig. S2). Recombinant CFHR3 and CFHR1 proteins are free of contaminating bands as demonstrated by silver staining (Supplemental Material, Fig. S1C). These data show that CFHR3 is a complement inhibitor.

CFHR3 neither dissociates nor 'freezes' activity of the C3 convertase

To define the regulatory function of CFHR3, the effect of CFHR3 on the C3 convertase activity was investigated. A C3 convertase was formed by incubating purified proteins C3b, factor B, factor D and properdin. Then the effect of CFHR3 used at increasing concentrations (10–50 μg/ml) on the C3 convertase stability was assayed by monitoring bound factor B. CFHR3 did not dissociate the C3 convertases as demonstrated by constant amounts of factor Bb bound to the preformed C3 convertase (Fig. 2B). Thus, CFHR3 lacks decay activity. Similarly, neither CFHR1 nor BSA did dissociate the preformed C3 convertases (Fig. 2B). As expected, factor H displayed decay acceleration activity and dissociated the C3bBb complex (Fig. 2B).

To determine whether CFHR3 'freezes' C3 convertase activity in a different way than by decay acceleration, the effect of CFHR3 on a preformed C3 convertase was measured

Table 1. Logistic regression analyses of rs1061170, rs2274700, Δ*CFHR3/CFHR1*, Δ*CFHR3* and Δ*CFHR1* using 530 AMD cases and 313 controls

Analyzed variation	Unadjusted		Adjusted for age, smoking, sex		Adjusted for age, smoking, sex and rs1061170		Adjusted for age, smoking, sex and rs2274700		Adjusted for age, smoking, sex, rs1061170 and rs2274700	
	OR (95% CI)	P_{ADD}	OR (95% CI)	P_{ADD}	OR (95% CI)	P_{ADD}	OR (95% CI)	P_{ADD}	OR (95% CI)	P_{ADD}
rs1061170:C	2.31 (1.86–2.85)	1.5×10^{-14}	2.19 (1.75–2.73)	5.0×10^{-12}	–	–	1.34 (0.99–1.82)	0.057	–	–
rs2274700:A	0.36 (0.28–0.45)	6.0×10^{-18}	0.36 (0.29–0.47)	6.1×10^{-16}	0.45 (0.33–0.63)	1.7×10^{-6}	–	–	–	–
Δ*CFHR3/CFHR1*	0.37 (0.28–0.49)	1.4×10^{-11}	0.36 (0.26–0.48)	3.6×10^{-11}	0.49 (0.35–0.69)	3.5×10^{-5}	0.67 (0.45–0.99)	0.044	0.67 (0.45–0.99)	0.044
Δ*CFHR3*	0.37 (0.28–0.49)	8.0×10^{-12}	0.36 (0.26–0.48)	2.7×10^{-11}	0.50 (0.36–0.70)	4.0×10^{-5}	0.67 (0.46–0.99)	0.045	0.67 (0.46–0.99)	0.045
Δ*CFHR1*	0.36 (0.28–0.48)	1.9×10^{-12}	0.35 (0.26–0.47)	3.5×10^{-12}	0.47 (0.34–0.66)	6.4×10^{-6}	0.60 (0.41–0.87)	0.0067	0.60 (0.42–0.88)	0.0077

The respective allele-specific OR (equal to the heterozygous genotype's OR) of the additive model is shown. The respective homozygote's OR can be directly obtained by the square of the heterozygote's OR.

by following C3a generation. In the presence of CFHR3, C3a levels remained constant and cleavage of C3 was unaffected (Fig. 2C). Also CFHR1 did not dissociate or 'freeze' the C3 convertase (Fig. 2C). In contrast in the presence of factor H, which exerts decay accelerating activity, C3a generation was reduced (Fig. 2C). The specificity of the effect was demonstrated by C3a generation in the absence of regulatory proteins and by the lack of cross-reactivity of the factor Bb antibody to C3b (Fig. 2C). Thus, CFHR3 does not 'freeze' C3 convertase activity upon cleavage of the substrate C3.

CFHR3 displays cofactor activity for the serine protease factor I (CFI)

As CFHR3 did not restrict C3 convertase activity, the role of CFHR3 as a cofactor for factor I in cleavage of C3b was investigated. CFHR3 was incubated with C3b and factor I, the reaction mixtures were separated by SDS–PAGE, transferred to a membrane and cleavage products of C3b were assayed. CFHR3 showed cofactor activity for factor I-mediated cleavage of C3b and cleavage products of 68 and 43 kDa as well as 41 kDa were generated. This effect was dose dependent (Fig. 2D). CFHR1 used at the same concentrations lacked cofactor activity and no C3b cleavage was detected (Fig. 2D, Table 2). Factor H, at low concentrations, displayed cofactor activity, and cleavage products of 68, 43 and 41 kDa were generated (Fig. 2D). Thus, CFHR3 acts as cofactor for factor I in the degradation of C3b.

CFHR3 and CFHR1 inhibit C5a generation

We next asked whether CFHR3 as a regulator of the C3 convertase affects C5a generation. C5b initiates the generation of the TCC, and C5a is a potent anaphylatoxin that attracts neutrophils to the side of infection and initiates and enhances inflammatory reactions. Thus, the role of CFHR3 on C5 convertase activity was followed by assaying C5a levels. CFHR3 was added to sheep erythrocytes that were incubated in complement active, factor H-depleted HP and C5a generation was monitored by ELISA. CFHR3 at a molar concentration of 1.6 μM reduced C5a generation by approximately 63% (Fig. 3A). Similarly, CFHR1 (1.8 μM) and factor H (0.5 μM) reduced C5a levels by about 60% (Fig. 3A).

CFHR3 and CFHR1 act anti-inflammatory

To explore whether the reduced C5a generation in response to CFHR3-mediated complement inhibition has a functional impact, the inflammatory effect of factor H-depleted plasma after addition of CFHR3 was assayed for neutrophil chemotaxis (Fig. 3A, column 2). CFHR3-treated plasma (1:4 dilution) inhibited neutrophil migration by 56% (Fig. 3B) when compared with BSA-treated plasma (Fig. 3B). Similarly, CFHR1- and factor H-treated plasma reduced neutrophil migration by 64% and by 81%, respectively (Fig. 3B). These data demonstrate that CFHR3 and CFHR1 reduce the generation of C5a and consequently block complement-mediated chemotaxis of neutrophils.

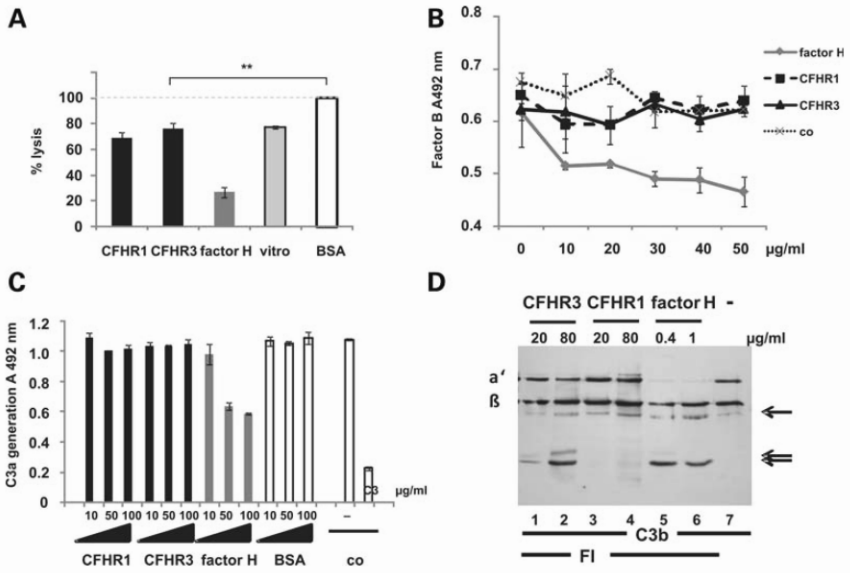

Figure 2. CFHR3 is a complement regulatory protein. (**A**) Factor H-depleted plasma was incubated with CFHR1 (80 μg/ml, 1.6 μM), CFHR3 (80 μg/ml, 1.8 μM), factor H (80 μg/ml, 0.5 μM), vitronectin (80 μg/ml, 1 μM) or with BSA (80 μg/ml) before addition of sheep red blood cells. Complement activation was determined by measuring hemolysis of erythrocytes at an absorbance of 415 nm. Data represent mean values of four independent assays. Hemolysis in depleted plasma with BSA was set to 100%. $^{**}P < 0.01$. (**B**) Decay of the C3 convertase by CFHR3 was determined by incubation of C3b with factor B and factor D in the presence of increasing concentrations (0–50 μg/ml) of CFHR3 (0–1 μM, triangles), CFHR1 (0–1.16 μM, squares), factor H (0–0.32 μM, diamonds), or BSA (crosses) and determination of bound factor Bb using a standard ELISA. Mean values ± standard deviations of three independent experiments are shown. (**C**) C3 convertase was generated by incubation of C3b with factor B, factor D and properdin and cleavage of C3 was followed in the presence of increasing concentrations (10–100 μg/ml) of CFHR3 (0.2–2 μM), CFHR1 (0.23–2.33 μM), factor H (0.06–0.64 μM) or BSA as indicated, by measuring generation of C3a by ELISA. Controls represent fluorescent signals of antibody binding to C3a or C3. Data represent mean values of three independent measurements. Standard deviations are shown. (**D**) C3b was incubated with factor I in the presence of 0.4 μM CFHR3 (lane 1) or 1.6 μM CFHR3 (lane 2), 0.4 μM CFHR1 (lane 3) or 1.8 μM CFHR1 (lane 4), 0.002 μM factor H (lane 5) or 0.006 μM factor H (lane 6), or without additional proteins (lane 7). Probes were separated by SDS–PAGE and immunoblotted using polyclonal C3b antiserum. Cleavage products of the C3b α′ chain are indicated by arrows.

CFHR3 and CFHR1 compete with factor H for binding to C3b

As CFHR3 like CFHR1 and factor H binds to C3b, we asked whether CFHR3 and CFHR1 affect factor H binding to C3b. Binding of constant amounts of factor H was assayed in the presence of increasing concentrations of CFHR3 or CFHR1. C3b bound proteins were quantified by ELISA using specific antibodies. CFHR3 competed with factor H, as indicated by decreased binding of factor H and increased binding of CFHR3 to C3b (Fig. 4A). Similarly, CFHR1 dose-dependently reduced factor H binding to C3b (Fig. 4B). Thus, CFHR3, CFHR1 and factor H, which bind to the same ligand C3b, compete for binding. This result also suggests a competitive binding to C3b in plasma.

CFHR3 and CFHR1 reduce factor H-mediated complement control

Based on the competitive binding of CFHR3, CFHR1 and factor H to C3b, we hypothesized that the absence of CFHR3 and CFHR1 in plasma influences factor H binding to C3b and thereby enhance complement regulation. Thus, decay acceleration activity of factor H was determined upon increasing the concentration of CFHR3 or CFHR1, which can replace factor H in C3b binding but which do not dissociate the C3 convertase. Decay acceleration activities were measured by bound factor Bb to the preformed C3 convertases. Fragment Bb binding was significantly increased when CFHR3 or CFHR1 was incubated with the preformed convertases in the presence of constant amounts of factor H (Fig. 4C). Thus, CFHR3 and CFHR1 modulate the C3 convertase activity in HP. In addition, the C3a level increased with elevated amounts of CFHR3 or CFHR1 in ΔCFHR3/CFHR1 plasma, but the level was reduced when factor H was added (Fig. 4D).

The modulating effect of CFHR3 and CFHR1 on complement activation was also tested in hemolytic assays using ΔCFHR3/CFHR1 plasma, chicken erythrocytes and increasing concentrations of CFHR3 or CFHR1. CFHR3 and CFHR1 significantly increased hemolysis of erythrocytes when compared with factor H or BSA (Fig. 4E).

DISCUSSION

Here, we show that the common CNP Δ*CFHR3/CFHR1* on chromosome 1q32 is protective for AMD. Moreover, we demonstrate that this effect is independent of the two prominent factor H risk determinants rs1061170 (CFH:Y402H, causative) and rs2274700 (CFH:A473A, protective). Functional

Table 2. CFHR3, CFHR1 and factor H exert specific complement regulatory activities

Activity	CFHR3	CFHR1	Factor H
Decay acceleration activity	−	−	+
C3 convertase activity	−	−	+
Cofactor activity	+	−	+
C5 convertase activity	(+)	+	(+)
TCC inhibition	nd	+	−

'+', effect; '−', no effect; nd, not determined; '()', secondary effect.

analyses allocate the risk-modifying mechanism of the CNP to the local balance between CFHR3, CFHR1 and factor H in complement regulation. CFHR3 and CFHR1 proteins are both complement regulators. CFHR3 acts as cofactor for factor I in inactivating C3b and CFHR1 inhibits the C5 convertase and the terminal complement pathway. Both CFHR3 and CFHR1 lack decay acceleration activity. The three proteins CFHR3, CFHR1 and factor H bind to similar sites on C3b and thus CFHR3 and CFHR1 compete with factor H for C3b binding. As a consequence, a homeostatic balance between CFHR3, CFHR1 and factor H determines complement activity which subsequently influences inflammation and progression of AMD.

The homozygous deletion of a chromosomal segment that includes gene loci *CFHR3* and *CFHR1* results in a complete deficiency of the corresponding proteins in plasma and was suggested a functional candidate for one of the two common protective *CFH* haplotypes (10,13). Furthermore, the associated AMD risk reduction in the corresponding haplotype was reported to be independent of the causative SNP rs1061170 which was confirmed in subsequent studies (12,28). These studies, however, indirectly assessed the impact of the deletion through its corresponding protective *CFH* haplotype. Most recently, Schmid-Kubista *et al*. (11) directly genotyped the Δ*CFHR3/CFHR1* in 252 AMD patients and 249 controls via MLPA. They confirmed that Δ*CFHR3/CFHR1* occurs on a single common protective *CFH* haplotype and the authors reasoned that the protective effect of Δ*CFHR3/CFHR1* may be partially explained by the absence of the rs1061170:C (His402) risk variant. When conditioning for rs1061170, Schmid-Kubista *et al*. (11) could not detect a statistically significant association between Δ*CFHR3/CFHR1* and AMD, thereby contradicting the findings of Hughes *et al*. (10).

In the present study, we genotyped Δ*CFHR3/CFHR1* in a large cohort of 530 AMD patients and 313 control persons. This provides sufficient statistical power, allowing the detection of an AMD protective effect of Δ*CFHR3/CFHR1* which remained statistically significant even after conditioning for rs1061170 and/or rs2274700. As a consequence, our findings suggest that Δ*CFHR3/CFHR1* by itself is an AMD risk factor independent of the known factor H variants.

Earlier studies defined rs2274700 as a major risk variant (3, 25), however our results clearly show that the minor allele of rs2274700 exerts a protective effect and occurs on two common protective haplotypes, but not on the common neutral or the common risk haplotypes. This is well in agreement with studies by Hughes *et al*. (10) and Spencer *et al*. (12)

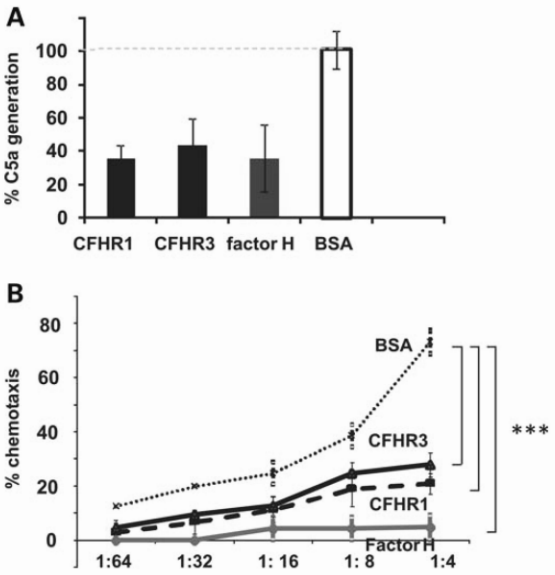

Figure 3. CFHR3 and CFHR inhibit C5a generation and complement-mediated chemotaxis of neutrophils. (**A**) CFHR3 inhibits complement activation and generation of C5a. Factor H-depleted plasma was incubated with CFHR1 (80 μg/ml, 1.6 μM), CFHR3 (80 μg/ml, 1.8 μM), factor H (80 μg/ml, 0.5 μM) or BSA and added to sheep red blood cells. C5a generation was determined by standard ELISA. Data are mean ± standard deviation of four independent experiments. C5a generation in BSA-treated plasma was set to 100%. (**B**) CFHR3 and CHFR1 inhibit complement-mediated migration of neutrophils. Serial dilutions of factor H-depleted plasma incubated with CFHR1 (1.6 μM), CFHR3 (1.8 μM), factor H (0.5 μM) or BSA were assessed for their potency to drive migration of BM-derived neutrophils. Data are mean ± standard deviation of three independent experiments and five measurements of each dilution, $^{***}P < 0.001$. Migration of neutrophils in response to factor H-treated plasma + buffer (1 × PBS) was set 100%.

who analyzed among other factor H variants, SNPs that were in high LD with rs2274700 ($r^2 > 0.8$) (25,29). Interestingly, when comparing the absolute effect sizes, rs2274700 appears to exceed the effect of rs1061170 (2.8-fold versus 2.2 risk alteration, respectively). Considering the minor allele frequencies of both risk variants revealed similar magnitude in the control sample (rs1061170: 40.6% versus rs2274700: 43.4%) thereby elevating this protective variant to the major risk determinant of this locus. Our association data suggest that the deletion or its tagged P2 haplotype explains part of the protective effect of rs2274700. Consequently, the remaining risk reduction might be due to the second protective haplotype P1 and eventually to its putatively functional SNP rs800292 (CFH:I62V). Nevertheless, we cannot exclude that both protective haplotypes together tagged by rs2274700 share a so far unknown, functional variant.

The Δ*CFHR3/CFHR1* deletion as an independent protective factor for AMD pathology suggests that CFHR3 and/or CFHR1 protein function confers risk to AMD. Therefore, we characterized CFHR3 functions and investigated how the absence of CFHR3 and CFHR1 proteins alter AMD risk. Here, we identify CFHR3 as a novel human complement regulator. CFHR3 acts as a cofactor for the serine protease factor

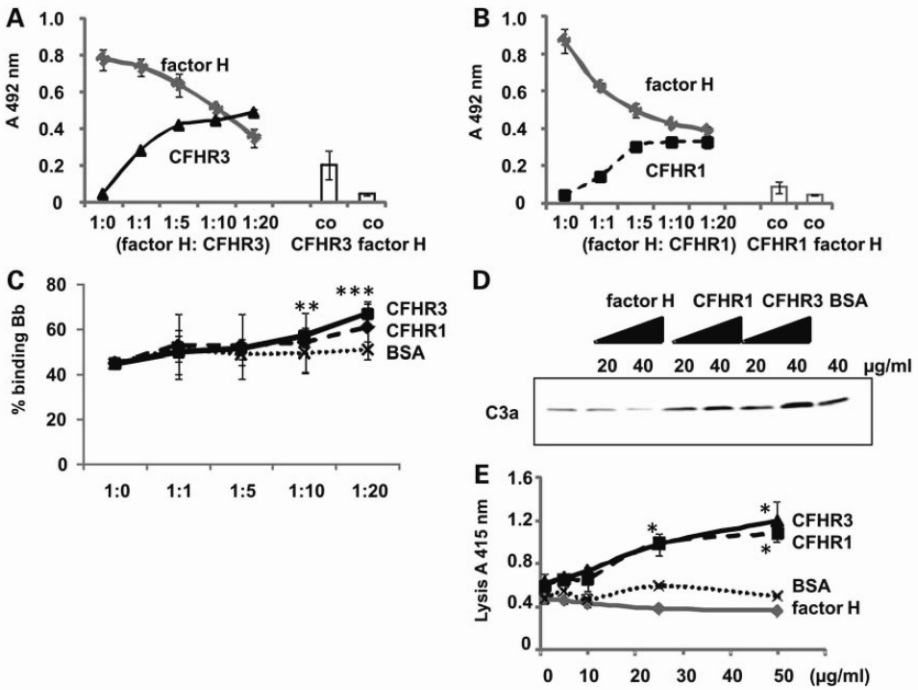

Figure 4. Competition between CFHR3, CFHR1 and factor H to C3b binding, C3 convertases and hemolytic activity in HP. (**A**) CFHR3 (triangles) or (**B**) CFHR1 (squares) replace factor H (diamonds) in C3b binding. Increasing amounts of CFHR3 (0.02–0.53 μM) or CFHR1 (0.03–0.61 μM) were incubated with constant amounts of factor H (0.03 μM) bound C3b. CFHR1 and factor H antibodies do not cross-react with C3b (co). Results represent mean values ± SD of three measurements. (**C**) C3 convertase was generated by C3b, FD and FB and properdin and decay acceleration was followed by factor Bb binding after incubation with constant amounts of factor H (0.03 μM) and increasing concentrations of CFHR3 (0.02–0.53 μM) or CFHR1 (0.03–0.61 μM), compared with BSA. Mean values ± standard deviations of six experiments are shown. $^{**}P < 0.01$ compared with buffer (100%). (**D**) ΔCFHR3/CFHR1 plasma C3 convertase activity was modulated under increasing concentrations (20 and 40 μg/ml) of CFHR3 (0.4 and 0.8 μM) or CFHR1 (0.46 and 0.93 μM). Factor H (0.13 and 0.26 μM) reduced C3a generation, but CFHR1 or CFHR3 enhanced C3a generation. BSA had no effect. Representative blot from three independent experiments is shown. (**E**) ΔCFHR3/CFHR1 plasma was incubated with chicken erythrocytes in the presence of increasing amounts (0–50 μg/ml) of recombinant CFHR1 (0–1 μM, squares), CFHR3 (0–1.16 μM, triangles), factor H (0–0.32 μM, diamonds) or BSA (crosses). Hemolysis of erythrocytes was determined by measuring absorbance at 415 nm. Increasing concentrations of CFHR1 increased hemolysis of chicken erythrocytes, in contrast to addition of similar amounts of factor H or BSA. Data represent results from three experiments. $^{*}<P = 0.05$, $^{**}P < 0.01$, compared with BSA.

I for degradation and inactivation of C3b. However, CFHR3-mediated cofactor activity is lower as that of factor H. Previous studies reported very low or no cofactor activity of CFHR3 alone, but an enhanced effect on cofactor activity of factor H (30). However, in these studies, radio-labeled C3b was used and possibly the labeling reaction affected conformation of the C3b molecule and consequently modified binding properties with interacting proteins. Wu *et al*. (31) reported that minor changes (e.g. single amino acid exchanges) induced conformational changes in C3b and alter binding characteristics, which have been reported for the severe kidney disease HUS (32).

CFHR3 displays cofactor activity for the serine protease factor I and inhibits hemolysis of sheep red blood cells in HP. Normally, sheep red blood cells are protected from complement-mediated lysis by attaching human factor H, CFHR3 and CFHR1 to their surface. However, sheep erythrocytes were lysed when incubated in factor H (also CFHR3 and CFHR1) depleted, complement active HP. In this setup, CFHR3 reduced hemolysis of red blood cells in a dose-dependent manner, which verifies the complement regulatory role of CFHR3. The plasma concentration of CFHR3 is 50–80 μg/ml (1–1.6 μM) (data not shown) and similar to CFHR1 (7).

CFHR3 and CFHR1 show inhibitory activities on hemolysis of sheep erythrocytes, however these effects are less pronounced than those of factor H. The lower activities are explained by lack of decay acceleration activity by CFHR3 and CFHR1. CFHR3 has cofactor activity and regulates the C3 convertase by promoting cleavage of C3b by factor I. Thus, CFHR3 as regulator of the C3 convertase and CFHR1 as regulator of the C5 convertase and the terminal complex inhibit complement activation at different levels. Despite these different regulatory actions, both proteins block the generation of the complement activation product C5a and subsequently inhibit chemoattractance of neutrophils. Therefore both CFHR3 and CFHR1 have anti-inflammatory activities. However, higher concentrations of CFHR3 and CFHR1 are necessary to obtain the same level of inhibition

as with factor H. Thus, deficiency of CFHR3 and CFHR1 in plasma due to genetic deletion of the encoding genes can result in a loss of complement regulatory functions.

How can such a loss of regulatory functions be protective to AMD? As shown in this study, CFHR3 and also CFHR1 compete with factor H for binding to C3b and thereby modulate local factor H-mediated complement activity. A similar replacement effect of factor H binding to C3b has been reported for a C-terminal fragment of factor H (ΔSCR19-20) (33). We conclude that binding of factor H to C3b is increased in case of CFHR3/CFHR1 deficiency. As factor H is a very potent regulator that exerts at least two regulatory functions, decay acceleration and cofactor activity, increased factor H binding to C3b also enhances complement regulation on the level of C3 convertase. This enhanced regulation by factor H is confirmed in hemolytic assays with CFHR3- and CFHR1-deficient HP. Addition of CFHR3 or CFHR1 enhances hemolysis of erythrocytes in contrast to the addition of factor H. However, these proteins represent complement regulators of the C3- and C5 convertase (Table 2) and presumably regulate complement activation at specific locations and ensure inactivation of the complement activation cascade. That deficiency of CFHR1 and CFHR3 results in a loss of regulatory functions is concluded as in DEAP-HUS this deficiency predisposes to the development of auto-antibodies to factor H in early ages (13,14). In HUS, Δ*CFHR3/CFHR1* is frequently associated with the presence of factor H auto-antibodies; however, a small fraction of HUS patients show CFHR3/CFHR1 deficiency but lack auto-antibodies to factor H. At present it is unclear, why in this group of patients the CFHR3/CFHR1 deficiency increased the risk for atypical HUS. Presumably CFHR3 and CFHR1 deficiency is combined with an unknown complement enhancing factor in these patients.

Two CFHR1 variants with three amino acid exchanges in SCR 3 (H36.1-YVQ and H36.2-HLE) were initially identified (20). The H36.1-YVQ variant has now been associated with an enhanced risk for the development of HUS (27). The H36.1-YVQ resembles the sequence of SCR 18 of factor H and is supposed to replace factor H from C3b with higher efficiency than the H36.2-HLE variant. Whether the frequency of the two CFHR1 variants also affects the risk of AMD has not been determined yet.

In summary, this study provides evidence that Δ*CFHR3/CFHR1* represents a loss of two complement regulators and affects local factor H binding, which results in increased factor H-mediated regulation. This is in agreement with our genetic data, which demonstrate that Δ*CFHR3/CFHR1* confers a protective effect against AMD development independent of the sequence variants of factor H. Thus, complement regulation predominantly by factor H acting at the level of C3 convertase is an advantage in the retina, but the imbalance among the regulators may also create novel risk factors for autoimmune diseases.

MATERIALS AND METHODS

Subjects and clinical assessment

Cases and controls were recruited at the Department of Ophthalmology, University of Bonn (112 cases, 68 controls) (26) and at the University Eye Clinic Würzburg (418 cases, 245 controls) (Frankonian AMD Study) (34). The total study comprised 530 non-familial AMD patients and 313 unrelated control individuals. The mean age at examination was 78.0 years for AMD patients (SD, 6.6 years; range: 56–95 years) and 77.2 years for controls (SD, 6.7 years; range: 60–99 years). The control subjects were free of macular changes such as drusen, pigmentary alterations or diabetic maculopathy. The study was approved by the Ethics Committee of the University of Würzburg and the University of Bonn and adhered to the tenets of the Declaration of Helsinki. All subjects were informed as to the nature of the study and signed a written consent prior to blood withdrawal.

