

# Investigation of the molecular background of alternative pathway dysregulation in thrombotic microangiopathies

Ph.D. thesis

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## 1. LIST OF ABBREVIATIONS

Acm – acetamidomethyl

ANC – absolute neutrophil count

ADAMTS13 – a disintegrin and metalloproteinase with a thrombospondin type 1 motif member 13

*ADAMTS13* – the gene encoding a disintegrin and metalloproteinase with a thrombospondin type 1 motif member 13

aHUS – atypical hemolytic uremic syndrome

AMD – Age related macular degeneration

Anti-FH antibody – anti-Factor H antibody

AP – alternative pathway

Arg – arginine

Asp – aspartic acid

Boc – tert-butyloxycarbonyl

C1-INH – C1-inhibitor

C1q – complement component 1q

C3 – complement component 3

*C3* – the gene encoding the complement component 3

C3a – complement component 3a

C3b – complement component 3b

C3bBbP – Alternative pathway C3 convertase stabilized by properdin

C3c – complement component 3c, cleavage product of the complement component 3b

C3d – complement component 3d, cleavage product of the complement component 3b

C3dg - complement component 3dg, cleavage product of the complement component 3b

C3G – C3 glomerulopathies

C3Nef - C3 Nephritic Factor

C4 – complement component 4

C4BP – C4b binding protein

C4c – complement component 4c, cleavage product of the complement component 4b

C4d – complement component 4d, cleavage product of the complement component 4b

*CD46* – the gene encoding the cluster of differentiation 46 or membrane cofactor protein

CD59 – cluster of differentiation 59  
*CFB* – the gene encoding the complement protein Factor B  
*CFH* – the gene encoding the complement protein Factor H  
*CFHR1-5* – the genes encoding Factor H-related proteins 1-5  
*CFI* – the gene encoding the complement protein Factor I  
CP – classical pathway  
CR – complement receptor  
CRIg – complement receptor of the immunoglobulin family  
CRP – C-reactive protein  
Cys – cysteine  
DAF – decay accelerating factor  
DAMP – danger associated molecular pattern  
*DGKE* – the gene encoding diacylglycerol kinase epsilon  
DMF – Dimethylformamide  
ELISA – enzyme-linked immunosorbent assay  
FB – complement Factor B  
FFP – fresh frozen plasma  
FH – Factor H  
FHR 1-5 – Factor H-related proteins 1-5  
FI – complement Factor I  
Fmoc/tBu - fluorenylmethyloxycarbonyl group/ tertiary butyl group  
FRETS-VWF73 – Fluorescence-Quenching Substrate for a disintegrin and metalloproteinase with a thrombospondin type 1 motif member 13  
GAG – glycosaminoglycan  
Glu – glutamic acid  
GPI – glycosylphosphatidylinositol  
His – histidine  
HSP60 – heat shock protein 60  
HUS – hemolytic uremic syndrome  
iC3b – inactive cleavage product of the complement component 3b  
iC4b – inactive cleavage product of the complement component 4b  
IgA – Immunoglobulin A

IgG – Immunoglobulin G  
IgG-HRP - Immunoglobulin G conjugated to horseradish peroxidase  
IgM – Immunoglobulin M  
LDH – lactate dehydrogenase  
LPS – lipopolysaccharide  
Lys – lysine  
MAC – membrane attack complex  
MAp19 – Map19 protein, smaller alternative splice product of the gene encoding mannose-binding lectin-associated serine protease-2  
MAp44 – mannose-binding lectin-associated protein of 44 kDa  
MASP-1, -2, -3 – mannose-binding lectin-associated serine proteases 1, 2 and 3  
MBL – mannose-binding lectin  
MCP – membrane cofactor protein  
MLPA – multiplex ligation-dependent probe amplification  
*MMACHC* – the gene encoding methylmalonic aciduria and homocystinuria type C protein  
MMACHC-TMA – thrombotic microangiopathy associated to methylmalonic aciduria and homocystinuria, cobalamin C complementation type  
NET – neutrophil extracellular trap  
NHS – normal human serum  
NLRP3 – NACHT, LRR and PYD domains-containing protein 3  
N $\alpha$ -Fmoc – N-(9-Fluorenylmethoxycarbonyl)  
OD – optical density  
O<sup>t</sup>Bu – tertiary butyl ester  
PAMP – pathogen associated molecular pattern  
Pbf – 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl  
PBS - phosphate-buffered saline  
PEX – plasma exchange therapy  
*PIGA* –phosphatidylinositol glycan anchor biosynthesis class A gene  
Pmc – 2,2,5,7,8-pentamethyl-chroman-6-sulphonyl  
PRM – pattern recognition molecule  
PTX3 – pentraxin-3