Genotyping

Genomic DNA was extracted from peripheral blood leukocytes according to established protocols. SNP genotyping was done by direct sequencing of PCR products: rs1061170:T>C with forward primer 5′-ATTTATCATTG TTATGGTCCTT-3′ and reverse primer 5′-GGTAAAACAA GGTGACATAAA-3′, rs2274700:G>A with forward primer: 5′-TGTTATTGATCATATGCTTGTCTTTT-3′ and reverse primer: 5′-GCTTTGTTCCTGCAGGTTTT-3′. Direct sequencing was performed with the 'Big Dye Terminator Cycle Sequencing Kit Version 1.1' (Applied Biosystems) according to the manufacturer's instructions. Reactions were analyzed with an 'ABI Prism Model 3130xl Sequencer' (Applied Biosystems). To test for genotyping accuracy, 3.5% of all genotypes were assessed in duplicate and showed 100% concordance.

Multiplex Ligation-dependent Probe Amplification (MLPA®)

We performed the MLPA assay 'SALSA MLPA KIT P236-A1 ARMD' according to the manufacturer's instruction (MRC-Holland, Amsterdam, The Netherlands). We used 150 ng DNA per sample at a concentration of 30 ng/μl. Four controls without the deletion of *CFHR3* and *CFHR1*, a negative control (no DNA) and two duplicates were included in each experiment. Fragment analyses were performed on ABI Prism Model 3130xl Genetic Analyzer (Applied Biosystems). CNVs were determined using 'SeqPilot Version 3.2.1.55' with the corresponding MLPA module and the default settings (JSI Medical Systems, Kippenheim, Germany). All duplicates resulted in concordant findings.

Statistical analysis

Deviations from Hardy-Weinberg equilibrium (HWE) were assessed for statistical significance using PLINK v1.07 (http://pngu.mgh.harvard.edu/purcell/plink/) (35); all genotypes were in HWE ($P > 0.001$). Pairwise LD coefficients r^2 and D' were calculated using Haploview 4.2 (www.broad.mit.edu/mpg/haploview) (36). Haplotype and genotype frequencies were calculated with UNPHASED 3.1.2 (http://www.mrc-bsu.cam.ac.uk/personal/frank/software/unphased/) (37). Logistic regression analysis and conditional analysis were performed assuming a log-additive genetic model implemented in PLINK v1.07.

Significant differences between two data groups in chemotaxis and binding assays were analyzed by the unpaired *t*-test. Values of $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$ were considered statistically significant.

Recombinant proteins and western blot analysis

Recombinant CFHR3 was expressed with the bacculovirus system as described (30) and CFHR1 in *Pichia pastoris* (7,38). All proteins were purified by Nickel chelate affinity chromatography (7). Recombinant proteins were separated by SDS–PAGE and visualized by silver staining. Normal HP and CFHR1 and CFHR3 deficient plasma (ΔCFHR3/CFHR1) were obtained from healthy volunteers, Jena, Germany, upon informed consent. Depletion of factor H and CFHR1 and CFHR3 was performed as described earlier (7). Briefly factor H-depleted HP was prepared by immunoabsorbance of factor H from normal HP (NHS). Polyclonal factor H antiserum (Merck Biosciences) was covalently coupled to a 1 ml HiTrap NHS (N-hydroxy succimide) column (GE Health Care) and incubated with NHS. Factor H depletion was confirmed by western blotting. HP was separated by SDS–PAGE, transferred to a membrane and immunoblotted using polyclonal antiserum to CFHR3 and CFHR1 as previously described (19). Vitronectin was purchased from BD Biosciences, C3b, C3d, factor H and Factor I from Comptech. A monoclonal antibody (4H9) to CFHR3 was generated.

Cofactor assay

Cofactor activity CFHR3, CFHR1 and factor H was analyzed by measuring factor I-mediated degradation of C3b following SDS–PAGE and western blot analysis. CFHR3 or CFHR1 (5 or 20 μg/ml, 0.1 or 0.4 μM) or factor H (0.4 or 1 μg/ml, 0.002–0.006 μM) were incubated with C3b (1 μg) and FI (0.1 μg) were added and cofactor activity was determined. C3b degradation products were visualized by using C3 antiserum (Comptech).

Hemolysis assays

CFHR3 (80 μg/ml, 1.6 μM), CFHR1 (80 μg/ml, 1.8 μM), factor H (80 μg/ml, 0.5 μM), vitronectin or BSA (each 80 μg/ml) was added to factor H- and CFHR3/CFHR1-depleted plasma (30%) and incubated at 37°C for 20 min with about 2×10^7 sheep erythrocytes in activation buffer C (20 mM Hepes, 144 mM NaCl, 7 mM $MgCl_2$, 10 mM EGTA, pH 7.4). Supernatants were recorded at 415 nm. In similar experiments, generation of complement activation products C3a and C5a was followed by ELISA (Quidel, DRG Diagnostics). Increasing amounts (1–50 μg/ml) of CFHR3 (0.02–1 μM), CFHR1 (0.02–1.16 μM), factor H (0.006–0.32 μM) or BSA were added to CFHR1- and CFHR3-deficient plasma from a healthy donor (20%) and incubated for 20 min at 37°C with chicken erythrocytes (5×10^6) which are more sensitive to hemolysis than sheep erythrocytes. Supernatants were recorded at 415 nm.

Chemotaxis assay

To obtain BM-derived neutrophils, femurs, tibias and humeri of BALB/c mice were flushed with sterile PBS. The migration of BM-derived neutrophils was determined using a 48-well microchemotaxis chamber (Neuro Probe) with polycarbonate filters (pore size, 3 μm). Two-fold serial dilutions (1:2 to 1:64) of CFHR3 (80 μg/ml, 1.6 μM), CFHR1 (80 μg/ml, 1.8 μM) or factor H (80 μg/ml, 0.5 μM)-treated human factor H deficient plasma activated by addition of sheep red blood cells were used and diluted using 2% BSA-GBSS (Sigma-Aldrich). Thirty microliters of the diluted plasma were added to the lower well to act as a chemoattractant for 50 μl of neutrophils (10^7 neutrophils per milliliter suspended in 2% BSA-GBSS buffer), which were added to the upper wells. Cells were allowed to migrate for 30 min at 37°C. Membranes were separated, fixed with methanol and stained with Diff-Quick (BaxterDade). The numbers of neutrophils per square micrometer migrating to the lower surface of the membrane were determined microscopically (≥20 high power fields, ×32 magnification).

ELISA

To investigate CFHR3 regulation of the C3 convertase, the C3 convertase was generated by incubation of C3b (5 μg/ml) and C3 (50 μg/ml) with factor D (2.5 μg/ml), properdin (2.5 μg/ml) and factor B (5 μg/ml) in activation buffer C (20 mM Hepes, 144 mM NaCl, 7 mM $MgCl_2$, 2 mM $NiCl_2$, 10 mM EGTA, pH 7.4). Activity of C3 convertase was determined by C3a generation after incubation of constant amounts of C3 (50 μg/ml) and increasing concentrations of CFHR3 (25 and 50 μg/ml, 0.5 and 1 μM), factor H (50 μg/ml, 0.32 μM) or 25 μg/ml human serum albumine (HSA). C3a concentrations were determined by ELISA (Quidel, USA). For the determination of C3 cleavage in plasma, ΔCFHR3/CFHR1 plasma (10%) was incubated with 20 or 40 μg/ml of CFHR3 (0.4 or 0.8 μM), CFHR1 (0.46 or 0.93 μM), factor H or BSA in complement activation buffer C. C3a generation was followed by western blot analysis.

In competition assays, C3b (5 μg/ml) was immobilized to the surface of a microtiter plate (nunc maxisorb) and incubated with constant amounts of factor H (5 μg/ml, 0.03 μM) and increasing concentrations of 1–30 μg/ml of CFHR3 (0.02–0.6 μM) or CFHR1 (0.02–0.69 μM) or BSA, or CFHR1 plus CFHR3 (each 1 to 20 μg/ml, 0.02–0.46 μM). To reflect physiological conditions, molar ratios of the proteins were used (factor H:CFHR of 1:0.27). Binding of the proteins to C3b was analyzed by flow cytometry using factor H SCR 1–4 antiserum (Alexis, Switzerland) for detection of factor H, monoclonal JHD10 (7) for detection of CFHR1 and monoclonal antibody 4H9 for detection of CFHR3.

For the determination of the decay acceleration activity of CFHR3, C3 convertases were formed with recombinant proteins on a microtiter plate surface by immobilization of C3b (10 μg/ml) and incubation with factor B (5 μg/ml) and factor D (2.5 μg/ml) generated under increasing concentrations (1–50 μg/ml) of CFHR3 (0.02–1 μM), CFHR1 (0.02–1.16 μM), factor H (0.006–0.32 μM) or BSA in activation buffer C (20 mM Hepes, 144 mM NaCl, 7 mM $MgCl_2$,

2 mM $NiCl_2$, 10 mM EGTA, pH 7.4). Decay acceleration was measured by determination of bound factor Bb to the formed C3 convertases using factor B antibodies (Comptech). Similarly, Bb binding was determined after constant amounts of factor H (5 μg/ml, 0.03 μM) together with increasing amounts (1–20 μg/ml in a molar ratio of factor H:CFHR of 1:0.27) of CFHR1 (0.02–0.4 μM) or CFHR3 (0.02–0.46 μM) were incubated with the assembled C3 convertases.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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REFERENCES

1. Pascolini, D., Mariotti, S.P., Pokharel, G.P., Pararajasegaram, R., Etya'ale, D., Negrel, A.D. and Resnikoff, S. (2004) 2002 global update of available data on visual impairment: a compilation of population-based prevalence studies. *Ophthalmic Epidemiol.*, **11**, 67–115.
2. Edwards, A.O., Ritter, R. 3rd, Abel, K.J., Manning, A., Panhuysen, C. and Farrer, L.A. (2005) Complement factor H polymorphism and age-related macular degeneration. *Science*, **308**, 421–424.
3. Hageman, G.S., Anderson, D.H., Johnson, L.V., Hancox, L.S., Taiber, A.J., Hardisty, L.I., Hageman, J.L., Stockman, H.A., Borchardt, J.D., Gehrs, K.M. *et al.* (2005) A common haplotype in the complement regulatory gene factor H (HF1/CFH) predisposes individuals to age-related macular degeneration. *Proc. Natl Acad. Sci.*, **102**, 7227–7232.
4. Haines, J.L., Hauser, M.A., Schmidt, S., Scott, W.K., Olson, L.M., Gallins, P., Spencer, K.L., Kwan, S.Y., Noureddine, M., Gilbert, J.R. *et al.* (2005) Complement factor H variant increases the risk of age-related macular degeneration. *Science*, **308**, 419–421.
5. Klein, R.J., Zeiss, C., Chew, E.Y., Tsai, J.Y., Sackler, R.S., Haynes, C., Henning, A.K., SanGiovanni, J.P., Mane, S.M., Mayne, S.T. *et al.* (2005) Complement factor H polymorphism in age-related macular degeneration. *Science*, **308**, 385–389.
6. Conley, Y.P., Jakobsdottir, J., Mah, T., Weeks, D.E., Klein, R., Kuller, L., Ferrell, R.E. and Gorin, M.B. (2006) CFH, ELOVL4, PLEKHA1 and LOC387715 genes and susceptibility to age-related maculopathy: AREDS and CHS cohorts and meta-analyses. *Hum. Mol. Genet.*, **15**, 3206–3218.
7. Heinen, S., Hartmann, A., Lauer, N., Wiehl, U., Dahse, H.M., Schirmer, S., Gropp, K., Enghardt, T., Wallich, R., Halbich, S. *et al.* (2009) Factor H-related protein 1 (CFHR-1) inhibits complement C5 convertase activity and terminal complex formation. *Blood*, **114**, 2439–2447.
8. Skerka, C., Lauer, N., Weinberger, A.A., Keilhauer, C.N., Sühnel, J., Smith, R., Schlötzer-Schrehardt, U., Fritsche, L., Heinen, S., Hartmann, A. *et al.* (2007) Defective complement control of factor H (Y402H) and FHL-1 in age-related macular degeneration. *Mol. Immunol.*, **44**, 3398–3406.
9. Yu, J., Wiita, P., Kawaguchi, R., Honda, J., Jorgensen, A., Zhang, K., Fischetti, V.A. and Sun, H. (2007) Biochemical analysis of a common human polymorphism associated with age-related macular degeneration. *Biochemistry*, **46**, 8451–8461.
10. Hughes, A.E., Orr, N., Esfandiary, H., Diaz-Torres, M., Goodship, T. and Chakravarthy, U. (2006) A common CFH haplotype, with deletion of CFHR1 and CFHR3, is associated with lower risk of age-related macular degeneration. *Nat. Genet.*, **38**, 1173–1177.
11. Schmid-Kubista, K.E., Tosakulwong, N., Wu, Y., Ryu, E., Hecker, L.A., Baratz, K.H., Brown, W.L. and Edwards, A.O. (2009) Contribution of copy number variation in the regulation of complement activation locus to development of age-related macular degeneration. *Invest. Ophthalmol. Vis. Sci.*, **50**, 5070–5079.
12. Spencer, K.L., Hauser, M.A., Olson, L.M., Schmidt, S., Scott, W.K., Gallins, P., Agarwal, A., Postel, E.A., Pericak-Vance, M.A. and Haines, J.L. (2008) Deletion of CFHR3 and CFHR1 genes in age-related macular degeneration. *Hum. Mol. Genet.*, **17**, 971–977.
13. Zipfel, P.F., Edey, M., Heinen, S., Jozsi, M., Richter, H., Misselwitz, J., Hoppe, B., Routledge, D., Strain, L., Hughes, A.E. *et al.* (2007) Deletion of complement factor H-related genes CFHR1 and CFHR3 is associated with atypical hemolytic uremic syndrome. *PLoS Genet.*, **3**, e41.
14. Jozsi, M., Licht, C., Strobel, S., Zipfel, S.L., Richter, H., Heinen, S., Zipfel, P.F. and Skerka, C. (2008) Factor H autoantibodies in atypical hemolytic uremic syndrome correlate with CFHR1/CFHR3 deficiency. *Blood*, **111**, 1512–1514.
15. Jozsi, M., Strobel, S., Dahse, H.M., Liu, W.S., Hoyer, P.F., Oppermann, M., Skerka, C. and Zipfel, P.F. (2007) Anti factor H autoantibodies block C-terminal recognition function of factor H in hemolytic uremic syndrome. *Blood*, **110**, 1516–1518.
16. Licht, C., Pluthero, F.G., Li, L., Christensen, H., Habbig, S., Hoppe, B., Geary, D.F., Zipfel, P.F. and Kahr, W.H. (2009) Platelet-associated complement factor H in healthy persons and patients with atypical HUS. *Blood*, **114**, 4538–4545.
17. Diaz-Guillen, M.A., Rodriguez de Cordoba, S. and Heine-Suner, D. (1999) A radiation hybrid map of complement factor H and factor H-related genes. *Immunogenetics*, **49**, 549–552.
18. Male, D.A., Ormsby, R.J., Ranganathan, S., Giannakis, E. and Gordon, D.L. (2000) Complement factor H: sequence analysis of 221 kb of human genomic DNA containing the entire fH, fHR-1 and fHR-3 genes. *Mol. Immunol.*, **37**, 41–52.
19. Skerka, C. and Zipfel, P.F. (2008) Complement factor H related proteins in immune diseases. *Vaccine*, **26**, I9–I14.
20. Skerka, C., Horstmann, R.D. and Zipfel, P.F. (1991) Molecular cloning of a human serum protein structurally related to complement factor H. *J. Biol. Chem.*, **266**, 12015–12020.
21. Skerka, C., Kuhn, S., Gunther, K., Lingelbach, K. and Zipfel, P.F. (1993) A novel short consensus repeat-containing molecule is related to human complement factor H. *J. Biol. Chem.*, **268**, 2904–2908.
22. Park, C.T. and Wright, S.D. (1996) Plasma lipopolysaccharide-binding protein is found associated with a particle containing apolipoprotein A-I, phospholipid, and factor H-related proteins. *J. Biol. Chem.*, **271**, 18054–18060.
23. Zipfel, P.F. and Skerka, C. (2009) Complement regulators and inhibitory proteins. *Nat. Rev.*, **9**, 729–740.
24. Francis, P.J., Schultz, D.W., Hamon, S., Ott, J., Weleber, R.G. and Klein, M.L. (2007) Haplotypes in the complement factor H (CFH) gene: associations with drusen and advanced age-related macular degeneration. *PloS ONE*, **2**, e1197.
25. Li, M., Atmaca-Sonmez, P., Othman, M., Branham, K.E., Khanna, R., Wade, M.S., Li, Y., Liang, L., Zareparsi, S., Swaroop, A. *et al.* (2006) CFH haplotypes without the Y402H coding variant show strong association with susceptibility to age-related macular degeneration. *Nat. Genet.*, **38**, 1049–1054.
26. Scholl, H.P., Charbel Issa, P., Walier, M., Janzer, S., Pollok-Kopp, B., Borncke, F., Fritsche, L.G., Chong, N.V., Fimmers, R., Wienker, T. *et al.* (2008) Systemic complement activation in age-related macular degeneration. *PloS ONE*, **3**, e2593.
27. Abarrategui-Garrido, C., Martinez-Barricarte, R., Lopez-Trascasa, M., de Cordoba, S.R. and Sanchez-Corral, P. (2009) Characterization of complement factor H-related (CFHR) proteins in plasma reveals novel

genetic variations of CFHR1 associated with atypical hemolytic uremic syndrome. *Blood*, **114**, 4261–4271.
28. Hageman, G.S., Hancox, L.S., Taiber, A.J., Gehrs, K.M., Anderson, D.H., Johnson, L.V., Radeke, M.J., Kavanagh, D., Richards, A., Atkinson, J. *et al.* (2006) Extended haplotypes in the complement factor H (CFH) and CFH-related (CFHR) family of genes protect against age-related macular degeneration: characterization, ethnic distribution and evolutionary implications. *Ann. Med.*, **38**, 592–604.
29. Maller, J., George, S., Purcell, S., Fagerness, J., Altshuler, D., Daly, M.J. and Seddon, J.M. (2006) Common variation in three genes, including a noncoding variant in CFH, strongly influences risk of age-related macular degeneration. *Nat. Genet.*, **38**, 1055–1059.
30. Hellwage, J., Jokiranta, T.S., Koistinen, V., Vaarala, O., Meri, S. and Zipfel, P.F. (1999) Functional properties of complement factor H-related proteins FHR-3 and FHR-4: binding to the C3d region of C3b and differential regulation by heparin. *FEBS Lett.*, **462**, 345–352.
31. Wu, J., Wu, Y.Q., Ricklin, D., Janssen, B.J., Lambris, J.D. and Gros, P. (2009) Structure of complement fragment C3b-factor H and implications for host protection by complement regulators. *Nat. Immunol.*, **10**, 728–733.
32. Fremeaux-Bacchi, V., Miller, E.C., Liszewski, M.K., Strain, L., Blouin, J., Brown, A.L., Moghal, N., Kaplan, B.S., Weiss, R.A., Lhotta, K. *et al.* (2008) Mutations in complement C3 predispose to development of atypical hemolytic uremic syndrome. *Blood*, **112**, 4948–4952.
33. Ferreira, V.P. and Pangburn, M.K. (2007) Factor H mediated cell surface protection from complement is critical for the survival of PNH erythrocytes. *Blood*, **110**, 2190–2192.
34. Rivera, A., Fisher, S.A., Fritsche, L.G., Keilhauer, C.N., Lichtner, P., Meitinger, T. and Weber, B.H. (2005) Hypothetical LOC387715 is a second major susceptibility gene for age-related macular degeneration, contributing independently of complement factor H to disease risk. *Hum. Mol. Genet.*, **14**, 3227–3236.
35. Purcell, S., Neale, B., Todd-Brown, K., Thomas, L., Ferreira, M.A., Bender, D., Maller, J., Sklar, P., de Bakker, P.I., Daly, M.J. *et al.* (2007) PLINK: a tool set for whole-genome association and population-based linkage analyses. *Am. J. Hum. Genet.*, **81**, 559–575.
36. Barrett, J.C., Fry, B., Maller, J. and Daly, M.J. (2005) Haploview: analysis and visualization of LD and haplotype maps. *Bioinformatics*, **21**, 263–265.
37. Dudbridge, F. (2008) Likelihood-based association analysis for nuclear families and unrelated subjects with missing genotype data. *Hum. Hered.*, **66**, 87–98.
38. Kunert, A., Losse, J., Gruszin, C., Huhn, M., Kaendler, K., Mikkat, S., Volke, D., Hoffmann, R., Jokiranta, T.S., Seeberger, H. *et al.* (2007) Immune evasion of the human pathogen *Pseudomonas aeruginosa*: elongation factor Tuf is a factor H and plasminogen binding protein. *J. Immunol.*, **179**, 2979–2988.

6 The C-terminal surface attachment region of Factor H targets the complement inhibitor compstatin to self surfaces and to sites of immunstress.

Manuskript in Vorbereitung für das *„Journal of Immunology“*

THE C-TERMINAL SURFACE ATTACHMENT REGION OF FACTOR H TARGETS THE COMPLEMENT INHIBITOR COMPSTATIN TO SELF SURFACES AND TO SITES OF IMMUNSTRESS[1]

Running title: the chimera COMP_CFH15-20 protects self surfaces

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Based on the increasing number of complement associated human diseases there is growing need for new complement inhibitors. Here we design and generate a novel complement inhibitor that links the cell surface recognition region of the human complement regulator Factor H with the established C3 inhibitor Compstatin. COMP_CFH15-20 which is composed of SCR15-20 of Factor H and the Compstatin sequence was expressed in yeast cells. The hybrid protein has several C3 interaction regions as shown by ELISA and surface plasmon resonance. This new inhibitor blocks complement amplification in fluid phase as shown by hemolytic assays using both rabbit and sheep erythrocytes. In addition the C-terminal surface recognition region targets the inhibitor to the surface of damaged nucleated cells, such as CHO cells upon complement challenge with complement active human serum that was depleted for the Factor H. Similarly the new inhibitor also binds to the surface of necrotic cells and blocks complement activation, as demonstrated by deposition of the activation fragment C5b on the necrotic surfaces. Thus, this new tagged complement inhibitor which allows targeted complement control at sites of immune stress lacks adverse effects occurring upon systemic complement inhibition. Thus COMP_CFH15-20 is a promising complement inhibitor whose therapeutic potential should be analysed in more detail.

The complement system is a central part of the innate immunity and an essential element for the elimination of invading microbes, clearance of damaged self cells, cellular homeostasis and also for modulating the adaptive immune response (1-3). Activation of the complement system occurs via three major pathways, the alternative pathway (AP), the classical pathway (CP) and the lectin pathway (LP). Once activated by immune complexes, by the inflammatory marker CRP or by carbohydrates the CP or LP form an amplification loop which utilizes the C3 convertase of the AP . The initial steps of the AP activation are non-discriminatory and occur on any surface. In its default setting, C3b is spontaneously and continuously activated by a “tickover” of C3 (4). In an uncontrolled manner the generated activation products have disastrous effects, initiate inflammation and form potent immune effector molecules. From side of the host this activation, in particular the activated effector compounds, must be targeted onto the surface of foreign invaders, (e.g. microbes) or to damaged self cells and need to be restricted on host surfaces (5). To this end the vertebrate organism uses a battery of potent and highly specific regulators and inhibitors that discriminate between activator- and non-activator surfaces. These regulators are distributed in fluid phase, such as plasma and body fluids or are expressed as integral membrane proteins to form non-activator surfaces (6, 7).

Factor H, the key regulator of the AP, is a 150 kDa abundant plasma protein that regulates the AP in fluid phase and that also attaches to cellular surfaces (8). Factor H is composed of 20 consecutive protein domains, termed short

consensus repeats (SCRs). The protein has two central functional regions located at the opposite ends of the protein (9). The four N-terminal domains SCR1-4 modulate the activity of the AP C3 convertase C3bBb (10, 11), the C-terminus, represented by SCR18-20, forms the central surface attachment and recognition region. This part of the protein contacts cellular surfaces via glycosaminoglycans, binds to heparin, and also to C3b and to C3d (12, 13). Factor H uses the C-terminal attachment region to bind e.g. to renal endothelial cells and to the glomerular basement membrane of the kidney or to the surface of retinal pigment epithelial cells and the Bruch's membrane of the retina. This recognition of self surfaces via the C-terminus is highly specific and directs the complement inhibitor to self surfaces where complement activation is initiated. Thus, by C-terminal binding of surfaces Factor H controls complement activation and discriminates precisely between activator- and non-activator surfaces (14).

The relevance of this surface attachment is highlighted by diseases associated with gene mutations within this region that result e.g. in the renal disease of atypical hemolytic uremic syndrome (aHUS). The majority of aHUS-associated gene mutations cluster in the C-terminal surface attachment region and consequently reduce Factor H surface binding (15, 16). Furthermore in the autoimmune DEAP-HUS subform, Factor H autoantibodies specifically bind the C-terminal region of Factor H and consequently block surface attachment(17). In the renal disease of type II membranoproliferative glomerulonephritis (MPGN II), the absence of Factor H results in local, uncontrolled complement activation onto the glomerular basement membrane and in pathology (18). Thus proper targeting of Factor H via the C-terminal surface attachment region is central for integrity of host tissues. Inappropriate binding or targeting is associated with enhanced local complement activation at cellular and biological surfaces and causes immune stress as well as uncontrolled inflammatory reactions and further damage (19).

Given the central role of complement for tissue homeostasis and for controlling inflammation any defective regulation of this cascade enhances cellular damage and can cause pathology (20). Understanding these disease mechanisms is relevant for therapeutic intervention. The various diseases scenarios demonstrate the need for a targeted complement control at sides that are vulnerable for complement-mediated damage. Local and targeted modulation of the complement is therefore a promising and attractive option for therapy (21-23). One major issue of complement control is whether inhibition is achieved systemically or locally.

Despite these first therapeutic achievements, applications of systemically acting complement inhibitors have potential side effects or hazards. Side effects due to systemic complement inhibition, in particular during long-term therapy or in immunocompromised patients, blocks any beneficial effects of the activated complement cascade such as the control of cellular homeostasis and the clearance of infectious agents/microbes. This may form a favorable environment for infectious microbes, allows inappropriate processing of immune complexes and developing of an inappropriate humoral immune response (24). Therefore an additional and highly relevant strategy is to target complement inhibitors to specific sites, i.e. to the sites of complement activation. One of such targeted inhibitory molecules links the human complement receptor 2 (CR2) with the N-terminal domains of Factor H (CR2-fH, Taligen Therapeutics) or CR2 to mouse Crry (CR2-Crry) (25, 26). The two hybrid proteins use their CR2 region to target the inhibitor to the site of complement activation and the regulatory region of Factor H for local complement inhibition for dissociating preformed C3 convertases and block of C3b formation.

Given the essential function of the C-terminal Factor H surface attachment region as a central sensor to distinguish between activator and non-activator surfaces, we aimed to utilize this recognition region as a tag to direct complement inhibitors to the side of complement activation, tissue damage or immunstress. Such a targeted inhibitor should allow to control complement activity at local sites, in particular at sites of immune stress or surface damage. To this end we generated a chimeric protein, composed of the C-terminal binding region of Factor H and the potent complement inhibitor compstatin. Compstatin is a cyclic 13-residue peptide, initially identified by screening a phage-display

library for peptides that inhibits complement by binding to native C3 and thereby block subsequent conversion, as well as the amplification of all three pathways (27, 28). In 2009 Potentia Pharmaceuticals successfully completed a phase 1 clinical trial of compstatin for treating age-related macular degeneration (AMD) (29, 30).

The novel Compstatin_Factor H_SCR15-20 (COMP_CFH 15-20) chimera presented here, was analyzed for C3/C3b and for cell surface binding. The inhibitory activities of the chimera were assayed in fluid phase and on cellular surfaces, using erythrocytes and living as well as necrotic nucleated cells. Once bound to the surface of living or necrotic CHO-cells COMP_CFH15-20 protected these nucleated cells effectively from complement-mediated damage. Due to the C-terminal Factor H tag COMP_CFH15-20 possess two binding sites for C3b, accompanied by an increased the affinity for C3b. Once bound to living or necrotic cells COMP_CFH15-20 was consistently more efficient as the untagged compstatin. The novel inhibitor COMP_CFH15-20 is therefore a promising new complement regulator which inhibits complement amplification specifically at sites of immune stress and abolishes the adverse effects occurring upon systemic complement inhibition.

MATERIAL AND METHODS

Proteins and antibodies-
Purified human Factor H, C3, C3b as well as goat antiserum against human Factor H, C5 and C7 were obtained from Comptech (Tyler, Texas, USA). Horseradish peroxidase (HRP) - conjugated swine anti-goat IgG (Dako, Hamburg, Germany) and AlexaFluor488 conjugated rabbit anti-goat, IgG (Invitrogen Karlsruhe, Germany) were used as secondary antibodies. As controls the peptides compstatin (amino acid sequence I_1-C_2-V_3-**W**$_4$-Q_5-D_6-W_7-G_8-**A**$_9$-H_{10}-R_{11}-C_{12}-T_{13} = variant 4W9A) and a linear inactive form were used (29).

Serum probes-
Normal human serum (NHS) was obtained from healthy volunteers, Jena, Germany, upon informed consent. The generation of Factor H depleted serum (HSΔ**CFH**) or C7 depleted serum (HSΔ**C7)** was performed by immune adsorbance as described (31).

Plasmid construction, protein expression and protein purification-
To generate a recombinant fusion protein that combines the C-terminal surface attachment region of Factor H with the potent inhibitor compstatin, the plasmid pBSV-SCR15-20 was used as template (32). To this end a forward 5' CTG CAG AT **ATT TGC GTT TGG CAG GAT TGG GGT GCG CAC CGT TGC ACC** GAA AAA ATT CCA TGT TCA CAA CCA CCT CAG 3' and a reverse 5' TCT AGA TT TCT TTT TGC ACA AGT TGG ATA CTC CAG 3' primer pair was used. (PstI and XbaI sites are underlined, the compstatin sequence is displayed bold). The reaction was performed using the Phusion High Fidelity PCR Kit (NEB, Frankfurt, Germany) with the following conditions: denaturation at 98 °C for 3 min, followed by 30 cycles of denaturation at 98 °C for 1 min, annealing at 68 °C for 1 min and extension at 72 °C for 1.5 min, and a final extension of 10 min at 72° C. The resulting 1143 bp DNA fragment contained the coding region for the complete peptide of compstatin fused to the Factor H SCR15-20 coding region, and is flanked by PstI and XbaI restriction sites. This DNA fragment was sub-cloned into *E. coli* cloning vector pCR4Blunt-TOPO (Invitrogen), amplified, isolated, treated with the appropriate restriction enzymes and subsequently cloned into the PstI and XbaI sites of the *Pichia pastoris* vector pPICZαB (Invitrogen). COMP_CFH15-20 was recombinantly expressed as a His-tagged protein in *P. pastoris* strain X33. Protein expression was induced with 1 % methanol. After 3 days of expression, the culture supernatant was harvested and the recombinant COMP_CFH15-20 was purified by nickel affinity chromatography as described (32). The recombinant Factor H deletion construct SCR15-20 was expressed in the baculovirus system (33).

SDS-Page and Western blotting-
Proteins were separated by SDS-PAGE and either visualized by silver staining or they were transferred onto a nitrocellulose membrane and identified with an appropriate antiserum in combination with the corresponding HRP-coupled secondary goat antiserum. Visualization occurred with the Chemiluminescence Gel

Documentation System MF-ChemBIS 3.2 (Biostep, Jahnsdorf, Germany).

Enzyme-linked immunosorband assay (ELISA-
MaxiSorb microtiter plate wells (Nunc, Wiesbaden, Germany) were coated with 5 µg / ml C3 or C3b over night at 4 °C. After blocking of non-specific binding sites with 2 % BSA in DPBS, increasing amounts of COMP_CFH15-20, Factor H and the SCR15-20 (2.5 –20 µg / ml) were added and bound proteins were detected with polyclonal Factor H antiserum. The reaction was developed with 1,2-phenylenediamine dihydrochloride (Dako) and the absorbency was measured at 492 nm.

Kinetic characterization of COMP_CFH15-20-
The interaction of COMP_CFH15-20 with C3b was analyzed by surface plasmon resonance on Biacore 3000 at 25°C. Unlabeled C3b was immobilized by thiol coupling on a sensor chip with a surface density of approx 1500 resonance units [RU]. An untreated flow cell was used as a reference. DPBS supplemented with 0.005 % Tween-20 was used as a running buffer, and data analysis was performed using Scrubber V2.0 (BioLogic Software, Campell, Australia). A kinetic experiment was performed by injecting each analyte (COMP_CFH15-20, SCR15-20 and the compstatin analog 4W9A) as a 1µM solution for 2 min using a flow rate of 10µl/min and following the dissociation for 3min. The signals were normalized by dividing each signal by the molecular weight of the corresponding analyte and further corrected for non-specific binding by subtracting the signals from the reference surface. For a detailed kinetic analysis a twofold serial dilution series from COMP_CFH15-20 (ranging from 2 to 1000nM) was prepared and injected in duplicates. Biosensor data were globally fitted into a two-site model. The equilibrium dissociation constant (K_D) was calculated from the equation $K_D = k_{off}/k_{on}$.

Cell culture-
Chinese hamster ovary cells (CHO, DSMZ ACC 110) and human umbilical vein endothelial cells (HUVEC, ATCC CRL-1730) were cultivated as monolayer's in DMEM medium (Lonza, Verviers, Belgium) supplemented with 10 % FCS. Before binding studies, cells were washed twice in DPBS (Lonza) and cultivated for additional 24 hrs in FCS-free medium. Detachment of the cells occurred by incubation with Accutase (PAA, Pasching, Austria) for 5 min at 37 °C. Necrosis was induced by treating detached cells at 65 °C for 45 min.
Protein binding to cellular surfaces-
Binding of COMP_CFH15-20 to living and necrotic CHO or HUVEC cells was examined by flow cytometry. Cells were washed twice in DPBS supplemented with 1 % BSA, then 20 µg / ml of COMP_CFH15-20, Factor H or SCR15-20 was added. After extensive washing bound proteins were detected with polyclonal Factor H antiserum and an Alexa488 labeled goat Factor H antiserum. 10.000 cells were routinely counted in a BD LSRII flow cytometer and analyzed with the FACSDiva- (BD Biosciences, Heidelberg, Germany) and FlowJo software (Tree Star, Ashland,OR, USA). Living intact cells were identified as propidium iodide negative cells and necrotic cells were identified as propidium iodide positive cells (BD Biosciences).

Hemolytic assay-
The complement regulatory activity of COMP_CFH15-20, was analyzed in hemolytic assays with HEPES buffer (20 mM HEPES, 144 mM NaCl, 7 mM $MgCl_2$, 10 mM EGTA, pH 7.5) using rabbit, as well as sheep erythrocytes (Rockland). Rabbit erythrocytes (rE) represent activator surfaces for NHS and are lysed due to TCC formation. $1x10^7$ rE were incubated with 7.5 % NHS and the effect of increasing amounts of COMP_CFH15-20 was assayed after 30 min at 37 °C. After centrifugation the fraction of lyzed cells was determinated by measuring the supernatant at 414 nm and also by assaying the generation of the complement activation product C5a by ELISA (OSTEOMedical GmbH, Bünde, Germany). In addition, deposition of C5 on the surface of rE incubated in HSΔ**C7** was detected with polyclonal C5 antiserum and examined by confocal microscopy using a laser scanning microscope LSM 510 META (Zeiss, Jena, Germany). Identical assays were performed with sheep erythrocytes (sE) that represent non-activator surfaces and remain intact when incubated in NHS. However, these cells are lyzed, when incubated in complement active HSΔ**CFH**. In the absence of Factor H, sE lyse and the replacement of the regulator has a dose dependent protective effect (31). $1x10^7$ sE were incubated with 30 % HSΔ**CFH** and increasing amounts of COMP_CFH15-20 for 30 min at

37 °C. In all hemolytic assays SCR15-20, the active compstatin analog 4W9A and a linear inactive compstatin were used as positive and negative controls, respectively.

Viability assay-
The protective effect of COMP_CFH15-20 on the metabolic capacity of cultivated CHO cells in the presence of HSΔ**CFH** was measured using the CellTiter-Blue® assay (Promega, Mannheim, Germany). The light absorbance of the CellTiter-Blue® reagent is changed by intracellular reduction of the non-fluorescent dye resazurin into the fluorescent dye resorufin. Therefore, 5×10^4 CHO cells were seeded into 96-well microplates (Nunc) and incubated for 2 days to allow adherence. The culture medium was replaced; cells were washed with HEPES buffer and then incubated for 1 h with 20 µg / ml COMP_CFH15-20, SCR15-20, the active and inactive compstatin analogs lacking the Factor H tag, to allow surface binding. After an additional washing procedure the cells were treated with 5 % NHS or HSΔ**CFH**. Then, the CellTiter-Blue® was added to the cells and incubated for 5 hours. During this time conversion of resazurin into resorufin was followed by measuring the absorbance of the probes at 570 nm in a microplate reader (Tecan, Crailsheim, Germany) every hour. The absorption maximum for resazurin is 605 nm and the absorption maximum for resorufin is 573 nm. Thus, the absorbance measurements at 570 nm and using 600 nm as a reference wavelength were used to monitor the results. Values are compared to blank well containing CellTiter-Blue® reagent without cells. During all incubation steps cells were kept at 37 °C in a humidified atmosphere and 5 % CO_2.

Complement inhibition on necrotic cells-
The complement regulative effect of COMP_CFH15-20 on the surface of necrotic CHO and HUVEC cells in the presence of HSΔ**CFH** was measured using flow cytometry. Therefore necrotic cells were washed in HEPES supplemented with 1 % BSA followed by a 0.5 h incubation step with 20 µg / ml and 40 µg / ml COMP_CFH15-20, SCR15-20, the active compstatin analog 4W9A and a linear inactive compstatin, to allow surface binding. After intensive washing steps the cells were treated with 10 % NHS or HSΔ**CFH** for additional 15 min at 30 °C. The deposition of the complement component C5b, and therefore the degree of complement activation on the necrotic cell surface, was detected with a polyclonal C5 antiserum and an appropriate Alexa488 labeled secondary antiserum.

Statistical analysis-
Significant differences between two groups were analyzed by the unpaired Student's t-test. The results were considered statistically significant at a P value of ≤0.05 (*), ≤0.01 (**), or ≤0.001 (***).

RESULTS

Protein expression and purification-
The recombinant fusion protein COMP_CFH15-20 was cloned and recombinantly expressed in the yeast *P. pastoris*. The fusion protein was purified by nickel chelate chromatography, separated by SDS-PAGE and analyzed by silver staining (Figure 1A). COMP_CFH15-20 was identified as a major band with a mobility of ca. 50 kDa (lane 1, arrow) and also as a smear of higher mobility. The SCR15-20 fragment was identified as a single band of 45 kDa band (lane 2) and Factor H as a 150 kDa band (lane 3). In parallel COMP_CFH15-20, SCR15-20 and Factor H were identified by immunoblotting using polyclonal Factor H antiserum together with a secondary HRP-labeled antiserum (Figure 1B). Also in this situation the smear was detected. The reason for this multimerization is currently unclear

COMP_CFH15-20 binds C3 and C3b-
Binding of COMP_CFH15-20 to the central complement components C3 and C3b was analyzed by ELISA. Both COMP_CFH15-20 and the untagged SCR15-20 bound dose-dependently to immobilized C3 (Figure 2A). COMP_CFH15-20 bound significantly stronger to C3 as compared to the C-terminal Factor H fragment SCR15-20, i.e. at 5 µg / ml ($p = 0.0156$), 10 µg / ml ($p = 0.0089$) and 20 µg / ml ($p = 0.0017$). In a parallel setting binding of COMP_CFH15-20, as well as SCR15-20 to immobilized C3b was assayed. Again, COMP_CFH15-20 bound with higher intensity to C3b, as SCR15-20 and binding was dose-dependent (Figure 2B). The stronger binding of COMP_CFH15-20 was significant at all examined concentrations, i.e. between 2.5 ($p = 0.0203$) and 20 µg / ml ($p = 0.0025$).

Due to the small molecular mass of compstatin (i.e. 1.5 kDa), it is not possible to immobilize the untagged compstatin efficiently onto an ELISA plate. In order to compare the C3b binding of the tagged (COMP_CFH15-20) and of the untagged compstatin, the interaction was analyzed by surface plasmon resonance. C3b was immobilized to the sensor surface by thiol coupling and COMP_CFH15-20 as well as the native compstatin analog 4W9A were applied as analytes at 1µM in fluid phase. COMP_CFH15-20 and 4W9A formed rapidly complexes and steady state levels were reached in less than 1 min (Figure 3A). The tagged COMP_CFH15-20 and 4W9A showed similar complex formation, but the two proteins showed different elution profiles. This difference is likely due to the additional C3b binding side of the tagged regulator. In this situation SCR15-20 bound relative weak to immobilized C3b. A similar difference was observed when the interaction of COMP_CFH15-20 and SCR15-20 was analyzed to immobilized C3b by ELISA (see above). A kinetic evaluation showed that COMP_CFH15-20 bound dose-dependently to C3 and formed rather stable C3b complexes (Figure 3B). The evaluation of the binging profile did not follow a 1:1 model of interaction and was fitted into a two-side model (Figure 3B, insert). This two point interaction, shows for the chimera COMP_CFH15-20, that each of the two C3 interaction regions can contact C3b. One interaction region is contained within SCR15-20 and the second interaction side by compstatin. The kinetic analyses reveals two K_D, one of relative high ($K_D = 2$ nM) and one of lower affinity ($K_D = 130$ nM) (Table 1). The low affinity interaction of 130 nM corresponds to compstatin 4W9A (34), and the high affinity interaction is probably the corresponding effect of 4W9A and SCR15-20. SCR15-20 had an affinity to C3b of K_D 5 µM (data not shown) which is in agreement with former results (35).

COMP_CFH15-20 inhibits complement-mediated lysis on activator surfaces-

The inhibitory activity of COMP_CFH15-20 was determined with rE, that represent activator surfaces and that are lysed upon incubation in NHS (Figure 4A, 0µg / ml). COMP_CFH15-20 inhibited lysis of rE and this effect was dose-dependent (Figure 4A). In contrast, SCR15-20 did not affect lysis of rE. The inhibitory effect of COMP_CFH15-20 was statistically significant in the presence 10 µg / ml ($p = 0.0233$) and 20 µg / ml ($p = 0.01$) protein. The untagged compstatin (4W9A) used at (20 µg / ml) also inhibited lysis, and the linear, inactive compstatin lacked lytic activity. In this assay and at a concentration of 20 µg / ml both the tagged inhibitor, i.e. COMP_CFH15-20 and the untagged inhibitor, i.e. compstatin 4W9A showed comparable effects.

The complement inhibitory effect of COMP_CFH15-20 was further analyzed by following the generation of the activation product C5a in the supernatant. COMP_CFH15-20, which reduced lysis of rE, also blocked C5a generation in a dose-dependent manner (Figure 4B). The reduction in C5a generation at concentrations of 10 µg / ml ($p = 0.0444$) and 20 µg / ml ($p = 0.001$) was statistically significant, as compared to SCR15-20. At a concentration of 20 µg / ml the chimera COMP_CFH15-20 and the native compstatin analog inhibited C5a generation to the same extend.

In addition the effect of COMP_CFH15-20 on C5b surface deposition was followed by immunofluorescence microscopy. For this assay complement active C7 depleted human serum (HSΔ**C7)** was used, which allows complement progression through the C5 convertase, C5b deposition, but which prevents TCC assembly and erythrocyte lysis. COMP_CFH15-20 inhibited C5b deposition on the erythrocyte surface, and no or rather weak fluorescence signal (Figure 4C). However, in the presence of SCR15-20 complement was activated, amplified and C5b was deposited onto the surface of the erythrocytes, as revealed by the strong fluorescence signal (Figure 4D). The untagged compstatin (4W9A) also blocked C5b surface deposition (Figure 4E) and in contrast the inactive linear form lacked an inhibitory effect (Figure 4F). Taken together the novel inhibitor COMP_CFH15-20 is functional active and blocks complement-mediated lysis of rabbit erythrocytes. In this system, which is largely based on fluid phase complement activation and control, both the tagged COMP_CFH15-20 and the untagged compstatin displayed comparable inhibitory activities.

COMP_CFH15-20 inhibits complement mediated lysis on non-activator surfaces-
Sheep erythrocytes (sE) represent non-activator surfaces and when incubated in NHS they are protected from complement-mediated damage, remain intact and are not lysed. However sE are lysed when incubated in complement active human serum, depleted for the regulator Factor H (HSΔCFH) (31). In this set up COMP_CFH15-20 prevented lysis of sheep erythrocytes and the effect was dose-dependent (Figure 5A). Compared to SCR15-20 the effect of COMP_CFH15-20 at 20 µg / ml the inhibitory difference was significant ($p = 0.0088$). Similarly COMP_CFH15-20 and the untagged compstatin showed comparable inhibitory activity.

In addition also the generation of C5a was assayed in the supernatants of the same probes. COMP_CFH15-20 also blocked C5a generation (Figure 5B). COMP_CFH15-20 showed a dose-dependent inhibitory effect and again at a concentration of 20 µg / ml the effect was more pronounced as that of the tag alone ($p = 0.0225$). Again in this assay, the effects of the tagged and untagged compstatin were comparable, because both inhibitors were applied in fluid phase.

COMP_CFH15-20 interact with intact as well as damaged nucleated cells-
In order to assay how or whether the tagged compstatin acts on and protects the surface of nucleated cells, first binding of COMP_CFH15-20 to CHO and to HUVEC cells was assayed by flow cytometry. Both COMP_CFH15-20 and SCR15-20 bound to intact CHO and to intact HUVEC cells with comparable intensities (Figure 6A and Supplementary Figure 1A). To directly confirm that COMP_CFH15-20 binds also to the surface of necrotic cells, binding of COMP_CFH15-20 to both necrotic CHO- and also HUVEC cells was analyzed. COMP_CFH15-20 and also SCR15-20 bound to necrotic CHO- as well as necrotic HUVEC cells. Binding was of comparable intensity (Figure 6B and Supplementary Figure 1B).

COMP_CFH15-20 prevents complement-mediated damage of living nucleated cells-
Intact nucleated cells use surface attached, as well as membrane bound regulators to control and block the continuous low level attack e.g. mediated by the AP. Therefore in the absence of surface attached regulators, when complement regulators are functionally inactivated, nucleated self cells are damaged by complement activation products (36). In order to assay the regulatory effect of COMP_CFH15-20 on the surface of intact, nucleated cells, CHO cells were used. These hamster cells lack human membrane bound complement regulators on their surface and therefore represent a very good model system to assay complement-mediated damage on a nucleated cell surfaces. The CHO cells were challenged with NHS or HSΔCFH to induced complement-mediated damage. The intracellular activity of the lactate dehydrogenase (LDH) was assayed which enzymatically converts the exogenously added and intracellular uptaken non-fluorescent dye resazurin into the fluorescent dye resorufin. The activity of LDH is a marker for cell metabolism and correlates with the amount of complement-mediated damage on intact nucleated cells (31).

First, CHO cells were treated with either NHS or HSΔCFH. The metabolic activity of cells incubated in NHS was stronger as compared to cells incubated in HSΔCFH (Figure 7A). Thus demonstrating that the absence of the fluid phase regulator Factor H affects complement control at the surface of these nucleated cells and that the metabolic activity can be used as a marker for complement stress at the cell surface. The difference between NHS and HSΔCFH was significant at all time points examined ($p = 0.0141$ after 1 h; $p = 0.0321$ after 2 h, $p = 0.0133$ after 3 h and $p \leq 0.01$ for all time points > 3 h). The protective effect of the soluble regulator Factor H on the surface of these nucleated, non-human cells was demonstrated by preincubating CHO cells with Factor H prior HSΔCFH challenge. Upon such pretreatment with Factor H the metabolic activity of CHO cells was increased and the effect was dose and time-dependent (Figure 7B). Factor H, used at 40 µg / ml restored the protective effect almost similar to NHS.

This system was now used to assay how efficient COMP_CFH15-20 protects the surface of nucleated cells. First CHO cells were incubated either with the tagged or the untagged compstatin. After washing, the cells were challenged with HSΔCFH and then the metabolic activity was assayed. Both inhibitors protected CHO cells form complement-mediated damage as revealed by the higher metabolic activity

(Figure 7C). In this assay, where mainly the surface action of the two inhibitors was compared, COMP_CFH15-20 was more efficient (up to 30 %), as compared to the untagged compstatin that acts predominantly in fluid phase. This difference was observed at all time points and the effect was significant at 1 h ($p = 0.01$) and at 2h ($p = 0.0278$). Both the SCR15-20 deletion fragment and also the linearized compstatin lacked this protective effect for CHO cells (data not shown). Thus, COMP_CFH15-20 acts specifically on the surface of nucleated cells.

COMP_CFH15-20 prevents C5b deposition on the surface of necrotic CHO cells-

Damaged cells such as necrotic or apoptotic cells change their membrane structure and loose membrane integrated complement regulators and consequently use attached fluid phase regulators for complement inhibition and control. The human fluid phase complement inhibitor Factor H, which has a central regulatory role on the apoptotic and necrotic cell surface, attaches to these surfaces via the C-terminal region. As the, COMP_CFH15-20 is equipped with the same C-terminal surface attachment region we analyzed how this tagged compstatin affects complement action on the necrotic cell surface. To this end necrotic CHO cells were challenged with NHS, or complement active HSΔCFH and deposition of C5b was compared via flow cytometry (Figure 8A). Upon incubation in HSΔCFH more C5b was deposited on the surface of necrotic CHO cells (MFI = 2049) as compared to cells incubated in NHS (MFI = 3040). This difference was statically significant ($p \leq 0.0001$). When necrotic CHO were preincubated with Factor H prior to HSΔCFH challenge C5b deposition was decreased and the effect was dose-dependent (Figure 8B). Thus Factor H has an essential protective effect at the cell surface as revealed by the reduced C5b deposition.

Next the inhibitory effect of the tagged compstatin was evaluated. Therefore C5b surface deposition observed upon incubation with HSΔCFH was set 100 %. First necrotic CHO cells were either incubated with COMP_CFH15-20, SCR15-20, the untagged compstatin (4W9A) or the linearized inactive compstatin variant to allow binding. After a washing step, the cells were challenged with HSΔCFH for 15 min and the C5b amount on the necrotic cell surface was analyzed. COMP_CFH15-20 efficiently inhibited deposition of C5b on the necrotic cell surface in a dose-dependent manner (Figure 8C). At the highest used concentration COMP_CFH15-20 reduced the C5b deposition back to NHS background level. The inhibitory effect of COMP_CFH15-20 (approx 40 %) is statistically stronger at concentration of 40 µg / ml ($p = 0.0226$) compared to SCR15-20 which showed only minor effects in C5b deposition (approx 8 %). Also 40 µg / ml of the compstatin analog 4W9A reduced the C5b deposition on CHO cells in the presence of HSΔCFH. However, the amount of C5b in the presence of the COMP_CFH15-20 was significantly lower compared to untagged compstatin ($p = 0.01$). Similar experiments were performed on the surface of necrotic HUVEC cells, and the observed effects were basically comparable (Supplementary Figure 2)

DISCUSSION

A still increasing list of human diseases is mediated by inappropriate action of complement components and inappropriate regulation. In particular defective action and regulation of the alternative complement pathway is a major cause for tissue injury for a variety of both, rare and common diseases (20). The increasing number and, in particular the more detailed understanding of the pathological mechanisms, of such complement-mediated human diseases, demonstrates the need for the design of specific complement inhibitors. In order to target complement inhibitors to sites of immune stress we designed and generated a chimeric protein that has the central C-terminal surface attachment region of the human complement inhibitor Factor H linked to compstatin, a potent inhibitor of the C3 convertase. This novel hybrid protein COMP_CFH15-20 efficiently blocked complement activation in fluid phase and also on non-activator surfaces, such as sheep erythrocytes and living- as well as necrotic nucleated cells. The Factor H tag as a sensor for self cells, modified cells and biomembranes directs the C3 inhibitor to sites of complement stress and the increased local concentration allows efficient complement control on damaged surfaces.

COMP_CFH15-20 was expressed in the *Pichia pastoris* expression system with a mobility of approx. 50 kDa (Figure 1). The

hybrid protein possess two C3 interaction sites, one provided by the SCR15-20 tag and one by compstatin, and bound consequently stronger to C3 and C3b as the SCR15-20 tag alone as shown by ELISA and surface plasmon resonance (Figure 2, Figure 3). The Factor H tag enhanced the (relative) affinity of the inhibitor for C3b in terms of a slower dissociation profile as compared to the untagged compstatin analog 4W9A (Figure 3A). The evaluation of the binging profile confirmed an interaction that did follow a 1:2 model (Figure 3B). Therefore, the increased affinity is explained by dual binding sites provided by compstatin as well as the SCR15-20 region, which both have a unique and separate C3b/C3d binding motif (Table 1). If these events are independent or cooperative events is difficult to distinguish. One possibility could be that part of the chimera binds via the compstatin part to C3b (low affinity site, K_D = 130nM) and part simultaneously to both sites (strong affinity K_D = 2nM).

Due to this dual activity COMP_CFH15-20 provide protection from complement amplification as shown by a reduced lysis of rE and sE (Figure 4A, Figure 5B), by a decreased generation of anaphylatoxin C5a (Figures 4B, Figure 5B) and by the blockade of C5b surface deposition on non-activator as well as activator surfaces (Figures 4C, Figure 8C). In particular on non activator surfaces COMP_CFH15-20 was more efficient as compared to the untagged compstatin analog 4W9A. This effect was observed when the same protein concentrations were used, despite of the large differences (33.3 fold) in the molecular mass of tagged vs untagged compstatin (50 vs. 1.5 kDa).

Complement control is essential on biomembranes, extracellular matrixes as well as apoptotic or necrotic cellular surfaces that naturally lack membrane integrated complement regulators such as CD46, CD55 or CD59. Such surfaces need to be protected by endogenous or synthetic fluid phase regulators that attach to their surfaces (37). The C-terminus surface attachment region, (i.e. SCRs 15-20) of the complement inhibitor Factor H recognizes such surfaces and discriminates precisely between activator- and non-activator surfaces and therefore directs the Factor H to host surfaces, when complement activation is initiated (12, 38). The SCR15-20 tag directs also the synthetic complement inhibitor compstatin to self surfaces and sites of immune stress, i.e. to living as well as necrotic cells (Figure 6A, Figure 6B and Supplementary Figure 1). The recruited inhibitor compstatin therefore blocks complement activation specifically on the surface of self-, and in particular modified-self surfaces. This protective effect is demonstrated; as cell bound COMP15-20 significantly enhanced the metabolic activity (viability) of CHO cells upon challenge with HSΔCFH. In this case the tagged compstatin was approx. 30 % more efficient as the untagged compstatin (4W9A) (Figure 7C). A similar protection was demonstrated when the protective effects were compared on the surface of necrotic surfaces of CHO and HUVEC cells (Figure 8C, Supplementary Figure 2). In this situation the protective effect of COMP15-20 was about 20 % higher as the untagged compstatin, although the smaller molecule was applied at a 33 fold higher molar concentration.

Reduced interaction of fluid phase regulators such as Factor H because of mutations, polymorphisms or autoantibodies are highly associated with aHUS (W1157R in SCR19, R1210C in SCR20), DEAP-HUS (autoantibodies) or AMD (Y402H in SCR7) (16, 17, 39, 40). Complete deficiency of Factor H in plasma causes unrestricted systemic C3 activation, systemic C3 consumption and results to thickening of the glomerular basement membrane, to renal damage and to MPGN-II (41). Here we show that the novel inhibitory protein COMP15-20 binds to self surfaces and to C3b and that this new complement inhibitor protects in particular modified-self surfaces from complement mediated damage. This potent inhibitory effect occurs at concentrations of 20 – 40 µg / ml (i.e. 0.4 - 0.8 µM), which are much lower than the Factor H levels in human plasma, which are 800 µg / ml (5.3 µM).

To proof these *in-vitro* results in-vivo studies are necessary as successfully shown for the tagged regulators CR2-fH and CR2-Crry (25, 26). In summary, with COMP_CFH15-20, we have developed a novel complement inhibitor that combines the C-terminal surface attachment region of Factor H with the potent, established inhibitor compstatin, to direct complement-inhibition specifically to sites of immune stress. The chimera has dual activities in terms of high

affinity binding to C3b binding and distinguishing activator and non-activator surfaces. COMP_CFH15-20, which controls complement locally at sites of immune stress in particular on damaged self surfaces, is more specific and more effective at these sites as compared to the untagged compstatin which acts systemically. Thus, this new tagged complement inhibitor allows targeted and precise complement control at sites of immune stress and the new inhibitor lacks the adverse effects occurring upon systemic complement inhibition. COMP_CFH15-20 is therefore a promising complement inhibitor whose therapeutic potential should be analysed in more detail.

REFERENCES

1. Walport, M. J. 2001. Complement. First of two parts. *N Engl J Med* 344:1058-1066.
2. Carroll, M. C. 2000. The role of complement in B cell activation and tolerance. *Adv Immunol* 74:61-88.
3. Ogden, C. A., and K. B. Elkon. 2006. Role of complement and other innate immune mechanisms in the removal of apoptotic cells. *Curr Dir Autoimmun* 9:120-142.
4. Pangburn, M. K., and H. J. Muller-Eberhard. 1983. Initiation of the alternative complement pathway due to spontaneous hydrolysis of the thioester of C3. *Ann N Y Acad Sci* 421:291-298.
5. Zipfel, P. F., M. Mihlan, and C. Skerka. 2007. The alternative pathway of complement: a pattern recognition system. *Adv Exp Med Biol* 598:80-92.
6. Turnberg, D., and M. Botto. 2003. The regulation of the complement system: insights from genetically-engineered mice. *Mol Immunol* 40:145-153.
7. Zipfel, P. F., and C. Skerka. 2009. Complement regulators and inhibitory proteins. *Nat Rev Immunol* 9:729-740.
8. Jozsi, M., and P. F. Zipfel. 2008. Factor H family proteins and human diseases. *Trends Immunol* 29:380-387.
9. Rodriguez de Cordoba, S., J. Esparza-Gordillo, E. Goicoechea de Jorge, M. Lopez-Trascasa, and P. Sanchez-Corral. 2004. The human complement factor H: functional roles, genetic variations and disease associations. *Mol Immunol* 41:355-367.
10. Pangburn, M. K., R. D. Schreiber, and H. J. Muller-Eberhard. 1977. Human complement C3b inactivator: isolation, characterization, and demonstration of an absolute requirement for the serum protein beta1H for cleavage of C3b and C4b in solution. *J Exp Med* 146:257-270.
11. Kuhn, S., and P. F. Zipfel. 1996. Mapping of the domains required for decay acceleration activity of the human factor H-like protein 1 and factor H. *Eur J Immunol* 26:2383-2387.
12. Ferreira, V. P., A. P. Herbert, H. G. Hocking, P. N. Barlow, and M. K. Pangburn. 2006. Critical role of the C-terminal domains of factor H in regulating complement activation at cell surfaces. *J Immunol* 177:6308-6316.
13. Oppermann, M., T. Manuelian, M. Jozsi, E. Brandt, T. S. Jokiranta, S. Heinen, S. Meri, C. Skerka, O. Gotze, and P. F. Zipfel. 2006. The C-terminus of complement regulator Factor H mediates target recognition: evidence for a compact conformation of the native protein. *Clin Exp Immunol* 144:342-352.
14. Meri, S., and M. K. Pangburn. 1990. Discrimination between activators and nonactivators of the alternative pathway of complement: regulation via a sialic acid/polyanion binding site on factor H. *Proc Natl Acad Sci U S A* 87:3982-3986.
15. Manuelian, T., J. Hellwage, S. Meri, J. Caprioli, M. Noris, S. Heinen, M. Jozsi, H. P. Neumann, G. Remuzzi, and P. F. Zipfel. 2003. Mutations in factor H reduce binding affinity to C3b and heparin and surface attachment to endothelial cells in hemolytic uremic syndrome. *J Clin Invest* 111:1181-1190.
16. Jozsi, M., S. Strobel, H. M. Dahse, W. S. Liu, P. F. Hoyer, M. Oppermann, C. Skerka, and P. F. Zipfel. 2007. Anti factor H autoantibodies block C-terminal recognition function of factor H in hemolytic uremic syndrome. *Blood* 110:1516-1518.

17. Zipfel, P. F., C. Mache, D. Muller, C. Licht, M. Wigger, and C. Skerka. 2010. DEAP-HUS: Deficiency of CFHR plasma proteins and autoantibody-positive form of hemolytic uremic syndrome. *Pediatr Nephrol* 10:2009-2019.
18. Licht, C., S. Heinen, M. Jozsi, I. Loschmann, R. E. Saunders, S. J. Perkins, R. Waldherr, C. Skerka, M. Kirschfink, B. Hoppe, and P. F. Zipfel. 2006. Deletion of Lys224 in regulatory domain 4 of Factor H reveals a novel pathomechanism for dense deposit disease (MPGN II). *Kidney Int* 70:42-50.
19. Ferreira, V. P., M. K. Pangburn, and C. Cortes. Complement control protein factor H: the good, the bad, and the inadequate. *Mol Immunol* 47:2187-2197.
20. Thurman, J. M., and V. M. Holers. 2006. The central role of the alternative complement pathway in human disease. *J Immunol* 176:1305-1310.
21. Mulligan, M. S., R. L. Warner, C. W. Rittershaus, L. J. Thomas, U. S. Ryan, K. E. Foreman, L. D. Crouch, G. O. Till, and P. A. Ward. 1999. Endothelial targeting and enhanced antiinflammatory effects of complement inhibitors possessing sialyl Lewisx moieties. *J Immunol* 162:4952-4959.
22. Smith, G. P., and R. A. Smith. 2001. Membrane-targeted complement inhibitors. *Mol Immunol* 38:249-255.
23. Rother, R. P., S. A. Rollins, C. F. Mojcik, R. A. Brodsky, and L. Bell. 2007. Discovery and development of the complement inhibitor eculizumab for the treatment of paroxysmal nocturnal hemoglobinuria. *Nat Biotechnol* 25:1256-1264.
24. Quigg, R. J. 2002. Use of complement inhibitors in tissue injury. *Trends Mol Med* 8:430-436.
25. Atkinson, C., H. Song, B. Lu, F. Qiao, T. A. Burns, V. M. Holers, G. C. Tsokos, and S. Tomlinson. 2005. Targeted complement inhibition by C3d recognition ameliorates tissue injury without apparent increase in susceptibility to infection. *J Clin Invest* 115:2444-2453.
26. Huang, Y., F. Qiao, C. Atkinson, V. M. Holers, and S. Tomlinson. 2008. A novel targeted inhibitor of the alternative pathway of complement and its therapeutic application in ischemia/reperfusion injury. *J Immunol* 181:8068-8076.
27. Sahu, A., B. K. Kay, and J. D. Lambris. 1996. Inhibition of human complement by a C3-binding peptide isolated from a phage-displayed random peptide library. *J Immunol* 157:884-891.
28. Sahu, A., A. M. Soulika, D. Morikis, L. Spruce, W. T. Moore, and J. D. Lambris. 2000. Binding kinetics, structure-activity relationship, and biotransformation of the complement inhibitor compstatin. *J Immunol* 165:2491-2499.
29. Ricklin, D., and J. D. Lambris. 2008. Compstatin: a complement inhibitor on its way to clinical application. *Adv Exp Med Biol* 632:273-292.
30. Potentia, P. 2009. POT-4 Drug Candidate for Age-Related Macular Degeneration Successfully Completes Phase I Clinical Trial - Prepares for Phase II. *Press Release*.
31. Heinen, S., A. Hartmann, N. Lauer, U. Wiehl, H. M. Dahse, S. Schirmer, K. Gropp, T. Enghardt, R. Wallich, S. Halbich, M. Mihlan, U. Schlotzer-Schrehardt, P. F. Zipfel, and C. Skerka. 2009. Factor H-related protein 1 (CFHR-1) inhibits complement C5 convertase activity and terminal complex formation. *Blood* 114:2439-2447.
32. Kuhn, S., C. Skerka, and P. F. Zipfel. 1995. Mapping of the complement regulatory domains in the human factor H-like protein 1 and in factor H1. *J Immunol* 155:5663-5670.
33. Kuhn, S., and P. F. Zipfel. 1995. The baculovirus expression vector pBSV-8His directs secretion of histidine-tagged proteins. *Gene* 162:225-229.
34. Magotti, P., D. Ricklin, H. Qu, Y. Q. Wu, Y. N. Kaznessis, and J. D. Lambris. 2009. Structure-kinetic relationship analysis of the therapeutic complement inhibitor compstatin. *J Mol Recognit* 22:495-505.
35. Hellwage, J., T. S. Jokiranta, M. A. Friese, T. U. Wolk, E. Kampen, P. F. Zipfel, and S. Meri. 2002. Complement C3b/C3d and cell surface polyanions are recognized by overlapping binding sites on the most carboxyl-terminal domain of complement factor H. *J Immunol* 169:6935-6944.
36. Zipfel, P. F., S. Heinen, M. Jozsi, and C. Skerka. 2006. Complement and diseases: defective alternative pathway control results in kidney and eye diseases. *Mol Immunol* 43:97-106.

37. Zipfel, P. F. 2001. Complement factor H: physiology and pathophysiology. *Semin Thromb Hemost* 27:191-199.
38. Pickering, M. C., E. G. de Jorge, R. Martinez-Barricarte, S. Recalde, A. Garcia-Layana, K. L. Rose, J. Moss, M. J. Walport, H. T. Cook, S. R. de Cordoba, and M. Botto. 2007. Spontaneous hemolytic uremic syndrome triggered by complement factor H lacking surface recognition domains. *J Exp Med* 204:1249-1256.
39. Jozsi, M., S. Heinen, A. Hartmann, C. W. Ostrowicz, S. Halbich, H. Richter, A. Kunert, C. Licht, R. E. Saunders, S. J. Perkins, P. F. Zipfel, and C. Skerka. 2006. Factor H and atypical hemolytic uremic syndrome: mutations in the C-terminus cause structural changes and defective recognition functions. *J Am Soc Nephrol* 17:170-177.
40. Skerka, C., N. Lauer, A. A. Weinberger, C. N. Keilhauer, J. Suhnel, R. Smith, U. Schlotzer-Schrehardt, L. Fritsche, S. Heinen, A. Hartmann, B. H. Weber, and P. F. Zipfel. 2007. Defective complement control of factor H (Y402H) and FHL-1 in age-related macular degeneration. *Mol Immunol* 44:3398-3406.
41. Appel, G. B., H. T. Cook, G. Hageman, J. C. Jennette, M. Kashgarian, M. Kirschfink, J. D. Lambris, L. Lanning, H. U. Lutz, S. Meri, N. R. Rose, D. J. Salant, S. Sethi, R. J. Smith, W. Smoyer, H. F. Tully, S. P. Tully, P. Walker, M. Welsh, R. Wurzner, and P. F. Zipfel. 2005. Membranoproliferative glomerulonephritis type II (dense deposit disease): an update. *J Am Soc Nephrol* 16:1392-1403.

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FOOTNOTES

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[3]Abbrevations used in this paper: AMD, age-related macular degeneration; AP, the alternative pathway; CHO, Chinese hamster ovary cells; CP, the classical pathway; HSΔCFH, human active serum depleted for Factor H; HSΔC7, human active serum depleted for complement component C7; HUVEC, human umbilical vein endothelial cells; LP, the lectin pathway; MPGN II, membranoproliferative glomerulonephritis type II; NHS, normal human serum; SCR, short consensus repeat; sE, sheep erythrocytes; rE, rabbit erythrocytes

TABLE

Table 1: Kinetic characterization of COMP_SCR15-20

protein	k_{on} ($10^6 M^{-1}s^{-1}$)	k_{off} ($10^{-3}s^{-1}$)	K_D (nM)
COMP_SCR15-20			
1	0.3 ±	39 ±	130 ±
2	1.4 ±	2.5 ±	2 ±
4W9A	0.4 ±	70 ±	158 ±
SCR15-20			5000

FIGURE LEGENDS

Figure 1: Generation and purification of COMP_CFH15-20. The sequence corresponding to compstatin fused to the surface attachment region of Factor H (i.e. SCRs 15-20) representing COMP_CFH15-20 was cloned into the expression vector pPICZαB of the yeast *P. pastoris* and transferred into yeast cells. After 3 days the culture supernatant was collected and recombinant protein COMP_CFH15-20 was purified by nickel affinity chromatography. The purified COMP_CFH15-20 protein, as well as the control proteins SCR15-20 and full length Factor H were separated by SDS-PAGE and detected either by **A)** silver staining or by **B)** western blotting using a polyclonal Factor H antiserum and a secondary HRP labeled antiserum. COMP_CFH15-20 is identified as a band of approx. 50 kDa (lane 1, arrow). The unmodified deletion fragment SCR15-20 had a mobility of 45 kDa (lane 2), Factor H a molar mass of 150 kDa (lane 3). The mobility of the proteins is indicated on the left of Figure 1A. One representative silver gel (A) and western blot (B) of five independent experiments is shown.

Figure 2: COMP_CFH15-20 binds to C3 and C3b. C3, C3b or heparin were coated onto a microtiter plate (each at 5 µg / ml), saturated with blocking buffer and then increasing amounts of either COMP_CFH15-20, SCR15-20 or the control protein BSA were added. Bound proteins were detected by using a HRP labeled polyclonal goat Factor H antiserum. **A)** COMP_CFH15-20 (black squares) bound dose-dependently to C3. At all concentrations > 2.5 µg / ml COMP_CFH15-20 showed significantly stronger binding to C3 as compared to the untagged deletion mutant SCR15-20 (open squares). **B)** COMP_CFH15-20 binds C3b significantly stronger as compared to SCR15-20 for all tested concentrations. For the binding studies BSA (dashed line, diamond) was used as a negative control and showed no binding to C3 and C3b. The figures A, B show one representative experiment of three independent experiments with three replicates each. Error bars indicate SD. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Figure 3: Kinetic characterization of COMP_CFH15-20. Kinetics of the interaction between C3b and COMP_CFH15-20 were analysed by surface plasmon resonance. Therefore C3b uniformly oriented due to immobilization by thiol coupling on a sensor chip and the analytes COMP_CFH15-20, SCR15-20 and the compstatin analog 4W9A were injected for 2min at a flow rate of 10µl/min followed by a 3 min dissociation phase. **A)** Kinetic ranking of the analytes. A constant concentration (1µM) of each analyte was injected and the resulting binding signals were normalized against molecular weight and overlaid to visualize relative differences in their association and dissociation phases. The SCR15-20 interaction (green) with C3b was rather weak, compared to COMP_CFH15-20 (red) and the compstatin analog 4W9A (blue). COMP_CFH15-20 showed a similar binding efficiency but an improved dissociated rate than 4W9A. **B)** Kinetic analysis of COMP_CFH15-20. Increasing concentrations of COMP_CFH15-20 were delivered to surface-bound C3b, whereas COMP_CFH15-20 showed a dose-depended and stable C3b binding. Processed surface plasmon resonance signals were globally fitted to 1:2 binding model and the kinetic rate constants k_{on} and k_{off} extracted (Table1).

Figure 4: COMP_CFH15-20 prevents complement activation on the surface of rabbit erythrocytes. Rabbit erythrocytes (rE), which represent activator surfaces, were incubated in 7.5 % complement active NHS together with increasing amounts of COMP_CFH15-20 or SCR15-20 for 30 min at 37 °C. The amount of lysed rE was determined by measuring the absorbance of the supernatant at 414 nm. **A)** COMP_CFH15-20 (black squares) prevented lysis of rE in a dose-dependent manner. At the highest concentration of 20 µg / ml lysis was inhibited completely. This protective effect was significantly stronger as compared to SCR15-20 (open squares), which did not affect lysis of rE. The compstatin analog 4W9A (black dot) showed a comparable protection of lysis as COMP_CFH15-20 whereas the linearized inactive compstatin (white dot) variant showed no protective effect on the lysis of rE. **B)** In parallel the C5a levels were assayed in the supernatant by ELISA. In the presence of COMP_CFH15-20 detection of C5a decreases with higher amounts of the inhibitor, whereas the C5a amount in the presence of SCR15-20 stays stable. The control peptide 4W9A completely prevented C5a generation. The linearized negative control peptide does not protect

from complement activation therefore a high C5a amount is detectable. The figures A-B show one representative experiment of three independent experiments with three replicates each. Error bars indicate SD. *, P < 0.05; **, P < 0.01; ***, P < 0.001. **C-F)** The deposition of C5b on the surface of rE incubated in complement active C7 depleted human serum (HSΔ**C7)** was assayed by immunofluorescence microscopy. After incubation of the rE with 7.5 % HSΔ**C7** together with 20 µg / ml COMP_CFH15-20, SCR15-20 as well as the active and inactive compstatin analogs for 30 min, C5b deposition was visualized using a polyclonal C5 antiserum followed by the appropriate AlexaFluor488 conjugated secondary antiserum. In the presence of COMP_CFH15-20 **(C)** only a weak C5 deposition detectable which is comparable to the compstatin analog 4W9A **(E)**. In the presence of the untagged SCR15-20 deletion mutant **(D)** rE were completely opsonized with C5 shown by the intense green fluorescence comparable to the linearized compstatin **(F)**. The images C – F are representative of three independent experiments. Bar = 20µm

Figure 5: COMP_CFH15-20 prevents complement activation on the non-activator surface of sheep erythrocytes. The inhibitory activity of COMP_CFH15-20 was also assayed for sheep erythrocytes (sE) that represent non-activator surfaces. **A)** sE are protected from complement mediated lysis when incubated with 30 % complement active human serum (first column), but are lysed when incubated in active human serum depleted for the inhibitor Factor H (HSΔCFH; blank value of each curve). Incubation of sE with COMP_SCR15-20 (black squares) in the presence of HSΔCFH for 30 min at 37 °C protected the erythrocytes from lysis. The effect was dose dependent. At the highest concentration of 20µg / ml lysis of sE was completely inhibited and showed a significantly stronger inhibition than the untagged SCR15-20 deletion fragment (open squares), which did not affect lysis. Compstatin analog 4W9A showed a protective effect (black dot) and the inactive compstatin peptide did not affect erythrocyte lysis (white dot) **B)** Generation of the complement activation product C5a was identified in the supernatant by ELISA. For all tested proteins and peptides the C5a level coincides with the values of the hemolytic assay. The figures A-B show one representative experiment of three independent experiments with three replicates each. Error bars indicate SD. *, P < 0.05; **, P < 0.01; ***, P < 0.001.

Figure 6: COMP_CFH15-20 binds to intact as well as damaged CHO cells. COMP_CFH15-20 and SCR15-20 bind to the surface of **A)** intact as well as to the surface of **B)** necrotic CHO cells. The cellular attachment of both proteins was assayed due to incubating the cells with the proteins and their detection with a polyclonal antiserum specific for the C-terminus of Factor H using flow cytometry. One representative experiment of three independent experiments with three replicates each is shown.

Figure 7: COMP_CFH15-20 enhances metabolic rate of living nucleated CHO cells upon complement challenge. A) Metabolic activity of CHO cells challenged with complement active human serum (NHS) was determined by uptake of the non fluorescent dye rezazurin and by following cytoplasmic conversion to the fluorescent dye resorufin. The resulting absorbance shift of the supernatant was measured at 570nm over a period of 5 h. Cells challenged with complement active human serum that was depleted for the inhibitor Factor H (HSΔCFH, open circles) showed less metabolic activity as cells in the presence of NHS (black circles). The effects were significantly different over the whole period of 5 h. **B)** Preincubation of Factor H (filled triangles) with CHO cells prior HSΔCFH treatment enhanced and improved metabolic activity in a dose-dependent manner. **C)** Preincubation of COMP_CFH15-20 (black squares) was more effective to protect CHO cells from complement mediated damage than preincubation with the untagged compstatin analog 4W9A (cross?). The CHO viability in the presence of COMP_CFH15-20 was significantly higher than in the presence of 4W9A after 1h and 2h. The viability value for CHO cells in the presence of NHS after 5h incubation was set to 100 %. The data represent mean values and standard deviation for three independent experiments. Error bars indicate SD. *, P < 0.05; **, P < 0.01; ***, P < 0.001.

Figure 8: COMP_CFH15-20 reduced deposition C5b onto the surface of necrotic CHO cells upon complement challenge. A) The absence of Factor H in human plasma results in complement activation and deposition of complement activation products on cellular surfaces. To compare the

amount of complement activation on the surface of necrotic CHO cells in the presence of NHS and HSΔCFH, C5b deposition was measured via flow cytometry. To this end CHO cells were challenged with either 10 % NHS or 10 %HSΔCFH for 15 min at 30 °C. In the presence of HSΔCFH (black histogram) the amount of cell bound C5b is significantly enhanced compared to cells incubated with NHS (gray histogram). A representative histogram profiles from four independent experiments are shown. **B)** Preincubation of increasing amounts of Factor H (filled triangles) with CHO cells prior HSΔCFH treatment reduced the C5b deposition in a dose-dependent manner. **C)** COMP_CFH15-20 (black squares) prevented C5b deposition in a dose-dependent manner. At the highest concentration of 40 µg / ml the amount of cell bound C5b was comparable to CHO cells incubated with NHS (gray column). This protective effect was stronger as compared to SCR15-20 (open squares). The compstatin analog 4W9A (black dot) showed also a reduced C5b deposition with the used concentration of approx.40µg / ml which was however not as strong as COMP_CFH15-20. The C5b deposition on CHO cells in the presence of HSΔCFH was set to 100 %. The data represent mean values and standard deviation for four independent experiments. Error bars indicate SD. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Unspecific antibody binding in the absence of any proteins is shown by the dotted line.

Figure 1

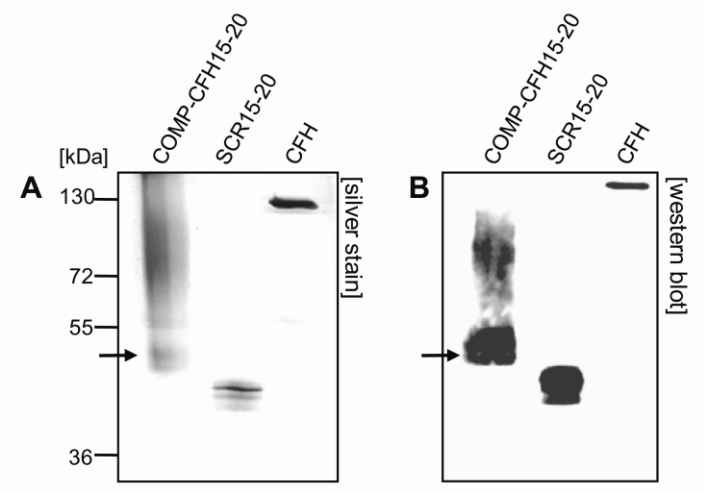


Figure 2

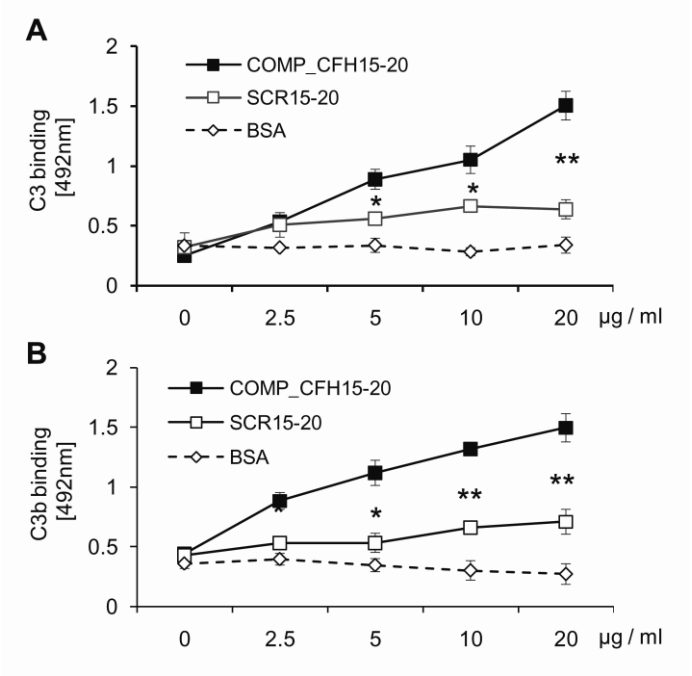


Figure 3

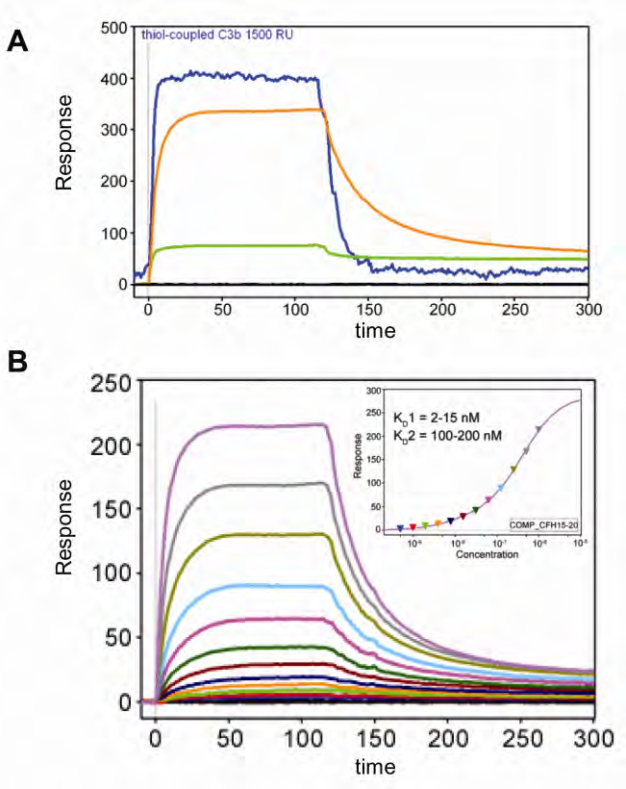


Figure 4

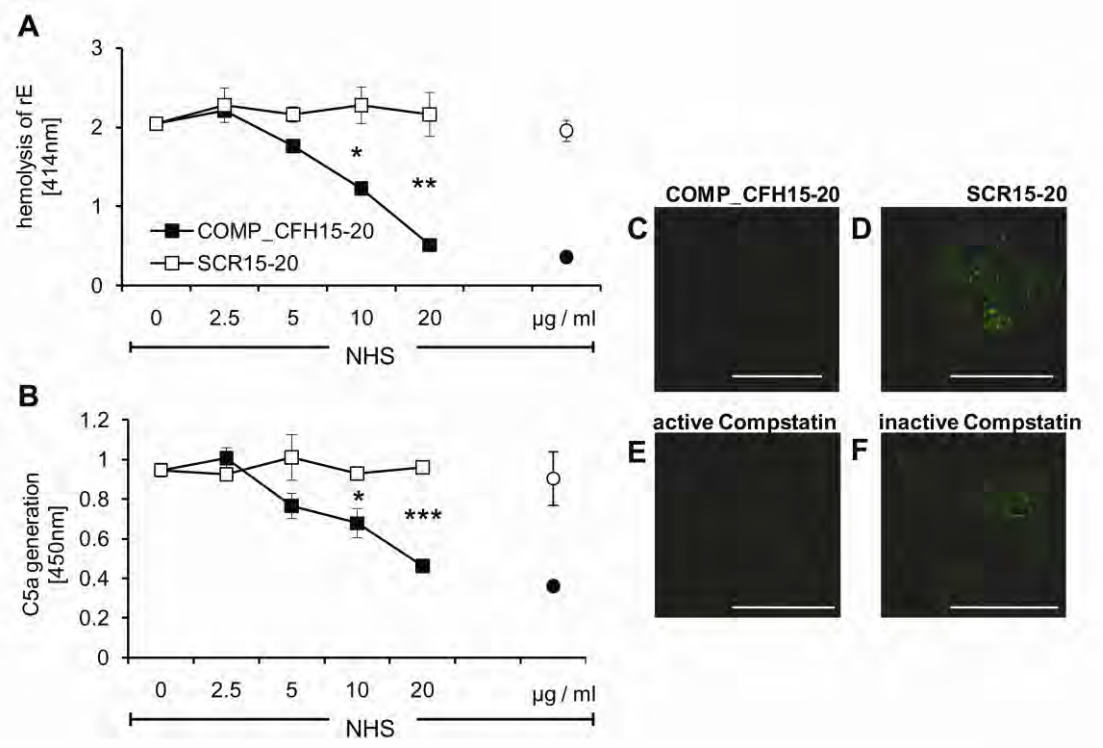
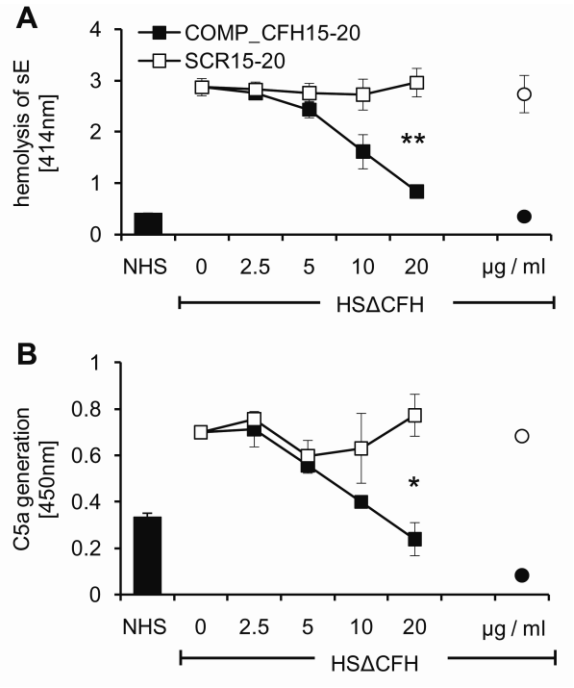


Figure 5



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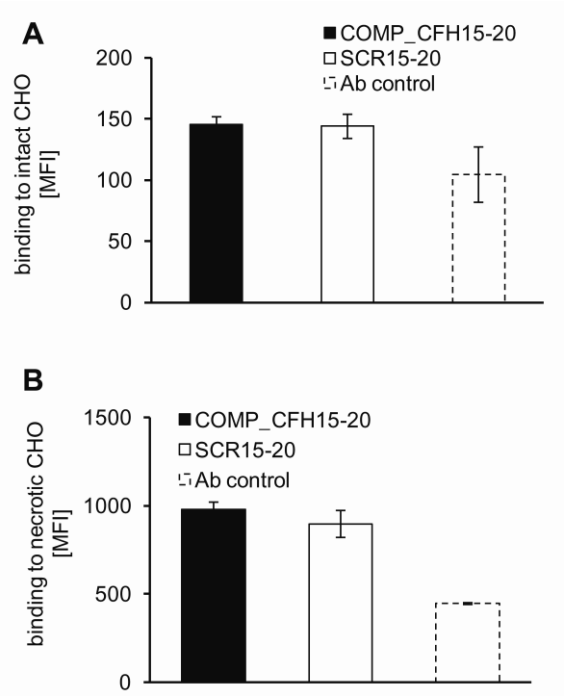


Figure 7

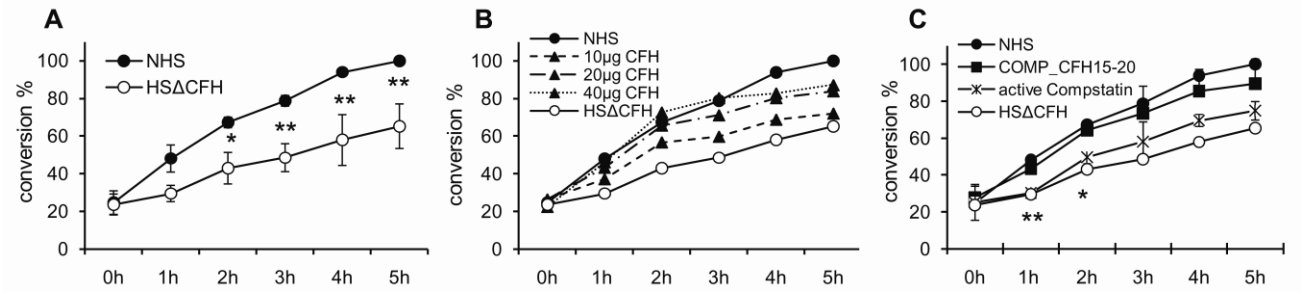
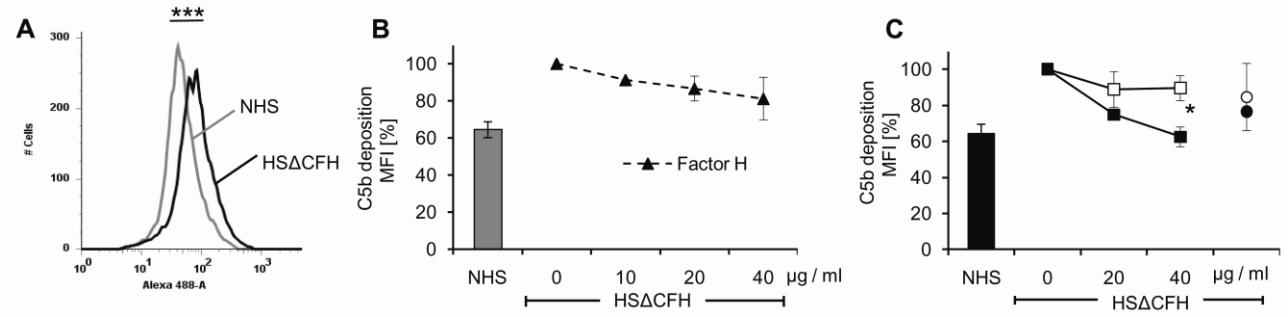


Figure 8



Diskussion

AMD – eine komplementassoziierte Erkrankung

Die altersabhängige Makuladegeneration ist eine multifaktorielle Erkrankung des Auges. Dabei kommt es durch lokale, chronische Entzündungsprozesse nahe der Retina zu einer Zerstörung der Fotorezeptoren der Makula und dementsprechend zu einem langsamen Verlust des zentralen Sehvermögens[17]. Lange waren die ursächlichen Faktoren der AMD-Entstehung unbekannt. Doch mit dem *Human Genome Project* und der damit verbundenen Erstellung von Datenbanken über SNPs des menschlichen Genoms, änderten sich die Voraussetzungen für genetische Untersuchungen der AMD entscheidend. Dies führte ab dem Jahre 2005 dazu, dass genomweite Assoziationsstudien sowie SNP-Feinkartierungen konkrete Risikogene auf Chromosom 1, 6, 10 und 19 identifiziert wurden. Eine zentrale Rolle in der AMD-Entstehung spielt demnach das Komplementsystem, in welchem Polymorphismen der Gene für *Faktor H*, *Faktor B*, *C2* und *C3* sowie die Defizienz der *CFHR1-/CFHR3*-Gene das individuelle Risiko beeinflussen (**Manuskript 1**). Lokale, z. T. auch systemische, Entzündungsereignisse der AMD werden dabei hauptsächlich durch die Dysregulation des alternativen Aktivierungsweges verursacht[75]. Diese neuen Erkenntnisse ergänzen frühere Konzepte, welche bisher die unverhältnismäßig hohe intraokulare Komplementaktivierung erklärten. Diese Konzepte basierten auf der Annahme, dass ein altersbedingter ineffizienter Transport von Nährstoffen und Abbauprodukten über den RPE-Choroid-Komplex zu einer Ansammlung von extrazellulärem Material führt[171]. Dessen Akkumulation generiert eine Aktivatoroberfläche, welche Komplementaktivierung, Amplifizierung und damit die Generation der Anaphylatoxine C3a und C5a erlaubt. Durch die kontinuierliche, spontane Aktivierung des alternativen Weges ist Komplementaktivierung, aber nicht nur sekundärer Verstärker eines bereits vorhandenen Schadens, sondern kann auch primär Zellschaden verursachen und demnach ausschlaggebend für die Initiierung einer Erkrankung sein[172]. Sollten die identifizierten AMD-assoziierten Polymorphismen der Komplementkomponenten Veränderungen in der Expression, Faltung oder Funktion hervorrufen, würden sie das empfindliche Gleichgewicht zwischen Komplementaktivierung und -inhibierung stören. In der Folge würde sich eine über Wochen, Monate oder gar Jahre andauernde unnötige Aktivierung, in der Manifestation von Drusen, der Degeneration des RPEs und der darüber liegenden Fotorezeptoren äußern.

Bei den seltenen Nierenerkrankungen des aHUS und der MPGN-II ist eine defekte Komplementregulation bereits mit der Pathogenese assoziiert[132]. Diese Erkrankungen sind mit mutiertem Faktor H, einer CFHR1-/CFHR3-Defizienz sowie Polymorphismen in Faktor B, Faktor I und C3 assoziiert. Interessanterweise zeigen diese Erkrankungen analoge pathopysiologische Veränderungen an der nephrologischen glomerulären Basalmembran, wie sie auch am RPE der Retina von AMD-Patienten vorkommen[143]. Der Austausch eines einzelnen Nukleotids in der DNA bzw. einer Aminosäure in der Proteinsequenz kann also die Funktion eines Proteins dahingehend verändern, dass die essenzielle Homöostase zwischen Komplementaktivierung und -inhibierung nicht mehr gewährleistet ist. Eine koordinierte, feinregulierte Kontrolle des Komplementsystems ist aber essenziell, um lokale Entzündungen bzw. Schäden autologen Gewebes im Auge zu verhindern[79].

Der AMD-assoziierte Polymorphismus in SCR 7 (Y402H), nicht aber in SCR 1 (V62I), hat Auswirkungen auf eine effektive Komplementregulation

Das *Faktor H*-Gen war das erste konkrete Risikogen, welches mit AMD assoziiert wurde. Zahlreiche, genetisch basierte Studien identifizierten extonische, aber auch intronische Faktor H-Polymorphismen, die das Krankheitsrisiko der AMD beeinflussen. Zwei der beschriebenen Polymorphismen resultieren in einer *„missense"*-Mutation, d. h. die Substitution eines Nukleotides im DNA-Strang führt zu einer Veränderung der Faktor H-Aminosäuresequenz. Die daraus resultierenden V62I- sowie Y402H-Polymorphismen sind, mit SCR 1 bzw. SCR 7, in funktionell relevanten Domänen von Faktor H und FHL1 lokalisiert. Bis dato waren die funktionellen und ggf. additiven Auswirkungen beider Polymorphismen auf die komplementregulative Funktion von Faktor H und FHL1 nahezu unerforscht.

Das **Manuskript 2** dieser Dissertation beschreibt, dass die Substitution an Aminosäureposition 62 (V → I) keine Auswirkungen auf die Interaktion von FHL1 mit C3b oder auf dessen Kofaktoraktivität hat. Hingegen beeinflusst die Substitution an Position 402 (Y → H) die Heparin- und die Zellbindung. Die protektive Y402-Variante des FHL1-Proteins band bis zu 25 % stärker an RPE-Zellen als die H402-Risikovariante. Die verstärkte Zellbindung von FHL1-Y402 beeinflusste gleichzeitig die Komplementregulation von FHL1 auf Zelloberflächen. Gebunden an Nichtaktivatoroberflächen war der FHL1-vermittelte Schutz vor Komplementaktivierung um 20 - 25 % für die Y402-Variante erhöht. Demzufolge hat, für den Regulator FHL1, nur die Substitution in SCR 7, nicht aber in SCR 1, funktionelle Auswirkungen auf eine optimale Komplementregulation. Genetische Studien aber zeigen eine signifikante Assoziation des V62I-Polymophismuses mit der AMD. Kausale Effekte auf Ebene der Transkription, der mRNA-Stabilität, der Translationseffizienz und sicherlich auch der Einfluss individueller Risikofaktoren sollten daher nicht ausgeschlossen und weiter untersucht werden.

Kristallstrukturanalysen sowie die Kernspinresonanzspektroskopie klärten die Molekülstruktur von Faktor H sowie seiner Interaktionspartner während der letzten zwei Jahre auf. Diese Analysen bestätigen, dass die Aminosäure an Position 62 von Faktor H / FHL1 kein Bestandteil der C3b-Bindedomäne in SCR 1 ist[173]. Zudem ist sie nicht an den Vorgängen der Kofaktoraktivität, sowie der beschleunigten Dissoziation der C3-Konvertase beteiligt. Die verborgene Lage innerhalb der SCR 1-Domäne macht Position 62 nur bedingt zugänglich für Liganden[174]. Bisherige Erkenntnisse über die Auswirkungen der V62I-Substitutionen im Faktor H-/ FHL1-Protein beschränken sich auf die geringfügige Erhöhung der Stabilität von SCR1, in Gegenwart der Aminosäure Isoleucin. Die einmalig beschriebenen funktionellen Unterschiede zwischen der I62- und V62-Variante von Faktor H[157] in Bezug auf die C3b-Interaktion, wurden sowohl in **Manuskript 2** als auch anderen Publikationen nicht bestätigt[175]. Im Gegensatz dazu ist die Aminosäure an Position 402 in SCR 7 oberflächenexponiert und entsprechend zugänglich für Heparin und GAGs[109,176]. Eine Gruppe positiv geladener Aminosäuren, die verantwortlich für die Interaktion mit negativ geladenen Strukturen der Zelloberfläche ist, befindet sich in

unmittelbarer Nähe der Aminosäure 402 (**Abbildung 8**). Der AMD-assoziierte Austausch von Tyrosin durch Histidin beeinträchtigt also unmittelbar die Zellbindungseigenschaften der Regulatoren Faktor H und FHL1[177,178].

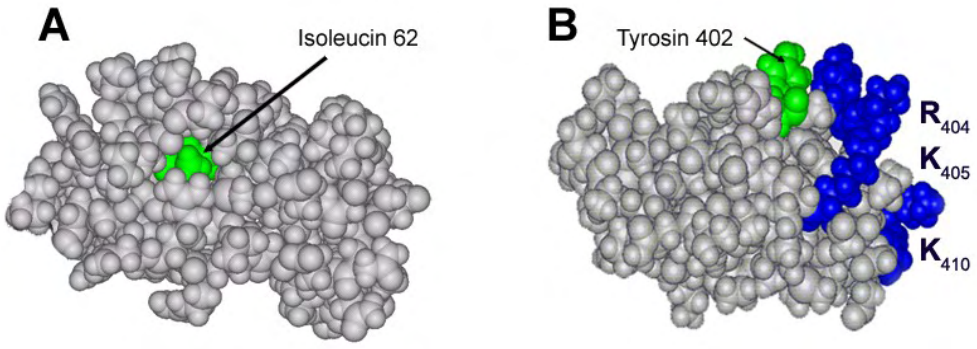


Abbildung 8: Dreidimensionale Darstellung von SCR 1 und SCR 7 des Faktor H-Proteins. A) Das Strukturmodell der Domäne 1 offenbart, dass die Aminosäure an Position 62 verborgen und schwer zugänglich für Liganden ist. Die mit einem reduzierten AMD-Risiko assoziierte Aminosäure Isoleucin ist grün dargestellt. **B)** Das Strukturmodell der Domäne 7 zeigt, dass die Position 402 oberflächenexponiert ist und sich in unmittelbarer Nähe von R404, K405 sowie K410 befindet (blau), positive geladene Aminosäuren, welche die Bindung an zelluläre Oberflächen vermitteln. Das protektive Tyrosin ist grün hervorgehoben.

Doch warum wirkt sich die reduzierte Zellbindung der H402-Risikovarianten speziell im Auge, nahe der Makula aus? Faktor H interagiert mit den sulfathaltigen GAGs Dermatansulfat sowie Heparansulfat[152]. Diese Bindung wird speziell durch SCR 7 vermittelt. Die Verteilung von GAGs ist spezifisch für jedes Gewebe und das Vorkommen bestimmter GAGs spielt eine entscheidende Rolle für die Vermittlung von bestimmten Proteinbindungen[179,180]. Immunhistochemische Studien im retinalen Gewebe zeigen, dass sowohl Dermatansulfat als auch Heparansulfat Bestandteile der BM sind. Zudem weisen die kollagenhaltigen, elastischen Fasern der BM unter der Makula eine reduzierte Integrität auf, d. h. die BM ist nahe der Makula dünner als in anderen Regionen der Retina[181]. Demzufolge gibt die Verteilung der GAGs sowie die topografische Eigenschaft der BM Hinweise darauf, warum eine reduzierte Komplementregulation, verursacht durch den Y402H-Polymorphismuses, vor allem im retinalen, makulärem Gewebe auftritt.

Das **Manuskript 2** beschreibt eine um 25 % reduzierte Komplementregulation auf Nichtaktivatoroberflächen in Gegenwart der H402-Risikovarianten. Das zeigt erstmals, dass die Beeinträchtigung der intraokularen Komplementregulation primär Zellschaden verursachen, d. h. AMD initiieren kann. Auf den ersten Blick erscheinen 25 % relativ wenig, um ernsthafte pathophysiologische Veränderungen im RPE-Choroid-Komplex hervorzurufen. Doch das Komplementsystem besitzt enormes Amplifikationspotenzial, welches besonders im immunprivilegiertem Auge reguliert werden muss[107]. Eine verminderte Regulation der C3-Konvertase durch Faktor H bzw. FHL1 resultiert in der Schädigung von gesundem, retinalem Gewebe[88]. Zudem beeinflussen AMD-assoziierte Polymorphismen des Faktor B-Proteins die Stabilität der C3-Konvertase und modifizieren so zusätzlich den Krankheitsverlauf[160]. Hält dieser Zustand über einen längeren Zeitraum an,

kommt es zu einem chronischen Entzündungsprozess, verbunden mit der Freisetzung von Anaphylatoxinen sowie der Ablagerung von Opsonin[26,147]. Dies resultiert in der Degeneration naheliegender RPE-Zellen, deren Bestandteile sich teilweise als Drusen, im subretinalen Raum, ablagern[52].

Der Y402H-Polymorphismus beeinflusst die Komplexbildung von Faktor H (sowie FHL1) mit dem Entzündungsmarker mCRP

Eine lokale Entzündungsreaktion nahe dem RPE, z. B. durch eine unkontrollierte Komplementaktivierung, führt auf Dauer zu einer Veränderung des RPE-Phänotyps. Die Abwandlung der Zellmorphologie geht einher mit Zytoplasmaaufquellung, gefolgt vom Anschwellen der Zellen sowie intrazellulärer Veränderung der Pigment- und Organellverteilung. Alles klassische Anzeichen, die dem nekrotischen Zelltod vorangehen[182]. Immunhistochemische sowie elektronenmikroskopische Analysen bestätigen, dass RPE-Zellen, vor allem jene die nahe Drusen lokalisiert sind, einen nekrotischen Phänotyp haben[27]. Der Verlust der Membranintegrität ist charakteristisch für das Endstadium der Nekrose, gefolgt vom Freiwerden zytoplasmatischer Bestandteile wie DNA, Histone oder Mitochondrien, welche alle in Drusen identifiziert wurden[50,183]. Weiterhin konnten degenerierte Zellwandbestandteile sowie ganze RPE-Zellen in Drusen detektiert werden, so dass die Formation von Drusen vonseiten des RPEs initiiert wird[52]. Degenerierte, nekrotische Zellen aktivieren die angeborene Immunabwehr und repräsentieren eine Aktivatoroberfläche, bei der es gerichtet zu Komplementaktivierung und Opsonisierung der Oberfläche mit C4b bzw. C3b kommt. **Manuskript 3** zeigt, dass die membranständigen Regulatoren CD46, CD55 gar nicht, CD59 nur noch in geringen Konzentrationen auf der RPE-Oberfläche exprimiert werden. Die Flüssigphaseregulatoren Faktor H, FHL1 oder C4BP müssen also deren Fehlen kompensieren, um die Amplifikation der Komplementkaskade auf C3-Ebene zu stoppen. Da das C4BP-Protein intraokular nicht synthetisiert wird, liegt es bei Faktor H und FHL1, die C3-Konvertase zu reglementieren, um damit eine nekrotisch vermittelte Entzündungsreaktion zu verhindern[76].

Ein wichtiger Entzündungsparameter ist das Akute-Phase-Protein CRP. CRP wurde in Drusen sowie dem umgebenden retinalen Gewebe nachgewiesen. Die detektierten CRP-Werte waren bei homozygoten Trägern der Faktor H-Risikovariante bis zu 2.5-fach stärker, als in Trägern der Nichtrisikovariante[155]. Weiterhin zeigten die H402-Risikovarianten von Faktor H sowie FHL1 in allen bisherigen Studien eine reduzierte Bindung an CRP[109,153,154,184]. Zwischen dem Y402H-Polymorphismus und dem Entzündungsmarker CRP existiert also ein Zusammenhang, der erstmals in **Manuskript 3** beschrieben wird. Das im Plasma zirkulierende CRP besteht aus fünf, nicht kovalent, verbundenen Untereinheiten. Gebunden an Oberflächen, durch Oxydation oder in saurem Milieu, dissoziiert die pentamere CRP-Form (pCRP) zu einer monomeren Form (mCRP)[185]. **Manuskript 3** beschreibt, dass die degenerierte Oberfläche von nekrotischen RPE-Zellen pCRP zu mCRP dissoziiert, und damit mCRP intraokular verfügbar ist. Bestätigt wird diese *in-vitro*-Beobachtung durch die erstmalige immunhistologische Unterscheidung von mCRP und pCRP innerhalb der RPE-BM-Choriocapillaris-Struktur. Dabei ist mCRP im Choroid, der BM sowie in Drusen

detektierbar, und gleichzeitig häufiger in den retinalen Geweben von AMD-Patienten als in gesunden Kontrollpersonen nachweisbar.

Faktor H und FHL1 interagieren ausschließlich mit mCRP über die SCRs 7, 8-11 sowie 19-20[100]. Daher präzisiert **Manuskript 3** die beschriebene reduzierte Bindung der H402-Risikovarianten an CRP eindeutig als mCRP-Interaktion. Innerhalb von SCR 7 existieren zwei Bindemotive für mCRP. Beide sind oberflächenexponiert. Die Bindung von mCRP an SCR 7 ist dabei abhängig von positiv geladenen Aminosäuren, denn beide Motive beinhalten das stark positiv geladene Lysin, ansonsten aber nur ungeladene Aminosäurereste. Motiv I, KFVQGK, liegt in direkter Nachbarschaft zu der AMD-assoziierten Position 402, was die reduzierte Bindung der Faktor H-/FHL1-Risikovarianten erklärbar macht (**Abbildung 9**). Die neu identifizierten mCRP-Bindemotive und die bereits beschriebene Heparinbindestelle von SCR 7, überlappen sich in den Aminosäuren K405 sowie K410. Die parallele Erkennung von mCRP und Polyanionen ist daher möglich, aber auch die gleichzeitige Beeinträchtigung der Oberflächen- und mCRP-Bindung durch die H402-Risikovarianten (**Manuskript 2 + 3**).

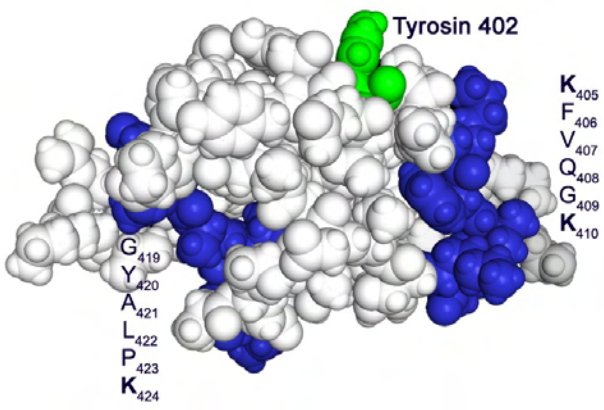


Abbildung 9: Darstellung der neu identifizierten mCRP-Bindedomänen in SCR 7. Innerhalb von SCR 7 existieren zwei lineare Bindedomänen, die mit mCRP interagieren (blau). Beide Regionen sind oberflächenexponiert und charakterisiert durch das Vorkommen von positiv geladenen Aminosäureresten, welche fett unterlegt sind. Motiv I, KFVQGK, überlappt teilweise mit der Heparinbinderegion (Abbildung 8) und liegt unmittelbar an der AMD-assoziierten Aminosäureposition 402.

Doch was sind die Auswirkungen einer reduzierten mCRP-Interaktion? mCRP bindet an nekrotische, degenerierte Zelloberflächen. Dabei interagiert mCRP mit Phosphatidylserin, einem intrazellulären Aminophospholipid, das während des Zelltodes an die Außenseite der Membran transloziert wird[100,186]. Da der nekrotische Zelltod mit einem Verlust der Membranintegrität einhergeht, ist besonders viel Phosphatidylserin auf der Oberfläche von nekrotischen Zellen lokalisiert. **Manuskript 3** zeigt, dass mCRP spezifisch an jene Stellen der nekrotischen Zellen bindet, welche das meiste Phosphatidylserin exponieren, d. h. dort wo der Membranschaden am größten ist. mCRP bildet mit Faktor H/FHL1 einen stabilen Komplex, in welchem beide Regulatoren ihre Funktion behalten. Zudem rekrutiert mCRP vermehrt Faktor H/FHL1 speziell an die geschädigte Membranregion und verstärkt dort die Faktor H-/FHL1-vermittelte C3b-Inaktivierung. Die höhere Dichte der Regulatoren sowie die erhöhte Generierung von iC3b haben den Effekt, dass nekrotische Zellen schneller durch

Komplementrezeptoren auf Makrophagen erkannt und effizienter phagozytiert werden. Bei den H402-Risikovarianten von Faktor H sowie FHL ist die Komplexbildung mit mCRP beeinträchtigt. Konsequenterweise werden die H402-Risikovarianten weniger stark an die nekrotische Zelloberfläche rekrutiert. Die reduzierte Zahl oberflächengebundener Regulatoren der C3-Konvertase beeinflusst die lokale Komplementregulation insofern, dass weniger C3b inaktiviert und infolgedessen weniger iC3b auf der Oberfläche abgelagert wird. Demnach wird durch die Y → H-Substitution die Aktivität der C3-Konvertase auf nekrotischen Zelloberflächen nicht optimal reguliert, führt zur Generierung des potenten Anaphylatoxins C5a sowie der Zusammenlagerung der TCC–Komplementkomponenten, die bereits in Drusen identifiziert wurden.

Zusätzlich zu der verstärkten Regulation der C3-Konvertase auf nekrotischen Zelloberflächen erhöhen stabile Faktor H–mCRP-Komplexe die Phagozytose und reduzieren die Produktion des inflammatorischen Zytokins TNF-α. TNF-α wird u. a. durch Makrophagen sekretiert, und dessen Menge ist entscheidend für das Ausmaß einer lokalen Entzündungsreaktion[187]. Die, durch Faktor H–mCRP-Komplexe, herabgesetzte TNF-α-Produktion von Makrophagen supprimiert somit aktiv eine Entzündung im umliegenden Gewebe. In Gegenwart der Faktor H-Risikovarianten ist, aufgrund der reduzierten mCRP-Komplexbildung, die TNF-α-Produktion durch Makrophagen nur geringfügig reduziert. Die lokale Entzündungsreaktion wird daher nicht supprimiert, sondern –im Gegenteil– weiter intensiviert. Das Vorhandensein der Faktor H-Risikovarianten vermittelt also keine anti-inflammatorische Eliminierung des nekrotischen RPE-Zellmaterials. Langfristig führt das zu einer zunehmenden Freisetzung von zytotoxischen sowie auto-immunogenen Zellbestandteilen, zur Vergrößerung von Drusen und zur Ausweitung der lokalen Entzündungsreaktion[53].

Zusammenfassend beschreibt **Manuskript 3** erstmals vier relevante Unterschiede zwischen den Risiko- (H402) und Nichtrisikovarianten (Y402): (I) reduzierte Komplexbildung mit mCRP, (II) vermindertes *Recruitment* von Faktor H/FHL1 an geschädigte Regionen der nekrotischen RPE-Zellmembran, (III) verringerte Regulation der C3-Konvertase sowie (IV) Verstärkung pro-inflammatorischer Prozesse durch die erhöhte TNF-α-Produktion von Makrophagen. Wird der natürliche Alterungsprozess von einer systemisch stattfindenden Entzündung begleitet (*inflamm-aging*), ist ein vollfunktionsfähiges Faktor H-Protein essenziell, um eine auftretende intraokulare Entzündungsreaktionen dauerhaft zu supprimieren. Bei Trägern der H402-Risikovariante ist die verfügbare Faktor H-Funktion nicht ausreichend, um die Akkumulierung von geschädigten RPE-Zellen bzw. deren drusenartige Ablagerung zu verhindern und resultiert in der Ausprägung des AMD-Phänotyps (**Abbildung 10**). Demzufolge hat die Variation an Aminosäure 402 von Y → H im Faktor H- und FHL1-Protein nicht nur das Potenzial primär Zellschaden zu verursachen, d. h. AMD zu initiieren (**Manuskript 2**), sondern ist auch verantwortlich für die nachfolgend unkontrollierten Entzündungsereignisse, die in Gegenwart nekrotischer RPE-Zellen entstehen (**Manuskript 3**).

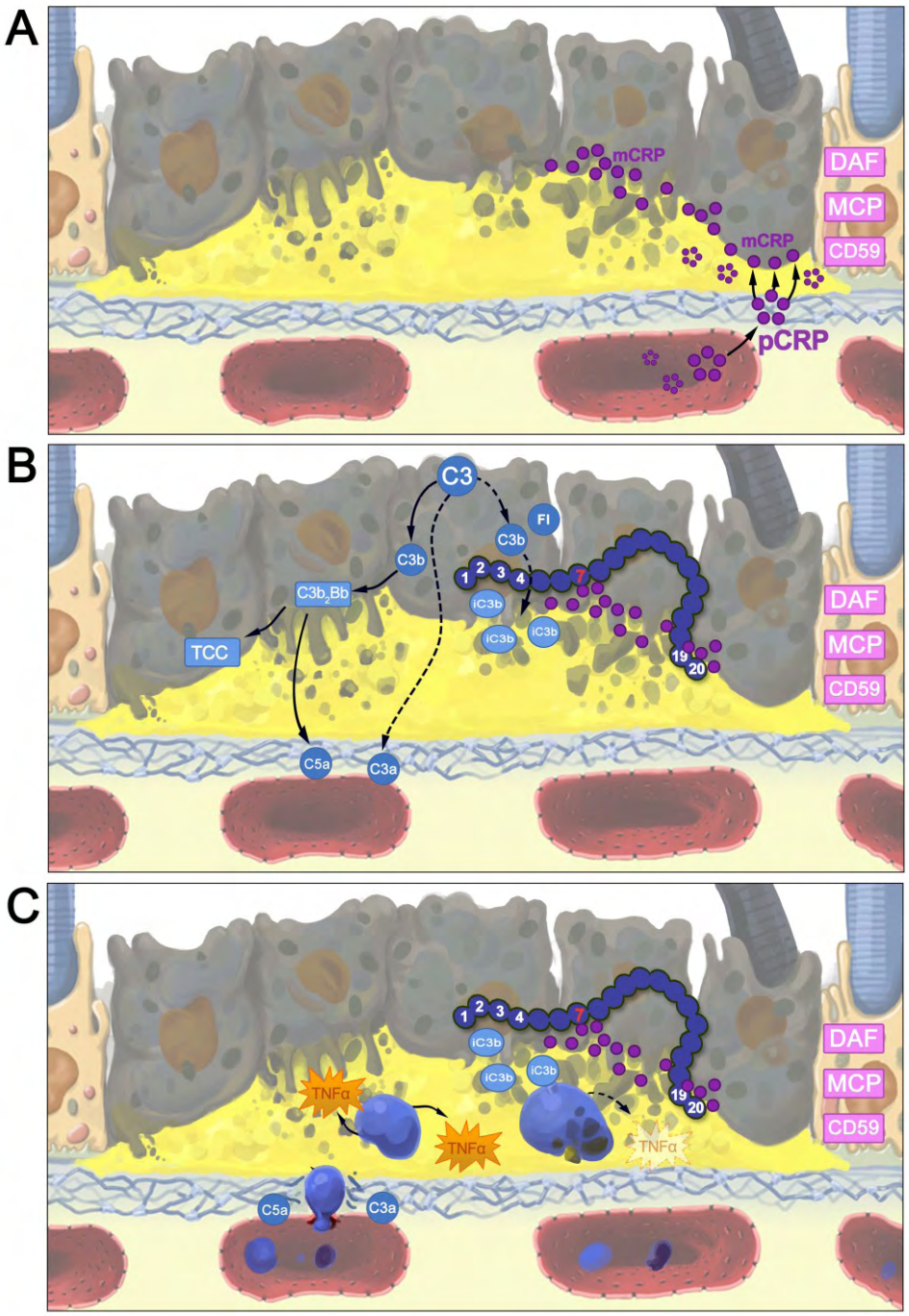


Abbildung 10: Komplementassoziierte intraokulare Entzündungsreaktion. A) Im Plasma zirkulierendes pCRP gelangt über die Choriocapillaris in den subpigmentepithelialen Raum. An nekrotischen RPE-Zellen wird pCRP zu mCRP dissoziiert (violette Kugeln). **B)** Faktor H bindet über die SCRs 7, 8-11(nicht dargestellt) und 19-20 an mCRP und wird an die RPE-Zelloberfläche rekrutiert. In Gegenwart der Y402-Variante (gestrichelte Linie) wird vermehrt iC3b generiert, was sich auf nekrotischen Zellen anreichert. In Gegenwart der H402-Risikovariante (durchgängige Linie) resultiert die ineffiziente Inhibierung der C3-Konvertase, in der Formation des TCC und der Generierung des Anaphylatoxins C5a. **C)** Die Anaphylatoxine locken chemotaktisch Makrophagen an die betroffene Region des RPEs. Stabile Faktor H–mCRP-Komplexe supprimieren deren TNF-α-Produktion und induzieren eine effiziente Phagozytose des zellulären Debris. In der Abwesenheit von stabilen Faktor H–mCRP-Komplexen, wird die TNF-α-Freisetzung nicht herabgesetzt und nekrotisches Zellmaterial nicht optimal entfernt.

Offen bleibt jedoch warum, diese Geschehnisse erst während eines fortgeschrittenen Alterungsprozesses auftreten und warum SCR 7 scheinbar die wichtigste Domäne für intraokulare Komplementregulation ist.

Das Auftreten altersabhängiger Erkrankungen wie der AMD hängt vom Genotyp des Einzelnen ab. Pro-inflammatorische Genotypen, wie z. B. der H402-kodierende Genotyp, sind von Vorteil um Infektionen zu widerstehen, resultieren aber im Alter in chronischen Entzündungsprozessen[7,9,188]. Während des normalen Alterungsprozesses erhöht der RPE-Choroid-Komplex zusätzlich die Expression von Immuneffektoren, wobei im Speziellen die Expression der Komplementaktivatoren C1q und C3 gesteigert wird[49]. Lebenslange photooxydative Prozesse der Retina resultieren zudem in der Ablagerung von Lipofuszin. Die Akkumulation von Lipofuszin im Zytoplasma der RPE-Zellen generiert aus dem RPE eine Aktivatoroberfläche auf der es ungehindert zur Aktivierung von Komplement kommt[189]. Oxydativ geschädigtes RPE vermindert die Expression des membranständigen Regulators CD55, und kann sich nur noch begrenzt vor Komplementaktivierung schützen. Das alternde Auge sowie zunehmender (photo-) oxydativer Stress kreieren demnach eine Mikroumwelt, die reich an immunologischen Aktivatoroberflächen ist, aber einen Mangel an Regulatoren aufweist[28,190]. Weitere Faktoren wie, die durch Rauchen freiwerdenden oxydativen Verbindungen und das damit verbundene reduzierte Faktor H-Plasmalevel, erhöhen weiter die Anfälligkeit des alternden RPE-Choroid-Komplexes für den AMD-Phänotyp[110,118]. Die Auswirkungen einer verminderten Kontrolle des Komplementsystems durch die Faktor H-/FHL1-Risikovarianten kommt also erst während des Alterungsprozesses, d. h. nicht vor dem 50. Lebensjahr, zum Tragen.

Für die Bedeutung der Domäne 7 innerhalb der intraokularen Komplementregulation sind verschiedene Erklärungen denkbar. SCR 7 ist eine multiple Bindedomäne[150], deren Bindestellen für mCRP und GAGs nicht identisch, aber überlappend sind, so dass die Interaktion von mindestens zwei Liganden beeinträchtigt ist. Zudem ist durch das alternative Spleißen des *Faktor H*-Gens neben Faktor H auch FHL1 betroffen, was die funktionellen Auswirkungen von Polymorphismen weiter intensiviert. Das Faktor H-Protein besitzt zwar mit SCR 8-11, SCR 13 sowie SCR 19-20 weitere Bindestellen für mCRP oder GAGs, doch findet die Interaktion der einzelnen Regionen tandemartig nacheinander statt. Über SCR 19-20 wird Faktor H gerichtet an die Oberfläche gebunden[191]. Die weiteren Interaktionspunkte sind essenziell, um die Bindung zu verstärken und zu stabilisieren. Demnach erklärt sich, warum in Gegenwart der H402-Risikovarianten die Interaktion mit mCRP oder Zelloberflächen nicht vollständig blockiert, sondern lediglich um 25 – 50 % reduziert sind.

Das Fehlen der CFHR1-/CFHR3-Proteine verstärkt die lokale Faktor H-Funktion

Insgesamt sind vier Haplotypen des *Faktor H*-Genclusters mit AMD assoziiert. Ein Risikohaplotyp, welcher die bereits beschriebene H402-Risikovariante kodiert, ein neutraler Haplotyp und zwei protektive Haplotypen, die mit einem reduzierten AMD-Risiko verbunden sind. Kennzeichnend für einen der beiden protektiven Haplotypen ist die

Defizienz der *CFHR1*- und *CFHR3*-Gene (Δ*CFHR1*/*CFHR3*)[12]. Das Fehlen der beiden Gene führt entweder zur Reduktion (heterozygote Defizienz) oder zu einer vollständigen Abwesenheit (homozygote Defizienz) der beider Proteine im Auge bzw. im Plasma.

Bis vor Kurzem war unklar, ob Δ*CFHR1*/*CFHR3* (bzw. der Haplotyp, welcher die Deletion trägt) das AMD-Risiko unabhängig beeinflusst, oder ob die Protektion zu mindestens partiell durch die Abwesenheit der H402-Risikovariante zustande kommt. Studien, die dieses Problem zu lösen versuchten, konnten –womöglich verursacht durch zu geringe Fallzahlen– keine klare Aussage treffen und waren nicht konsistent[192-194]. In **Manuskript 5** dieser Dissertation wurde eine Kohorte von 530 AMD-Patienten und 313 Kontrollpersonen auf Δ*CFHR1*/*CFHR3* sowie sechs Faktor H-Polymorphismen genotypisiert. Die große Fallzahl hatte genügend statistische Aussagekraft, so dass selbst nach logistischer Regression der Variablen Y402H, Alter, Rauchen und Geschlecht, der protektive Effekt von Δ*CFHR1*/*CFHR3* erhalten blieb. **Manuskript 5** beschreibt, dass die Anwesenheit der *CFHR1*- und *CFHR3*-Gene ein Risikofaktor ist, der unabhängig von Faktor H-Polymorphismen oder individuellen Risiken agiert. Die Abwesenheit der beiden Plasmaproteine CFHR1 und CFHR3 ist also von Vorteil für die Verhütung der AMD. Um aber den zugrunde liegenden Mechanismus aufzudecken, war es wichtig, die bis dato unbekannten Funktionen des CFHR1- sowie des CFHR3-Proteins zu identifizieren.

CFHR1 ist ein Komplementregulator, welcher die Aktivität der C5-Konvertase sowie die Zusammenlagerung des TCC kontrolliert (**Manuskript 4**). Es verhindert, durch seine N-terminale Interaktion mit C5 bzw. C5b6, die C5-Spaltung. Dementsprechend ist die Generation des Anaphylatoxins C5a, die Formation und Insertion des TCC sowie die Zytolyse blockiert. CFHR1 ist der erste beschriebene Regulator der Faktor H-Proteinfamilie, der die C5- nicht aber die C3-Konvertase beeinflusst und dadurch speziell auf Ebene der terminalen Prozesse wirkt. CFHR3 ist ebenfalls ein Komplementregulator. **Manuskript 5** beschreibt CFHR3 erstmals als Regulator der C3-Konvertase mit Kofaktoraktivität für die Serinprotease Faktor I. Frühere Studien beschrieben bereits eine verstärkte Faktor H-Kofaktoraktivität in Gegenwart von CFHR3[104]. Die zu geringen CFHR3-Konzentrationen deuten darauf hin, dass damals die Funktion von CFHR3 nicht genauer verifiziert wurde. Als Regulator der C3-Konvertase kontrolliert CFHR3 gleichzeitig auch indirekt den Aufbau der C5-Konvertase. Die Generierung von C5a wird, wie in Anwesenheit von CFHR1 (direkt) und Faktor H (indirekt), blockiert, so dass in der Konsequenz die C5a-vermittelte Chemoattraktion von neutrophilen Granulozyten verhindert wird. CFHR1 und CFHR3 vermitteln demnach anti-inflammatorische Aktivität. Verglichen mit Faktor H sind die Aktivitäten von CFHR1 und CFHR3 weitaus geringer, d. h. höhere Konzentrationen sind notwendig, um eine entsprechende Inhibierung wie durch Faktor H zu erreichen. Die geringere Aktivität der beiden neuen Komplementregulatoren wird höchstwahrscheinlich durch das Fehlen der sog. „*decay-accelerating activity*“, d. h. der Fähigkeit eine C3-Konvertase zu dissoziieren, hervorgerufen.

Die Abwesenheit von CFHR1 und CFHR3 führt also zu einem Verlust von Komplementregulatoren. Wie aber kann sich das Fehlen von Komplementregulatoren, vor

allem in einer sensitiven Umgebung wie dem immunprivilegiertem Auge, positiv auf den Krankheitsverlauf der AMD auswirken? Die einzelnen SCRs von CFHR1, CFHR3 und Faktor H besitzen untereinander eine hohe Sequenzidentität von bis zu 100 %[158]. Die C-terminalen SCRs 3-5 von CFHR1 sind nahezu homolog zu den SCRs 18-20 von Faktor H und interagieren, wie Faktor H, mit den Liganden C3b, Heparin und zellulären Oberflächen. CFHR1 differenziert demnach zwischen Aktivator- und Nichtaktivatoroberflächen. In ihrem physiologischen Konzentrationsverhältnis von 1:3 konkurrieren CFHR1 und Faktor H um die Bindung an Heparin sowie C3b, und Faktor H wird durch CFHR1 verdrängt. Folglich ist die Faktor H-vermittelte Regulation der C3-Konvertase reduziert, während sich gleichzeitig die Regulation der C5-Konvertase durch CFHR1 erhöht (**Manuskript 4**). Auch CFHR3 zeigt Sequenzhomologien mit Faktor H, bindet C3b und ist in der Lage, Faktor H kompetitiv von C3b zu verdrängen. Dies resultiert in einer reduzierten Faktor H-vermittelten *„decay-accelerating activity“* (**Manuskript 5**). Die Gegenwart der Regulatoren CFHR1 und CFHR3 verändert also die lokale Komplementregulation (**Abbildung 11**). Die Interaktion von Faktor H mit den Liganden C3b, Heparin und vermutlich auch mit humanen Zellen ist, in Abwesenheit von CFHR1 und CFHR3, erhöht. Dadurch verstärkt sich die lokale Kontrolle der C3-Konvertase durch die Faktor H-vermittelte *„decay-accelerating activity“* eines bereits generierten C3bBb-Komplexes und durch die vermehrte C3b-Inaktivierung. Erhöhte iC3b-Generierung und die damit verbundene gesteigerte Phagozytose von iC3b-opsonierten nekrotischen, retinalen Ablagerungen, scheint v. a. in der Retina von Vorteil (**Manuskript 3**).

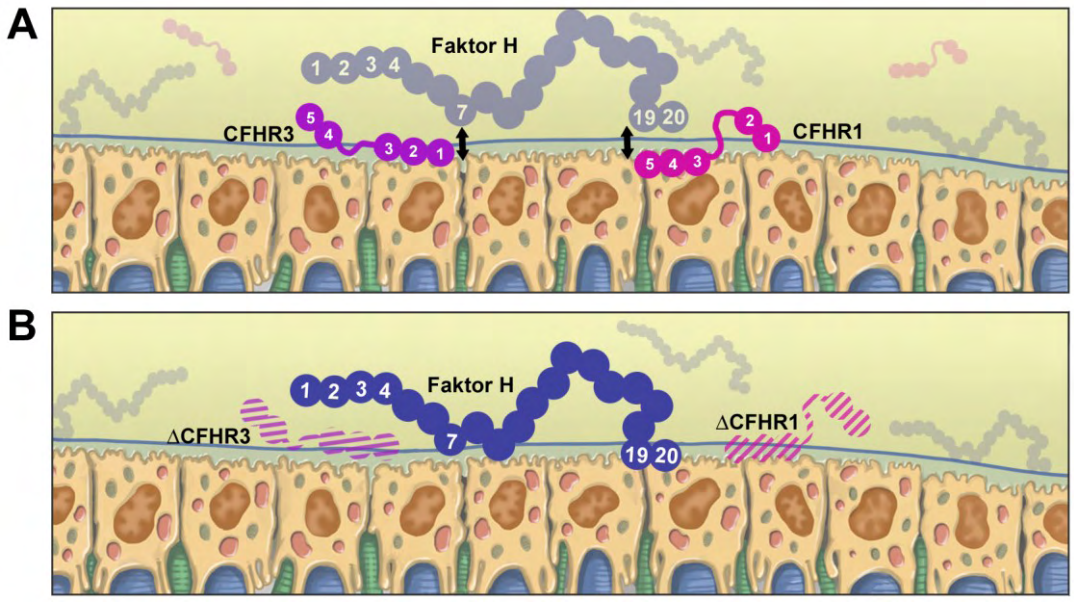


Abbildung 11: CFHR1 und CFHR3 kompetitieren mit Faktor H. A) In Gegenwart von CFHR1(rosa) und CFHR3 (violett) wird die Bindung von Faktor H an Zelloberflächen bzw. gemeinsame Liganden geschwächt. Die hohe Sequenzidentität der SCRs 1-2 von CFHR3 mit den SCRs 6-7 von Faktor H bzw. SCRs 3-5 von CFHR1 mit den SCRs 18-20 von Faktor H, ermöglichen die kompetitive Verdrängung von Faktor H, und resultieren in einer reduzierten Faktor H-vermittelten Regulation. **B)** Die Abwesenheit der CFHR1- und CFHR3-Proteine im Plasma (schraffierte Formen) verstärkt die natürliche Faktor H-Interaktion mit seinen Liganden, wodurch sich die lokale Kontrolle der C3-Konvertase erhöht.

Während Δ*CFHR1*/*CFHR3* protektiv für die AMD ist, stellt sie gleichzeitig einen Risikofaktor für das Krankheitsbild des DEAP-HUS dar[195]. Bei DEAP-HUS korreliert die Abwesenheit der CFHR1- und CFHR3-Proteine mit dem Vorhandensein von Autoantikörpern, die gegen den C-Terminus von Faktor H gerichtet sind[138]. Die Autoantikörper verhindern die Bindung von Faktor H an Endothelzellen, vermindern dadurch die lokale Komplementregulation, was in Komplementaktivierung und renalen Entzündungsreaktionen resultiert[140]. Homozygote Defizienzen eines Gens sind häufig assoziiert mit der Entwicklung von Autoantikörpern gegen das fehlende Protein[196]. Eventuell richten sich die Faktor H-Autoantikörper ursprünglich gegen CFHR1, behindern jedoch durch die Kreuzreaktion mit dem homologen C-Terminus die normale Funktion von Faktor H. Die AMD ist nicht mit dem Auftreten von Antikörpern gegen Faktor H assoziiert[197]. Die Defizienz des CFHR1- und des CFHR3-Proteins erhöht also vermutlich die Effizienz von Faktor H, sofern sich keine Autoantikörper bilden.

Die Ergebnisse aus den **Manuskripten 4 + 5** verdeutlichen, dass sich CFHR1, CFHR3 und Faktor H in einem empfindlichen Gleichgewicht befinden. Veränderungen der einzelnen Proteinkonzentrationen modifizieren die Homöostase von Komplementaktivierung und –regulation, so dass ein Ungleichgewicht den risikomodifizierenden Charakter von Δ*CFHR1*/*CFHR3* erklärt. Die Faktor H-vermittelte Reglementierung der C3-Konvertase ist von Vorteil in der Retina, d. h. sie wird durch das Fehlen von CFHR1 und CFHR3 verstärkt, kreiert aber gleichzeitig ein Risiko für Autoimmunerkrankungen wie DEAP-HUS.

Gerichtete Komplementregulierung auf Oberflächen durch COMP_CFH15-20

Die Erkenntnis, dass ein ineffizient kontrollierter alternativer Komplementweg ursächlich für eine Vielzahl von chronisch entzündlichen Erkrankungen –wie die AMD– ist, liefert die Voraussetzung für die Entwicklung von therapeutisch applizierbaren Komplementinhibitoren[198]. Zum gegenwärtigen Zeitpunkt sind noch keine Präparate auf dem Markt, welche die aktuellen Erkenntnisse der AMD-Forschung aufnehmen und therapeutisch in die Komplementkaskade eingreifen. Einige Komplementinhibitoren befinden sich aber bereits in tierexperimentellen sowie klinischen Studien. Ein zukünftiges Präparat muss vor allem eine hohe Spezifität für den entsprechenden Angriffspunkt, d. h. möglichst keine Kreuzreaktionen mit anderen Komplementkomponenten aufweisen, das Komplementsystem kontrolliert modulieren und eine angemessene Halbwertszeit besitzen. Die Intervention auf Ebene von C3 ist dabei von größtem Interesse, da an diesem Punkt alle drei Aktivierungswege amplifiziert sowie Anaphylatoxine generiert werden.

Das Peptid Compstatin interagiert direkt mit C3, blockiert die Abspaltung von C3a und ist zudem hochselektiv für humanes C3[166]. Die bereits beendete Phase-I-Studie von Compstatin prüfte erfolgreich die Sicherheit und Verträglichkeit im Menschen. Dennoch korreliert die Applikation des systemisch wirkenden Inhibitors Compstatin auch mit Risiken. In **Manuskript 6** wurde daher ein Inhibitor entwickelt, welcher sich die Eigenschaft von Faktor H zunutze macht, zwischen Aktivator- („Fremd“) und Nichtaktivatoroberflächen

(„Selbst“) zu unterscheiden, um den Inhibitor Compstatin spezifisch an körpereigene Oberflächen zu rekrutieren.

Die systemische Inhibition der Komplementaktivierung blockiert u. a. die Markierung und Entfernung von Immunkomplexen sowie die Eliminierung von Mikroorganismen[199]. Die hier angestrebte, neuartige Strategie ist, Komplementinhibitoren so zu modifizieren, dass diese nicht systemisch, sondern gezielt an Orten chronischer Komplementaktivierung wirken. Die medizinische Relevanz –im Falle von AMD– ist es, Inhibitoren an geschädigte RPE-Zellen zu rekrutieren und die Effizienz der lokalen Komplementregulation zu erhöhen. Eine Funktion, die unter physiologischen Bedingungen durch den endogenen Faktor H vermittelt wird. Essenziell für die gerichtete Bindung von Faktor H an zelluläre Oberflächen ist dessen C-Terminus, welcher primär verantwortlich für die Unterscheidung von Aktivator- und Nichtaktivatoroberflächen ist[200].

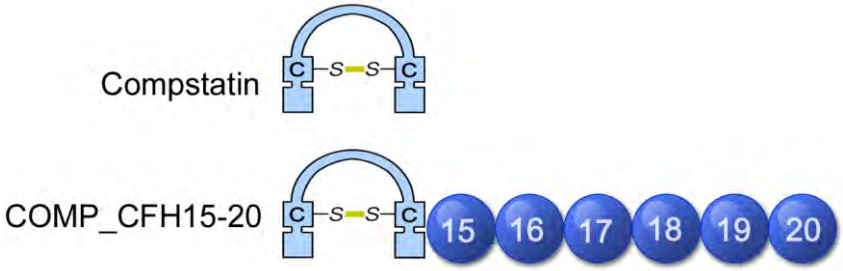


Abbildung 12: Darstellung des chimären Proteins COMP_CFH15-20. Der ursprüngliche, ungetaggte Komplementinhibitor Compstatin besteht aus 13 Aminosäuren. Die ringförmige, aktive Form von Compstatin, entsteht durch Ausbildung einer Disulfidbrücke (- S-S -) zwischen zwei Cysteinresten (C). Für die Generierung des neuartigen Komplementinhibitors COMP_CFH15-20 wurde an das Peptid Compstatin der oberflächenbindende C-Terminus SCR15-20 von Faktor H kloniert. COMP_CFH15-20 differenziert nun zwischen Aktivator- und Nichtaktivatoroberflächen und reguliert die Amplifizierung des Komplementsystems gezielt an Orten von Immunstress.

In **Manuskript 6** wurde ein chimäres Protein generiert, welches die C-terminalen Domänen SCR15-20 von Faktor H mit dem potenten, etablierten Komplementinhibitor Compstatin kombiniert (**Abbildung 12**). Der neuartige Inhibitor COMP_CFH15-20 besitzt mit Compstatin und den Domänen SCR19-20 nun zwei unabhängige Interaktionspunkte für den Liganden C3b. Die gesteigerte Aktivität von COMP_CFH15-20 für C3 sowie C3b blockiert den Amplifizierungsprozess in Form von reduzierter C5a-Generation sowie herabgesetzter C5b-Ablagerung auf Nichtaktivatoroberflächen. Die relative Aktivität von COMP_CFH15-20 ist dabei stärker als die des Inhibitors Compstatin allein. COMP_CFH15-20 interagiert über die Domänen SCR 15-20 mit lebenden sowie mit nekrotischen Zellen. Der verknüpfte Inhibitor Compstatin blockiert daher die Komplementamplifikation nun spezifisch auf zellulären Oberflächen. **Manuskript 6** veranschaulicht, dass die Bindung von COMP_CFH15-20, in Gegenwart eines aktiven –aber regulatordezimierten– Komplementsystems, die Überlebensfähigkeit von intakten Zellen erhöht und die C5b-Ablagerung auf nekrotischen Zelloberflächen reduziert. Gebunden an Oberflächen, erzeugt COMP_CFH15-20 einen bis zu 30 % stärkeren Schutz vor Komplementaktivierung als das systemisch wirkende, nichtoberflächenbindende Compstatin. Durch COMP_CFH15-20 wird

die Amplifikation des Komplementsystems spezifisch auf lebenden sowie nekrotischen Zellen verhindert. Von Bedeutung ist, dass die eingesetzten Konzentrationen von max. 40 µg/ml (0,8 µM) des neuartigen Chimärs, um ein Vielfaches geringer sind als die physiologische Plasmakonzentration von Faktor H (400 – 800 µg/ml d. h. 2,6 – 5.3 µM). Es handelt sich also um ein hochaktiv wirkendes, gerichtetes Therapeutikum.

Unter physiologischen Bedingungen dient die Aktivierung des Komplementsystems dazu, degenerierte, körpereigene Zellen sowie Mikroorganismen zu markieren und mittels Phagozytose zu entfernen[42,43]. Die Komplementaktivierung mit ihren Effektorfunktionen ist also unerlässlich für die Aufrechterhaltung der Gewebshomöostase sowie bei der Eliminierung von Mikroorgansimen. Der Einsatz systemisch wirkender Komplementinhibitoren ist daher bei immunsupprimierten Patienten oder in der Langzeitanwendung mit Risiken verbunden. In Gegenwart von COMP_CFH15-20 wird der klassische Weg ungehindert, durch das Initiatorprotein C1q aktiviert. Die Interaktion mit CRP, Polyanionen oder DNA aktiviert die C1q-assoziierten Serinproteasen, diese spalten die Komplementkomponenten C4 sowie C2, und die Fragmente C4b und C2a lagern sich kovalent an Oberflächenstrukturen an. Durch die gerichtete C4b-CR1-Interaktion, werden degenerierte Zellen und Immunkomplexe weiterhin von Makrophagen erkannt und eliminiert[201].

Zusammenfassend verbindet das neu entwickelte Chimär COMP_CFH15-20 die oberflächendifferenzierenden Eigenschaften der C-terminalen Region von Faktor H mit dem potenten Komplementinhibitor Compstatin. COMP_CFH15-20 blockiert spezifisch die Amplifikationsprozesse des Komplementsystems auf Nichtaktivatoroberflächen, und unterstützt dadurch, die, durch Polymorphismen, Mutationen sowie Defizienzen, beeinträchtigte Komplementregulation. **Manuskript 6** charakterisiert also einen vielversprechenden Kandidaten, um komplementverursachte Erkrankungen, wie die AMD, therapeutisch zu behandeln. Potenzielle Nebenwirkungen systemisch wirkender Komplementinhibitoren würden reduziert. Erste Testungen weiterer modifizierter Regulatoren zeigen bereits im Maus- und Primatenauge, dass ein sukzessiver Rückgang in der Drusenbildung sowie dem Einsprossen neu gebildeter choroidaler Kapillaren möglich ist[169,202]. Demnach etablieren sich neue Ansätze für die frühzeitige Behandlung der AMD. Durch den demografischen Wandel der westlichen Gesellschaft, und den damit verbundenen erhöhten Neuerkrankungen, wird der Bedarf an neuartigen, effektiven Komplementregulatoren in den nächsten Jahren weiter steigen.

Schlussfolgerungen

Die altersabhängige Makuladegeneration ist eine komplexe genetische Erkrankung mit starken individuellen Einflüssen. Das Erkrankungsrisiko ist assoziiert mit genetischen Varianten verschiedener Komplementgene. Vor allem die gehäuft auftretenden Polymorphismen und Defizienzen im *Faktor H*-Gencluster beeinflussen maßgeblich die Prädisposition der AMD. Ausgehend von der ursprünglichen Fragestellung, ob die beiden Polymorphismen, V62I sowie Y402H, die Funktion von Faktor H bzw. FHL1 beeinflussen, zeigt diese Promotionsarbeit, dass die Y402H-Variation, nicht aber die V62I-Variation, die Effektivität der beiden Regulatoren beeinträchtigt. Die Variation an Aminosäure 402 von Y → H verringert die intraokulare Komplementregulation dahingehend, dass die Regulatoren nicht mehr effektiv mit Zelloberflächen interagieren (**Manuskript 2**). Das, durch die H402-Risikovarianten, unzureichend kontrollierte Komplementsystem verursacht einen primären RPE-Zellschaden und konnte als ursprünglicher Trigger der AMD definiert werden. Zudem zeigt diese Promotionsarbeit erstmals, dass die Y → H-Substitution an Position 402 an den intraokularen Entzündungsereignissen beteiligt ist (**Manuskript 3**). Das Vorhandensein der Faktor H-Risikovarianten verhindert die Opsonisierung sowie anti-inflammatorische Eliminierung von nekrotischem RPE-Zellmaterial, was in der drusenartigen Ablagerung degenerierter RPE-Zellen und in der Ausprägung des AMD-Phänotyps resultiert. Des Weiteren konnten innerhalb dieser Promotionsarbeit die bisher unbekannten Funktionen der CFHR1- und CFHR3-Proteine identifiziert werden. Dies ermöglichte die Beantwortung der Frage, warum die Defizienz der entsprechenden *CFHR1-/CFHR3*-Gene sich positiv auf den Krankheitsverlauf auswirkt. CFHR1 und CFHR3 befinden sich mit Faktor H in einem empfindlichen Gleichgewicht, in welchem Veränderungen der einzelnen Proteinkonzentrationen die Komplementregulierung auf Oberflächen modifiziert. Die Abwesenheit beider Proteine führt zu einer Erhöhung der Faktor H-Konzentration, was im Speziellen, auf retinalen Oberflächen, ein Vorteil ist (**Manuskript 4 + 5**).

Die Erkenntnisse dieser Promotionsarbeit sowie die Ergebnisse von genetisch-, strukturell- oder proteinbasierten Studien der letzten fünf Jahre demonstrieren, dass eine modifizierte Komplementaktivierung entscheidend für die Entstehung der AMD ist. Dabei ist u. a. das Komplementsystem ein primärer Verursacher des anfänglichen RPE-Choroid-Schadens. Aber auch andere Prozesse wie mikrobielle Infektionen, photooxydativer Stress, Veränderungen in der Lipidzusammensetzung der BM, Ablagerung von Lipofuscin oder choroidale Ischämie spielen eine wichtige Rolle in der Initierungsphase der AMD. Bei Personen, die aufgrund des Vorhandenseins von genetischen Risikovarianten, die Aktivierung des Komplemensystems nicht ausreichend regulieren können, kommt es zu einer chronischen Aktivierung von Komplement, verbunden mit einer lokalen Entzündungsreaktion und dem langsamen Verlust des zentralen Sehvermögens.

Die Kombination aller AMD-assoziierter Komplementvarianten von Faktor H, Faktor B, C2, C3 sowie der CFHR1-/CFHR3-Defizienz, beeinflusst nicht nur das intraokulare, sondern sehr wahrscheinlich auch das systemische Komplementsystem. Denn obwohl einige der

genetischen Varianten mit einem reduzierten AMD-Risiko assoziiert sind, existiert –bezogen auf die systemische Wirkung– immer auch ein Kompromiss. So ist die Q32-Variante von Faktor B zwar mit einem reduzierten Risiko für AMD verbunden, erhöht gleichzeitig aber die Anfälligkeit von Infektionen[160]. Die risikobehaftete H402-Variante von Faktor H hingegen wurde als protektiver Faktor für das systemisch inflammatorische Responsesyndrom beschrieben[203]. Demnach prädisponieren unterschiedliche Proteinkonformationen für verschiedene Krankheiten und haben, wie im Fall der CFHR1-/CFHR3-Defizienz, antagonistische Auswirkungen in ihrem homozygoten Vorkommen. Es existiert also keine Proteinvariante, die ausschließlich positive oder negative Effekte erzielt, sondern es existieren vielmehr Varianten, die abhängig vom Einfluss anderer Gene, der Umwelt oder Mikroorgansimen, verschiedene Reaktionen hervorrufen. Das Aufgreifen dieses Zwiespaltes wird zukünftig entscheidend für die Entwicklung neuer Therapien sein, die gezielt das Komplementsystem beeinflussen (**Manuskript 6**).

Die Entdeckung konkreter Risikogene in der Epidemiologie der AMD war einer der ersten Erfolge genomweiter Assoziationsstudien, die dem *Human Genome Project* folgten. Die erstmalige Beschreibung der funktionellen Auswirkungen von AMD-assoziierten Polymorphismen sowie der Defizienzen des *Faktor H*-Genclusters innerhalb dieser Promotionsarbeit, wird dazu beitragen, die Ursachen der AMD besser zu verstehen und neuartige, therapeutische Konzepte zu entwickeln.

Literaturverzeichnis

1. Rodriguez-Rodero, S., Fernandez-Morera, J.L., Fernandez, A.F., Menendez-Torre, E. & Fraga, M.F. Epigenetic regulation of aging. *Discov Med* **10**, 225-233 (2010).
2. Probst-Hensch, N.M. Chronic age-related diseases share risk factors: do they share pathophysiological mechanisms and why does that matter? *Swiss Med Wkly* **140**, w13072 (2010).
3. Franceschi, C., *et al.* Inflamm-aging. An evolutionary perspective on immunosenescence. *Ann N Y Acad Sci* **908**, 244-254 (2000).
4. Candore, G., *et al.* Biology of longevity: role of the innate immune system. *Rejuvenation Res* **9**, 143-148 (2006).
5. Franceschi, C., *et al.* Neuroinflammation and the genetics of Alzheimer's disease: the search for a pro-inflammatory phenotype. *Aging (Milano)* **13**, 163-170 (2001).
6. Van Den Biggelaar, A.H., *et al.* Inflammation underlying cardiovascular mortality is a late consequence of evolutionary programming. *Faseb J* **18**, 1022-1024 (2004).
7. Wick, G., Berger, P., Jansen-Durr, P. & Grubeck-Loebenstein, B. A Darwinian-evolutionary concept of age-related diseases. *Exp Gerontol* **38**, 13-25 (2003).
8. Krabbe, K.S., Pedersen, M. & Bruunsgaard, H. Inflammatory mediators in the elderly. *Exp Gerontol* **39**, 687-699 (2004).
9. Caruso, C., *et al.* Looking for immunological risk genotypes. *Ann N Y Acad Sci* **1019**, 141-146 (2004).
10. Venter, J.C., *et al.* The sequence of the human genome. *Science* **291**, 1304-1351 (2001).
11. Edwards, A.O., *et al.* Complement factor H polymorphism and age-related macular degeneration. *Science* **308**, 421-424 (2005).
12. Hughes, A.E., *et al.* A common CFH haplotype, with deletion of CFHR1 and CFHR3, is associated with lower risk of age-related macular degeneration. *Nat Genet* **38**, 1173-1177 (2006).
13. Gold, B., *et al.* Variation in factor B (BF) and complement component 2 (C2) genes is associated with age-related macular degeneration. *Nat Genet* **38**, 458-462 (2006).
14. Yates, J.R., *et al.* Complement C3 variant and the risk of age-related macular degeneration. *N Engl J Med* **357**, 553-561 (2007).
15. Corder, E.H., *et al.* Gene dose of apolipoprotein E type 4 allele and the risk of Alzheimer's disease in late onset families. *Science* **261**, 921-923 (1993).
16. Doria, A., Patti, M.E. & Kahn, C.R. The emerging genetic architecture of type 2 diabetes. *Cell Metab* **8**, 186-200 (2008).
17. Jager, R.D., Mieler, W.F. & Miller, J.W. Age-related macular degeneration. *N Engl J Med* **358**, 2606-2617 (2008).
18. Klein, R., Peto, T., Bird, A. & Vannewkirk, M.R. The epidemiology of age-related macular degeneration. *Am J Ophthalmol* **137**, 486-495 (2004).
19. Vingerling, J.R., Klaver, C.C., Hofman, A. & de Jong, P.T. Epidemiology of age-related maculopathy. *Epidemiol Rev* **17**, 347-360 (1995).
20. Mitchell, P., Smith, W., Attebo, K. & Wang, J.J. Prevalence of age-related maculopathy in Australia. The Blue Mountains Eye Study. *Ophthalmology* **102**, 1450-1460 (1995).
21. Pauleikhoff, D., Barondes, M.J., Minassian, D., Chisholm, I.H. & Bird, A.C. Drusen as risk factors in age-related macular disease. *Am J Ophthalmol* **109**, 38-43 (1990).

22. Bird, A.C., *et al.* An international classification and grading system for age-related maculopathy and age-related macular degeneration. The International ARM Epidemiological Study Group. *Surv Ophthalmol* **39**, 367-374 (1995).

23. Pascolini, D., *et al.* 2002 global update of available data on visual impairment: a compilation of population-based prevalence studies. *Ophthalmic Epidemiol* **11**, 67-115 (2004).

24. Marx, J. Genetics. A clearer view of macular degeneration. *Science* **311**, 1704-1705 (2006).

25. Friedman, D.S., *et al.* Prevalence of age-related macular degeneration in the United States. *Arch Ophthalmol* **122**, 564-572 (2004).

26. Johnson, L.V., Leitner, W.P., Staples, M.K. & Anderson, D.H. Complement activation and inflammatory processes in Drusen formation and age related macular degeneration. *Exp Eye Res* **73**, 887-896 (2001).

27. Anderson, D.H., Mullins, R.F., Hageman, G.S. & Johnson, L.V. A role for local inflammation in the formation of drusen in the aging eye. *Am J Ophthalmol* **134**, 411-431 (2002).

28. Hollyfield, J.G., *et al.* Oxidative damage-induced inflammation initiates age-related macular degeneration. *Nat Med* **14**, 194-198 (2008).

29. Grehn, F. *Augenheilkunde*, (Springer Medizin Verlag, Heidelberg, 2008).

30. F.G. Holz, D.P., R.F. Spaide, A.C. Bird. *Alterabhängige Makuladegeneration*, (Springer Verlang, Berlin Heidelberg New York, 2004).

31. Frennesson, C., Nilsson, U.L. & Nilsson, S.E. Colour contrast sensitivity in patients with soft drusen, an early stage of ARM. *Doc Ophthalmol* **90**, 377-386 (1995).

32. Tolentino, M.J., Miller, S., Gaudio, A.R. & Sandberg, M.A. Visual field deficits in early age-related macular degeneration. *Vision Res* **34**, 409-413 (1994).

33. Streilein, J.W. Ocular immune privilege: therapeutic opportunities from an experiment of nature. *Nat Rev Immunol* **3**, 879-889 (2003).

34. Anderson, D.H., Stern, W.H., Fisher, S.K., Erickson, P.A. & Borgula, G.A. The onset of pigment epithelial proliferation after retinal detachment. *Invest Ophthalmol Vis Sci* **21**, 10-16 (1981).

35. Niederkorn, J.Y. & Lynch, M.G. Reconsidering the immunologic privilege and lymphatic drainage of the anterior chamber of the eye. *Transplant Proc* **21**, 259-260 (1989).

36. Streilein, J.W. & Niederkorn, J.Y. Induction of anterior chamber-associated immune deviation requires an intact, functional spleen. *J Exp Med* **153**, 1058-1067 (1981).

37. D'Orazio, T.J. & Niederkorn, J.Y. A novel role for TGF-beta and IL-10 in the induction of immune privilege. *J Immunol* **160**, 2089-2098 (1998).

38. Griffith, T.S., Brunner, T., Fletcher, S.M., Green, D.R. & Ferguson, T.A. Fas ligand-induced apoptosis as a mechanism of immune privilege. *Science* **270**, 1189-1192 (1995).

39. Sugita, S. & Streilein, J.W. Iris pigment epithelium expressing CD86 (B7-2) directly suppresses T cell activation in vitro via binding to cytotoxic T lymphocyte-associated antigen 4. *J Exp Med* **198**, 161-171 (2003).

40. Ferguson, T.A. & Griffith, T.S. A vision of cell death: Fas ligand and immune privilege 10 years later. *Immunol Rev* **213**, 228-238 (2006).

41. Mondino, B.J., Ratajczak, H.V., Goldberg, D.B., Schanzlin, D.J. & Brown, S.I. Alternate and classical pathway components of complement in the normal cornea. *Arch Ophthalmol* **98**, 346-349 (1980).

42. Walport, M.J. Complement. First of two parts. *N Engl J Med* **344**, 1058-1066 (2001).

43. Walport, M.J. Complement. Second of two parts. *N Engl J Med* **344**, 1140-1144 (2001).

44. Strauss, O. The retinal pigment epithelium in visual function. *Physiol Rev* **85**, 845-881 (2005).

45. Delori, F.C., Goger, D.G. & Dorey, C.K. Age-related accumulation and spatial distribution of lipofuscin in RPE of normal subjects. *Invest Ophthalmol Vis Sci* **42**, 1855-1866 (2001).

46. Boulton, M. & Dayhaw-Barker, P. The role of the retinal pigment epithelium: topographical variation and ageing changes. *Eye (Lond)* **15**, 384-389 (2001).

47. Bergmann, M., Schutt, F., Holz, F.G. & Kopitz, J. Inhibition of the ATP-driven proton pump in RPE lysosomes by the major lipofuscin fluorophore A2-E may contribute to the pathogenesis of age-related macular degeneration. *Faseb J* **18**, 562-564 (2004).

48. Bird, A.C. Doyne Lecture. Pathogenesis of retinal pigment epithelial detachment in the elderly; the relevance of Bruch's membrane change. *Eye (Lond)* **5 (Pt 1)**, 1-12 (1991).

49. Chen, H., Liu, B., Lukas, T.J. & Neufeld, A.H. The aged retinal pigment epithelium/choroid: a potential substratum for the pathogenesis of age-related macular degeneration. *PLoS One* **3**, e2339 (2008).

50. Crabb, J.W., *et al.* Drusen proteome analysis: an approach to the etiology of age-related macular degeneration. *Proc Natl Acad Sci U S A* **99**, 14682-14687 (2002).

51. Mullins, R.F., Russell, S.R., Anderson, D.H. & Hageman, G.S. Drusen associated with aging and age-related macular degeneration contain proteins common to extracellular deposits associated with atherosclerosis, elastosis, amyloidosis, and dense deposit disease. *Faseb J* **14**, 835-846 (2000).

52. Farkas, T.G., Sylvester, V. & Archer, D. The ultrastructure of drusen. *Am J Ophthalmol* **71**, 1196-1205 (1971).

53. Hageman, G.S., *et al.* An integrated hypothesis that considers drusen as biomarkers of immune-mediated processes at the RPE-Bruch's membrane interface in aging and age-related macular degeneration. *Prog Retin Eye Res* **20**, 705-732 (2001).

54. Sunness, J.S., *et al.* Visual function abnormalities and prognosis in eyes with age-related geographic atrophy of the macula and good visual acuity. *Ophthalmology* **104**, 1677-1691 (1997).

55. A randomized, placebo-controlled, clinical trial of high-dose supplementation with vitamins C and E, beta carotene, and zinc for age-related macular degeneration and vision loss: AREDS report no. 8. *Arch Ophthalmol* **119**, 1417-1436 (2001).

56. Tomany, S.C., *et al.* Risk factors for incident age-related macular degeneration: pooled findings from 3 continents. *Ophthalmology* **111**, 1280-1287 (2004).

57. Coleman, H.R., Chan, C.C., Ferris, F.L., 3rd & Chew, E.Y. Age-related macular degeneration. *Lancet* **372**, 1835-1845 (2008).

58. Ferris, F.L., 3rd, Fine, S.L. & Hyman, L. Age-related macular degeneration and blindness due to neovascular maculopathy. *Arch Ophthalmol* **102**, 1640-1642 (1984).

59. Guidelines for using verteporfin (visudyne) in photodynamic therapy to treat choroidal neovascularization due to age-related macular degeneration and other causes. *Retina* **22**, 6-18 (2002).

60. Rosenfeld, P.J., *et al.* Ranibizumab for neovascular age-related macular degeneration. *N Engl J Med* **355**, 1419-1431 (2006).

61. Augustin, A.J., Puls, S. & Offermann, I. Triple therapy for choroidal neovascularization due to age-related macular degeneration: verteporfin PDT, bevacizumab, and dexamethasone. *Retina* **27**, 133-140 (2007).

62. Owens, S.L., *et al.* Prophylactic laser treatment hastens choroidal neovascularization in unilateral age-related maculopathy: final results of the drusen laser study. *Am J Ophthalmol* **141**, 276-281 (2006).

63. Klein, R., *et al.* Prevalence of age-related macular degeneration in 4 racial/ethnic groups in the multi-ethnic study of atherosclerosis. *Ophthalmology* **113**, 373-380 (2006).

64. Delcourt, C., Diaz, J.L., Ponton-Sanchez, A. & Papoz, L. Smoking and age-related macular degeneration. The POLA Study. Pathologies Oculaires Liees a l'Age. *Arch Ophthalmol* **116**, 1031-1035 (1998).

65. Khan, J.C., *et al.* Smoking and age related macular degeneration: the number of pack years of cigarette smoking is a major determinant of risk for both geographic atrophy and choroidal neovascularisation. *Br J Ophthalmol* **90**, 75-80 (2006).

66. Hyman, L., Schachat, A.P., He, Q. & Leske, M.C. Hypertension, cardiovascular disease, and age-related macular degeneration. Age-Related Macular Degeneration Risk Factors Study Group. *Arch Ophthalmol* **118**, 351-358 (2000).

67. Seddon, J.M., *et al.* Dietary carotenoids, vitamins A, C, and E, and advanced age-related macular degeneration. Eye Disease Case-Control Study Group. *Jama* **272**, 1413-1420 (1994).

68. Seddon, J.M., Ajani, U.A. & Mitchell, B.D. Familial aggregation of age-related maculopathy. *Am J Ophthalmol* **123**, 199-206 (1997).

69. Seddon, J.M., Cote, J., Page, W.F., Aggen, S.H. & Neale, M.C. The US twin study of age-related macular degeneration: relative roles of genetic and environmental influences. *Arch Ophthalmol* **123**, 321-327 (2005).

70. Haines, J.L., *et al.* Complement factor H variant increases the risk of age-related macular degeneration. *Science* **308**, 419-421 (2005).

71. Klein, R.J., *et al.* Complement factor H polymorphism in age-related macular degeneration. *Science* **308**, 385-389 (2005).

72. Li, M., *et al.* CFH haplotypes without the Y402H coding variant show strong association with susceptibility to age-related macular degeneration. *Nat Genet* **38**, 1049-1054 (2006).

73. Dewan, A., *et al.* HTRA1 promoter polymorphism in wet age-related macular degeneration. *Science* **314**, 989-992 (2006).

74. Fritsche, L.G., *et al.* Age-related macular degeneration is associated with an unstable ARMS2 (LOC387715) mRNA. *Nat Genet* **40**, 892-896 (2008).

75. Thurman, J.M. & Holers, V.M. The central role of the alternative complement pathway in human disease. *J Immunol* **176**, 1305-1310 (2006).

76. Anderson, D.H., *et al.* The pivotal role of the complement system in aging and age-related macular degeneration: hypothesis re-visited. *Prog Retin Eye Res* **29**, 95-112 (2010).

77. Carroll, M.C. The role of complement in B cell activation and tolerance. *Adv Immunol* **74**, 61-88 (2000).

78. Ehrlich P, M.J. Zur Theorie der Lysinwirkung. *Berliner klinische Wochenschrift* **36**, 6-9 (1899).

79. Zipfel, P.F. & Skerka, C. Complement regulators and inhibitory proteins. *Nat Rev Immunol* **9**, 729-740 (2009).

80. Mollnes, T.E., *et al.* Essential role of the C5a receptor in E coli-induced oxidative burst and phagocytosis revealed by a novel lepirudin-based human whole blood model of inflammation. *Blood* **100**, 1869-1877 (2002).

81. DiScipio, R.G., Daffern, P.J., Jagels, M.A., Broide, D.H. & Sriramarao, P. A comparison of C3a and C5a-mediated stable adhesion of rolling eosinophils in postcapillary venules and transendothelial migration in vitro and in vivo. *J Immunol* **162**, 1127-1136 (1999).

82. Klos, A., *et al.* The role of the anaphylatoxins in health and disease. *Mol Immunol* **46**, 2753-2766 (2009).

83. Schifferli, J.A., Ng, Y.C. & Peters, D.K. The role of complement and its receptor in the elimination of immune complexes. *N Engl J Med* **315**, 488-495 (1986).

84. Trouw, L.A., Blom, A.M. & Gasque, P. Role of complement and complement regulators in the removal of apoptotic cells. *Mol Immunol* **45**, 1199-1207 (2008).

85. Bhakdi, S. & Tranum-Jensen, J. Molecular nature of the complement lesion. *Proc Natl Acad Sci U S A* **75**, 5655-5659 (1978).

86. Hawlisch, H. & Kohl, J. Complement and Toll-like receptors: key regulators of adaptive immune responses. *Mol Immunol* **43**, 13-21 (2006).

87. Dunkelberger, J.R. & Song, W.C. Role and mechanism of action of complement in regulating T cell immunity. *Mol Immunol* **47**, 2176-2186 (2010).

88. Zipfel, P.F., Mihlan, M. & Skerka, C. The alternative pathway of complement: a pattern recognition system. *Adv Exp Med Biol* **598**, 80-92 (2007).

89. Pangburn, M.K. & Muller-Eberhard, H.J. Initiation of the alternative complement pathway due to spontaneous hydrolysis of the thioester of C3. *Ann N Y Acad Sci* **421**, 291-298 (1983).

90. Pangburn, M.K. & Muller-Eberhard, H.J. The alternative pathway of complement. *Springer Semin Immunopathol* **7**, 163-192 (1984).

91. Pangburn, M.K., Schreiber, R.D. & Muller-Eberhard, H.J. C3b deposition during activation of the alternative complement pathway and the effect of deposition on the activating surface. *J Immunol* **131**, 1930-1935 (1983).

92. Ziccardi, R.J. The first component of human complement (C1): activation and control. *Springer Semin Immunopathol* **6**, 213-230 (1983).

93. Dodds, A.W., Ren, X.D., Willis, A.C. & Law, S.K. The reaction mechanism of the internal thioester in the human complement component C4. *Nature* **379**, 177-179 (1996).

94. Nagasawa, S. & Stroud, R.M. Cleavage of C2 by C1s into the antigenically distinct fragments C2a and C2b: demonstration of binding of C2b to C4b. *Proc Natl Acad Sci U S A* **74**, 2998-3001 (1977).

95. Ohta, M., Okada, M., Yamashina, I. & Kawasaki, T. The mechanism of carbohydrate-mediated complement activation by the serum mannan-binding protein. *J Biol Chem* **265**, 1980-1984 (1990).

96. Thiel, S., *et al.* A second serine protease associated with mannan-binding lectin that activates complement. *Nature* **386**, 506-510 (1997).

97. Muller-Eberhard, H.J. The membrane attack complex of complement. *Annu Rev Immunol* **4**, 503-528 (1986).

98. Tschopp, J., Podack, E.R. & Muller-Eberhard, H.J. Ultrastructure of the membrane attack complex of complement: detection of the tetramolecular C9-polymerizing complex C5b-8. *Proc Natl Acad Sci U S A* **79**, 7474-7478 (1982).

99. Poon, I.K., Hulett, M.D. & Parish, C.R. Molecular mechanisms of late apoptotic/necrotic cell clearance. *Cell Death Differ* **17**, 381-397 (2010).

100. Mihlan, M., Stippa, S., Jozsi, M. & Zipfel, P.F. Monomeric CRP contributes to complement control in fluid phase and on cellular surfaces and increases phagocytosis by recruiting factor H. *Cell Death Differ* **16**, 1630-1640 (2009).

101. Sahu, A. & Lambris, J.D. Structure and biology of complement protein C3, a connecting link between innate and acquired immunity. *Immunol Rev* **180**, 35-48 (2001).

102. Janeway, C.A., Jr. & Medzhitov, R. Innate immune recognition. *Annu Rev Immunol* **20**, 197-216 (2002).

103. Blom, A.M., Kask, L., Ramesh, B. & Hillarp, A. Effects of zinc on factor I cofactor activity of C4b-binding protein and factor H. *Arch Biochem Biophys* **418**, 108-118 (2003).

104. Hellwage, J., *et al.* Functional properties of complement factor H-related proteins FHR-3 and FHR-4: binding to the C3d region of C3b and differential regulation by heparin. *FEBS Lett* **462**, 345-352 (1999).

105. Zipfel, P.F., Heinen, S., Jozsi, M. & Skerka, C. Complement and diseases: defective alternative pathway control results in kidney and eye diseases. *Mol Immunol* **43**, 97-106 (2006).

106. Zipfel, P.F. Complement factor H: physiology and pathophysiology. *Semin Thromb Hemost* **27**, 191-199 (2001).

107. Sohn, J.H., Kaplan, H.J., Suk, H.J., Bora, P.S. & Bora, N.S. Chronic low level complement activation within the eye is controlled by intraocular complement regulatory proteins. *Invest Ophthalmol Vis Sci* **41**, 3492-3502 (2000).

108. Bora, N.S., Gobleman, C.L., Atkinson, J.P., Pepose, J.S. & Kaplan, H.J. Differential expression of the complement regulatory proteins in the human eye. *Invest Ophthalmol Vis Sci* **34**, 3579-3584 (1993).

109. Skerka, C., *et al.* Defective complement control of factor H (Y402H) and FHL-1 in age-related macular degeneration. *Mol Immunol* **44**, 3398-3406 (2007).

110. Wu, Z., Lauer, T.W., Sick, A., Hackett, S.F. & Campochiaro, P.A. Oxidative stress modulates complement factor H expression in retinal pigmented epithelial cells by acetylation of FOXO3. *J Biol Chem* **282**, 22414-22425 (2007).

111. Rodriguez de Cordoba, S., Esparza-Gordillo, J., Goicoechea de Jorge, E., Lopez-Trascasa, M. & Sanchez-Corral, P. The human complement factor H: functional roles, genetic variations and disease associations. *Mol Immunol* **41**, 355-367 (2004).

112. Jozsi, M. & Zipfel, P.F. Factor H family proteins and human diseases. *Trends Immunol* **29**, 380-387 (2008).

113. Misasi, R., *et al.* Human complement factor H: an additional gene product of 43 kDa isolated from human plasma shows cofactor activity for the cleavage of the third component of complement. *Eur J Immunol* **19**, 1765-1768 (1989).

114. Norman, D.G., *et al.* Three-dimensional structure of a complement control protein module in solution. *J Mol Biol* **219**, 717-725 (1991).

115. Zipfel, P.F., *et al.* Factor H family proteins: on complement, microbes and human diseases. *Biochem Soc Trans* **30**, 971-978 (2002).

116. Nilsson, U.R. & Mueller-Eberhard, H.J. Isolation of Beta If-Globulin from Human Serum and Its Characterization as the Fifth Component of Complement. *J Exp Med* **122**, 277-298 (1965).

117. Ripoche, J., Day, A.J., Harris, T.J. & Sim, R.B. The complete amino acid sequence of human complement factor H. *Biochem J* **249**, 593-602 (1988).

118. Esparza-Gordillo, J., *et al.* Genetic and environmental factors influencing the human factor H plasma levels. *Immunogenetics* **56**, 77-82 (2004).

119. Jokiranta, T.S., Hellwage, J., Koistinen, V., Zipfel, P.F. & Meri, S. Each of the three binding sites on complement factor H interacts with a distinct site on C3b. *J Biol Chem* **275**, 27657-27662 (2000).

120. Jokiranta, T.S., *et al.* Binding of complement factor H to endothelial cells is mediated by the carboxy-terminal glycosaminoglycan binding site. *Am J Pathol* **167**, 1173-1181 (2005).

121. Pangburn, M.K., Atkinson, M.A. & Meri, S. Localization of the heparin-binding site on complement factor H. *J Biol Chem* **266**, 16847-16853 (1991).

122. Losse, J., Zipfel, P.F. & Jozsi, M. Factor H and factor H-related protein 1 bind to human neutrophils via complement receptor 3, mediate attachment to Candida albicans, and enhance neutrophil antimicrobial activity. *J Immunol* **184**, 912-921 (2010).

123. Luo, S., Poltermann, S., Kunert, A., Rupp, S. & Zipfel, P.F. Immune evasion of the human pathogenic yeast Candida albicans: Pra1 is a Factor H, FHL-1 and plasminogen binding surface protein. *Mol Immunol* **47**, 541-550 (2009).

124. Reuter, M., Caswell, C.C., Lukomski, S. & Zipfel, P.F. Binding of the human complement regulators CFHR1 and factor H by streptococcal collagen-like protein 1 (Scl1) via their conserved C termini allows control of the complement cascade at multiple levels. *J Biol Chem* **285**, 38473-38485 (2010).

125. Hallstrom, T., *et al.* Haemophilus influenzae interacts with the human complement inhibitor factor H. *J Immunol* **181**, 537-545 (2008).

126. Kuhn, S. & Zipfel, P.F. Mapping of the domains required for decay acceleration activity of the human factor H-like protein 1 and factor H. *Eur J Immunol* **26**, 2383-2387 (1996).

127. Friese, M.A., *et al.* Different regulation of factor H and FHL-1/reconectin by inflammatory mediators and expression of the two proteins in rheumatoid arthritis (RA). *Clin Exp Immunol* **121**, 406-415 (2000).

128. Skerka, C., Horstmann, R.D. & Zipfel, P.F. Molecular cloning of a human serum protein structurally related to complement factor H. *J Biol Chem* **266**, 12015-12020 (1991).

129. Skerka, C., Kuhn, S., Gunther, K., Lingelbach, K. & Zipfel, P.F. A novel short consensus repeat-containing molecule is related to human complement factor H. *J Biol Chem* **268**, 2904-2908 (1993).

130. Mihlan, M., *et al.* Human complement factor H-related protein 4 binds and recruits native pentameric C-reactive protein to necrotic cells. *Mol Immunol* **46**, 335-344 (2009).

131. McRae, J.L., *et al.* Human factor H-related protein 5 has cofactor activity, inhibits C3 convertase activity, binds heparin and C-reactive protein, and associates with lipoprotein. *J Immunol* **174**, 6250-6256 (2005).

132. Zipfel, P.F. Complement and immune defense: from innate immunity to human diseases. *Immunol Lett* **126**, 1-7 (2009).

133. Noris, M. & Remuzzi, G. Atypical hemolytic-uremic syndrome. *N Engl J Med* **361**, 1676-1687 (2009).

134. Appel, G.B., *et al.* Membranoproliferative glomerulonephritis type II (dense deposit disease): an update. *J Am Soc Nephrol* **16**, 1392-1403 (2005).

135. Jozsi, M., *et al.* Factor H and atypical hemolytic uremic syndrome: mutations in the C-terminus cause structural changes and defective recognition functions. *J Am Soc Nephrol* **17**, 170-177 (2006).

136. Caprioli, J., *et al.* The molecular basis of familial hemolytic uremic syndrome: mutation analysis of factor H gene reveals a hot spot in short consensus repeat 20. *J Am Soc Nephrol* **12**, 297-307 (2001).

137. Manuelian, T., *et al.* Mutations in factor H reduce binding affinity to C3b and heparin and surface attachment to endothelial cells in hemolytic uremic syndrome. *J Clin Invest* **111**, 1181-1190 (2003).

138. Zipfel, P.F., *et al.* DEAP-HUS: Deficiency of CFHR plasma proteins and autoantibody-positive form of hemolytic uremic syndrome. *Pediatr Nephrol* **10**, 2009-2019 (2010).

139. Jozsi, M., *et al.* Anti factor H autoantibodies block C-terminal recognition function of factor H in hemolytic uremic syndrome. *Blood* **110**, 1516-1518 (2007).

140. Jozsi, M., *et al.* Factor H autoantibodies in atypical hemolytic uremic syndrome correlate with CFHR1/CFHR3 deficiency. *Blood* **111**, 1512-1514 (2008).

141. West, C.D., Witte, D.P. & McAdams, A.J. Composition of nephritic factor-generated glomerular deposits in membranoproliferative glomerulonephritis type 2. *Am J Kidney Dis* **37**, 1120-1130 (2001).

142. Mullins, R.F., Aptsiauri, N. & Hageman, G.S. Structure and composition of drusen associated with glomerulonephritis: implications for the role of complement activation in drusen biogenesis. *Eye (Lond)* **15**, 390-395 (2001).

143. D'Souza, Y.B. & Short, C.D. The eye--a window on the kidney. *Nephrol Dial Transplant* **24**, 3582-3584 (2009).

144. Licht, C., *et al.* Deletion of Lys224 in regulatory domain 4 of Factor H reveals a novel pathomechanism for dense deposit disease (MPGN II). *Kidney Int* **70**, 42-50 (2006).

145. Williams, D.G. C3 nephritic factor and mesangiocapillary glomerulonephritis. *Pediatr Nephrol* **11**, 96-98 (1997).

146. Hageman, G.S., *et al.* A common haplotype in the complement regulatory gene factor H (HF1/CFH) predisposes individuals to age-related macular degeneration. *Proc Natl Acad Sci U S A* **102**, 7227-7232 (2005).

147. Nozaki, M., *et al.* Drusen complement components C3a and C5a promote choroidal neovascularization. *Proc Natl Acad Sci U S A* **103**, 2328-2333 (2006).

148. Seddon, J.M., Gensler, G., Milton, R.C., Klein, M.L. & Rifai, N. Association between C-reactive protein and age-related macular degeneration. *Jama* **291**, 704-710 (2004).

149. Scholl, H.P., *et al.* Systemic complement activation in age-related macular degeneration. *PLoS One* **3**, e2593 (2008).

150. Giannakis, E., *et al.* A common site within factor H SCR 7 responsible for binding heparin, C-reactive protein and streptococcal M protein. *Eur J Immunol* **33**, 962-969 (2003).

151. Clark, S.J., *et al.* His-384 allotypic variant of factor H associated with age-related macular degeneration has different heparin binding properties from the non-disease-associated form. *J Biol Chem* **281**, 24713-24720 (2006).

152. Clark, S.J., *et al.* Impaired binding of the age-related macular degeneration-associated complement factor H 402H allotype to Bruch's membrane in human retina. *J Biol Chem* **285**, 30192-30202 (2010).

153. Laine, M., *et al.* Y402H polymorphism of complement factor H affects binding affinity to C-reactive protein. *J Immunol* **178**, 3831-3836 (2007).

154. Sjoberg, A.P., *et al.* The factor H variant associated with age-related macular degeneration (His-384) and the non-disease-associated form bind differentially to C-reactive protein, fibromodulin, DNA, and necrotic cells. *J Biol Chem* **282**, 10894-10900 (2007).

155. Johnson, P.T., *et al.* Individuals homozygous for the age-related macular degeneration risk-conferring variant of complement factor H have elevated levels of CRP in the choroid. *Proc Natl Acad Sci U S A* **103**, 17456-17461 (2006).

156. Schaumberg, D.A., *et al.* A prospective assessment of the Y402H variant in complement factor H, genetic variants in C-reactive protein, and risk of age-related macular degeneration. *Invest Ophthalmol Vis Sci* **47**, 2336-2340 (2006).

157. Tortajada, A., *et al.* The disease-protective complement factor H allotypic variant Ile62 shows increased binding affinity for C3b and enhanced cofactor activity. *Hum Mol Genet* **18**, 3452-3461 (2009).

158. Skerka, C. & Zipfel, P.F. Complement factor H related proteins in immune diseases. *Vaccine* **26 Suppl 8**, I9-14 (2008).

159. McKay, G.J., *et al.* Further assessment of the complement component 2 and factor B region associated with age-related macular degeneration. *Invest Ophthalmol Vis Sci* **50**, 533-539 (2009).

160. Montes, T., Tortajada, A., Morgan, B.P., Rodriguez de Cordoba, S. & Harris, C.L. Functional basis of protection against age-related macular degeneration conferred by a common polymorphism in complement factor B. *Proc Natl Acad Sci U S A* **106**, 4366-4371 (2009).

161. Botto, M., Fong, K.Y., So, A.K., Koch, C. & Walport, M.J. Molecular basis of polymorphisms of human complement component C3. *J Exp Med* **172**, 1011-1017 (1990).

162. Fagerness, J.A., *et al.* Variation near complement factor I is associated with risk of advanced AMD. *Eur J Hum Genet* **17**, 100-104 (2009).

163. Dinu, V., Miller, P.L. & Zhao, H. Evidence for association between multiple complement pathway genes and AMD. *Genet Epidemiol* **31**, 224-237 (2007).

164. Narendra, U., Pauer, G.J. & Hagstrom, S.A. Genetic analysis of complement factor H related 5, CFHR5, in patients with age-related macular degeneration. *Mol Vis* **15**, 731-736 (2009).

165. Sahu, A., Kay, B.K. & Lambris, J.D. Inhibition of human complement by a C3-binding peptide isolated from a phage-displayed random peptide library. *J Immunol* **157**, 884-891 (1996).

166. Ricklin, D. & Lambris, J.D. Compstatin: a complement inhibitor on its way to clinical application. *Adv Exp Med Biol* **632**, 273-292 (2008).

167. Potentia, P. POT-4 Drug Candidate for Age-Related Macular Degeneration Successfully Completes Phase I Clinical Trial - Prepares for Phase II. *Press Release* (2009).

168. Rother, R.P., Rollins, S.A., Mojcik, C.F., Brodsky, R.A. & Bell, L. Discovery and development of the complement inhibitor eculizumab for the treatment of paroxysmal nocturnal hemoglobinuria. *Nat Biotechnol* **25**, 1256-1264 (2007).

169. Rohrer, B., *et al.* A targeted inhibitor of the alternative complement pathway reduces angiogenesis in a mouse model of age-related macular degeneration. *Invest Ophthalmol Vis Sci* **50**, 3056-3064 (2009).

170. Davis, A.E., 3rd. Mechanism of angioedema in first complement component inhibitor deficiency. *Immunol Allergy Clin North Am* **26**, 633-651 (2006).

171. Zarbin, M.A. Age-related macular degeneration: review of pathogenesis. *Eur J Ophthalmol* **8**, 199-206 (1998).

172. Donoso, L.A., Kim, D., Frost, A., Callahan, A. & Hageman, G. The role of inflammation in the pathogenesis of age-related macular degeneration. *Surv Ophthalmol* **51**, 137-152 (2006).

173. Wu, J., *et al.* Structure of complement fragment C3b-factor H and implications for host protection by complement regulators. *Nat Immunol* **10**, 728-733 (2009).

174. Hocking, H.G., *et al.* Structure of the N-terminal region of complement factor H and conformational implications of disease-linked sequence variations. *J Biol Chem* **283**, 9475-9487 (2008).

175. Kelly, U., *et al.* Heparan sulfate, including that in Bruch's membrane, inhibits the complement alternative pathway: implications for age-related macular degeneration. *J Immunol* **185**, 5486-5494 (2010).

176. Ormsby, R.J., *et al.* Functional and structural implications of the complement factor H Y402H polymorphism associated with age-related macular degeneration. *Invest Ophthalmol Vis Sci* **49**, 1763-1770 (2008).

177. Herbert, A.P., *et al.* Structure shows that a glycosaminoglycan and protein recognition site in factor H is perturbed by age-related macular degeneration-linked single nucleotide polymorphism. *J Biol Chem* **282**, 18960-18968 (2007).

178. Prosser, B.E., *et al.* Structural basis for complement factor H linked age-related macular degeneration. *J Exp Med* **204**, 2277-2283 (2007).

179. Bernfield, M., *et al.* Functions of cell surface heparan sulfate proteoglycans. *Annu Rev Biochem* **68**, 729-777 (1999).

180. Kreuger, J., Spillmann, D., Li, J.P. & Lindahl, U. Interactions between heparan sulfate and proteins: the concept of specificity. *J Cell Biol* **174**, 323-327 (2006).

181. Chong, N.H., *et al.* Decreased thickness and integrity of the macular elastic layer of Bruch's membrane correspond to the distribution of lesions associated with age-related macular degeneration. *Am J Pathol* **166**, 241-251 (2005).

182. Hotchkiss, R.S., Strasser, A., McDunn, J.E. & Swanson, P.E. Cell death. *N Engl J Med* **361**, 1570-1583 (2009).

183. Henriquez, M., Armisen, R., Stutzin, A. & Quest, A.F. Cell death by necrosis, a regulated way to go. *Curr Mol Med* **8**, 187-206 (2008).

184. Ormsby, R.J., *et al.* Localization of the third heparin-binding site in the human complement regulator factor H1. *Mol Immunol* **43**, 1624-1632 (2006).

185. Eisenhardt, S.U., Thiele, J.R., Bannasch, H., Stark, G.B. & Peter, K. C-reactive protein: how conformational changes influence inflammatory properties. *Cell Cycle* **8**, 3885-3892 (2009).

186. Krysko, O., De Ridder, L. & Cornelissen, M. Phosphatidylserine exposure during early primary necrosis (oncosis) in JB6 cells as evidenced by immunogold labeling technique. *Apoptosis* **9**, 495-500 (2004).

187. Locksley, R.M., Killeen, N. & Lenardo, M.J. The TNF and TNF receptor superfamilies: integrating mammalian biology. *Cell* **104**, 487-501 (2001).

188. Haapasalo, K., *et al.* Complement factor H allotype 402H is associated with increased C3b opsonization and phagocytosis of Streptococcus pyogenes. *Mol Microbiol* **70**, 583-594 (2008).

189. Zhou, J., Jang, Y.P., Kim, S.R. & Sparrow, J.R. Complement activation by photooxidation products of A2E, a lipofuscin constituent of the retinal pigment epithelium. *Proc Natl Acad Sci U S A* **103**, 16182-16187 (2006).

190. Thurman, J.M., *et al.* Oxidative stress renders retinal pigment epithelial cells susceptible to complement-mediated injury. *J Biol Chem* **284**, 16939-16947 (2009).

191. Hellwage, J., *et al.* Complement C3b/C3d and cell surface polyanions are recognized by overlapping binding sites on the most carboxyl-terminal domain of complement factor H. *J Immunol* **169**, 6935-6944 (2002).

192. Raychaudhuri, S., *et al.* Associations of CFHR1-CFHR3 deletion and a CFH SNP to age-related macular degeneration are not independent. *Nat Genet* **42**, 553-555; author reply 555-556 (2010).

193. Schmid-Kubista, K.E., *et al.* Contribution of copy number variation in the regulation of complement activation locus to development of age-related macular degeneration. *Invest Ophthalmol Vis Sci* **50**, 5070-5079 (2009).

194. Spencer, K.L., *et al.* Deletion of CFHR3 and CFHR1 genes in age-related macular degeneration. *Hum Mol Genet* **17**, 971-977 (2008).

195. Zipfel, P.F., *et al.* Deletion of complement factor H-related genes CFHR1 and CFHR3 is associated with atypical hemolytic uremic syndrome. *PLoS Genet* **3**, e41 (2007).

196. Walport, M.J. Complement and systemic lupus erythematosus. *Arthritis Res* **4 Suppl 3**, S279-293 (2002).

197. Dhillon, B., *et al.* Complement factor h autoantibodies and age-related macular degeneration. *Invest Ophthalmol Vis Sci* **51**, 5858-5863 (2010).

198. Ricklin, D. & Lambris, J.D. Complement-targeted therapeutics. *Nat Biotechnol* **25**, 1265-1275 (2007).

199. Quigg, R.J. Use of complement inhibitors in tissue injury. *Trends Mol Med* **8**, 430-436 (2002).

200. Schmidt, C.Q., Herbert, A.P., Hocking, H.G., Uhrin, D. & Barlow, P.N. Translational mini-review series on complement factor H: structural and functional correlations for factor H. *Clin Exp Immunol* **151**, 14-24 (2008).

201. McGreal, E. & Gasque, P. Structure-function studies of the receptors for complement C1q. *Biochem Soc Trans* **30**, 1010-1014 (2002).

202. Chi, Z.L., Yoshida, T., Lambris, J.D. & Iwata, T. Suppression of drusen formation by compstatin, a peptide inhibitor of complement C3 activation, on cynomolgus monkey with early-onset macular degeneration. *Adv Exp Med Biol* **703**, 127-135.

203. Agbeko, R.S., *et al.* Genetic variability in complement activation modulates the systemic inflammatory response syndrome in children. *Pediatr Crit Care Med* **11**, 561-567.

Anhänge

Eigenständigkeitserklärung

Hiermit erkläre ich, dass mir die geltende Promotionsordnung der Biologisch Pharmazeutischen Fakultät der Friedrich-Schiller-Universität Jena bekannt ist, ich die vorliegende Dissertation selbst angefertigt habe und alle benutzten Hilfsmittel, persönliche Mitteilungen und verwendete Quellen angegeben habe.

Alle Personen, die mich bei der Auswahl des Materials sowie bei der Herstellung der Manuskripte unterstützt haben, habe ich benannt.

Ich habe nicht die Hilfe eines Promotionsberaters in Anspruch genommen.

Dritte Personen haben weder unmittelbar noch mittelbar geldwerte Leistungen von mir für Arbeiten erhalten, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen.

Die vorliegende Arbeit wurde weder in der Vergangenheit noch gegenwärtig als Dissertation an einer anderen Hochschule eingereicht. Ebenso wenig wurde diese Dissertation als Prüfungsarbeit für eine staatliche oder wissenschaftliche Prüfung eingereicht.

Jena, den 05.08.2011

Nadine Lauer

Vorträge, Poster, Auszeichnungen

Vorträge

5th International Complement Therapeutics Conference, June 2011 Rhodos, Greece.
Titel: Use of the C-terminal surface attachment region of Factor H to target compstatin to self surfaces and to sites of immune stress.
ausgezeichnet mit einem Student Travel Award

IV International Workshop on Thrombotic Microangiopathies, October 2009, Weimar, Germany;
Titel: Factor H-protein variants affect complement control on damaged cells.

XXII European Meeting on Complement in Human Disease, September 2009 Visegrád, Hungary
Titel: Factor H-related genes and age-related macular degeneration.

Treffen der Regionalgruppe Thüringen von ProRetina e.V., 15. August 2009, Jena
Titel: Was verbindet das Hans-Knöll-Institut mit unserer Patientengruppe? Wie erlebten Sie als wissenschaftliches Mitglied des HKI das internationale Forschungskolloquium der ProRetina e.V?

Ist International Conference on Inflammation and Retinal Disease, June 2009 Crete, Greece.
Titel: Y402H polymorphism affects mCRP binding and leads to a diminished complement control on the surface of necrotic ARPE cells.
ausgezeichnet mit einem Student Travel Award

XXII International Complement Workshop, October 2008 Basel, Switzerland
Titel: The AMD-associated variants of the Factor H protein affect protein function and complement control.

Posterpräsentationen

7. ProRetina Forschungskolloquium, April 2011 Potsdam, Germany
Titel: Imbalance of complement regulatory proteins CFHR1, CFHR3 and factor H influences risk for age related macular degeneration (AMD).

7. ProRetina Forschungskolloquium, April 2011 Potsdam, Germany
Titel: The new chimeric alternative complement inhibitor COMP_SCR15-20 protects self-surfaces from complement-mediated damage.

XXIII International Complement Workshop, August 2010 New York City, USA
Titel: Complement regulation at necrotic cell lesions is impaired by the AMD-associated Factor H - H402 risk variant.
Mol Immunol 2010; 47 (13): 2211
ausgezeichnet mit einem Student Travel Award

XXIII International Complement Workshop, August 2010 New York City, USA
Titel: Imbalance of complement regulatory proteins CFHR1, CFHR3 and factor H influences risk for age related macular degeneration (AMD).
Mol Immunol 2010; 47 (13): 2211

6. ProRetina Forschungskolloquium April, 2010 Potsdam, Germany
Titel: Complement regulation at necrotic cell lesions is impaired by the AMD-associated Factor H - H402 risk variant.

XXII European Meeting on Complement in Human Disease, September 2009 Visegrád, Hungary
Titel: AMD-associated Y402H-polymorphism affects binding of the complement regulator mCRP and results in an altered complement control on necrotic ARPE-19 cells
Mol Immunol 2009; 46 (14): 2856

5. ProRetina Forschungskolloquium, April 2009 Potsdam, Germany
Titel: Frequency of the complement Factor H-related genes within a german AMD cohort.
ausgezeichnet mit einem Posterpreis

5. ProRetina Forschungskolloquium, April 2009 Potsdam, Germany
Titel: AMD-associated Y402H-polymorphism of complement Factor H affects complement control on the surface of necrotic ARPE cells

XXII International Complement Workshop, October 2008 Basel, Switzerland
Titel: The two AMD-associated variants of the Factor H protein (V62I and Y402H) affect protein function and complement control
Mol Immunol 2008; 45 (16): 4116

4. ProRetina Forschungskolloquium, April 2008 Potsdam, Germany
Titel: The Y402H-polymorphism in CFH and FHL 1 affect mCRP binding
ausgezeichnet mit einem Posterpreis

Stipendien und Patente

Stipendien

Reisestipendium;
vergeben durch den **D**eutschen **A**kademischen **A**ustausch **D**ienst (DAAD) für die Teilnahme am XXIII International Complement Workshop, August 2010 New York City, USA

Reisekostenzuschuss;
vergeben durch die **D**eutsche **G**esellschaft **f**ür **I**mmunologie (DGFI) für die Teilnahme am XXIII International Complement Workshop, August 2010 New York City, USA

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Patentanmeldungen

Zipfel PF, Skerka C, Lambris JD, **Lauer N**, Heinen S,
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Publikationsliste

Skerka C, **Lauer N**, Weinberger AWA, Keilhauer CN, Sühnel J, Smith R, Schlötzer–Schrehardt U, Fritsche L, Heinen S, Hartmann A, Weber BHF, Zipfel PF (2007)
Defective complement control of Factor H (Y402H) and FHL-1 in age related macular degeneration.
Mol Immunol 44 (13): 3398-3406

Hallström T, Zipfel PF, Blom AM, **Lauer N**, Forsgren A, Riesbeck K. (2008)
Haemophilus influenzae interacts with the human complement inhibitor factor H.
J Immunol 181(1):537-545

Heinen S, Hartmann A, **Lauer N**, Wiehl U, Dahse HM, Schirmer S, Gropp K, Enghardt T, Wallich R, Hälbich S, Mihlan M, Schlötzer-Schrehardt U, Zipfel PF, Skerka C (2009)
Factor H related protein 1 (CFHR-1) inhibits complement C5 convertase activity and terminal complex formation.
Blood 114(12):2439-47
ausgezeichnet mit dem Medac Forschungspreis 2009

Zipfel PF, **Lauer N**, Skerka C (2010)
The role of complement in age-related macular degeneration.
Adv Exp Med Biol. 703:9-24.

Fritsche LG*, **Lauer N***, Hartmann A, Stippa S, Keilhauer CN, Oppermann M, Pandey MK, Köhl J, Zipfel PF, Weber BH, Skerka C (2010)
An imbalance of human complement regulatory proteins CFHR1, CFHR3 and factor H influences risk for age-related macular degeneration.
Hum Mol Genet. 19(23):4694-704
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Lauer N, Mihlan M, Hartmann A, Schlötzer-Schrehardt U, Keilhauer CN, Scholl HPN, Charbel-Issa P; Holz F, Weber BH, Skerka C, Zipfel PF:
Complement regulation at necrotic cell lesions is impaired by the AMD associated Factor H - H402 risk variant.
Manuskript in Überarbeitung, *J Immunol*

Lauer N, Böhme J, Dahse HM, Skerka C, Zipfel PF.
The AMD-associated variation at position 62 does not, but the exchange at position 402 does affect surface regulation of the complement inhibitor FHL1.
Manuskript in Überarbeitung, *J Biol Chem*

Weismann D, Hartvigsen K, **Lauer N**, Bennett KL, Scholl HPN, Charbel-Issa P, Cano M, Skerka C, Superti-Furga G, Handa JT, Zipfel PF, Witztum JL, Binder CJ
Complement factor H binds malondialdehyde-epitopes and protects from oxidative-stress induced inflammation – implications for age-related macular degeneration.
Manuskript in Überarbeitung, *Nat Med*

Lauer N, Ricklin D, Heinen S, Tzekou A, Lambris JD, Zipfel PF.
Use of the C-terminal surface attachment region of Factor H to target compstatin to self surfaces and to sites of immune stress.
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Nadine Lauer

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