RCA – regulators of complement activation  
sC5b-9 – soluble form of the membrane attack complex, consisting of complement components 5b to 9  
SCR – short consensus repeat  
Ser – serine  
SFTL – a unique amino acid sequence on the C-terminal end of the Factor H-like protein 1 consisting of serine, phenylalanine, threonine and leucine  
STEC – Shiga toxin-producing *Escherichia coli*  
STEC-HUS – Shiga toxin-producing *Escherichia coli*-associated hemolytic uremic syndrome  
Stx – Shiga-toxin  
<sup>t</sup>Bu – tertiary butyl group  
Tert-butyl ether – tertiary butyl ether  
*THBD* – the gene encoding thrombomodulin  
THBD – thrombomodulin  
Thr – threonine  
TMA – thrombotic microangiopathy  
TMB - 3,3',5,5'-tetramethylbenzidine  
TTP – thrombotic thrombocytopenic purpura  
Tyr – tyrosine  
ULVWF – ultra-large form of von Willebrand Factor  
WBC – white blood cell count

## **2. INTRODUCTION**

### **2.1. Pathophysiology of the complement system**

#### **2.1.1. Physiological activation of the complement system and significance of the alternative pathway**

The complement system is an integral part of the innate immune system that contributes to the elimination of invading pathogens, immune complexes and maintains continuous clearance of redundant cellular debris together with the regulation of the innate immune response (Kavanagh et al. 2013). Complement activation is triggered by the recognition of molecular patterns characteristic to microorganisms or modified host tissues (pathogen and danger associated molecular patterns, PAMPs and DAMPs, respectively). Binding of such activators induces the initiation of the proteolytic cascade of complement that ultimately leads to the elimination of the activator either through opsonization or direct lysis, in addition to the simultaneous alarm of both innate and adaptive immunity (Ricklin et al. 2010, Bajic et al. 2015). Complement activation may occur via the simultaneous or distinct initiation of three major pathways, the classical, the lectin and the alternative pathways (CP, LP and AP, respectively). While each of the activation pathways has its specific triggers of activation and plays a well-defined role in the effector functions of complement, full-blown activation of the individual pathways converges on the level of the C3 molecule, cleavage of which initiates the mutual, terminal pathway of complement and leads to the attack and lysis of the target molecule.

The CP of complement is initiated via the surface binding of the C1 complex, a flexible assembly formed by the pattern recognition molecule (PRM) C1q (Wallis et al. 2010) and a heterotetramer of the serine proteases C1r2s2 (Gaboriaud et al. 2014). Target recognition of C1q, either through direct binding of PAMPs and DAMPs, or via the recognition of surface-bound PRMs including immunoglobulins and pentraxins (Ricklin et al. 2010), triggers autoactivation of C1r and thus cleavage of C1s (Gaboriaud et al. 2004, Mortensen et al. 2017). Activated C1s in turn will be capable of the cleavage of the C4 and C2 molecules. The subsequent generation of C4a and C4b exposes a previously hidden thioester bond on C4 that allows for the covalent surface attachment of C4b (Ricklin et al. 2010), hence opsonization of the target tissue, whereas cleavage

of C4b-bound C2 molecules results in the formation of the CP C3 convertase C4bC2a (Mortensen et al. 2017).

The activation of the lectin pathway is initiated in a similar manner as that of the CP. Even though, primary activation triggers of the LP are surface glycoprotein and glycolipid patterns characteristic to invading microorganisms (Bajic et al. 2015), recognition of host cell organelles, e.g. mitochondria (Brinkmann et al. 2013) may also provide an activation trigger of the LP. Target surfaces are recognized by mannose-binding lectin (MBL), collectins and ficolins, specific PRMs of the LP (Ricklin et al. 2010). Target binding of the LP PRMs activates the MBL-associated serine proteases (MASPs) via the intermolecular cleavage of MASP homodimers complexed to distinct PRMs (Mortensen et al. 2017). This leads to the downstream cleavage of complement factors C4 and C2, thus aiding the formation of the LP C3-convertase (C4bC2a). Since activation of MASP-2 is inevitable for the cleavage of both C2 and C4 (Matsushita et al. 2000, Rossi et al. 2001) and MASP-1 is the exclusive activator of MASP-2 (Heja et al. 2012), the two serine proteases have to be positioned in close proximity for the induction of the downstream proteolytic activity of MASP-2. This resembles a major difference between the initiation of the CP and LP. Whereas CP protease activation is induced by conformational changes within the C1 complex, initiation of the LP requires inter-complex protease activity, thus accentuating the role of the glycan pattern in the clustering and correct orientation of the MASP/PRM complexes (Degn et al. 2014, Mortensen et al. 2017).

The AP plays a dual role in the activation of complement. On the one hand, it has a baseline, continuous activation, whereas on the other hand the AP may amplify the intensity of complement activation launched through the CP and LP (Sanchez-Corral et al. 2018). The baseline activation of the AP is initiated by the spontaneous hydrolysis of C3 in the fluid phase. In the end product of hydrolysis (C3(H<sub>2</sub>O)) the thioester bond of the C3 molecule becomes accessible and allows the binding of Factor B (FB). Then, Factor D-mediated cleavage of FB activates the preformed AP C3 pro-convertase (C3bB or C3(H<sub>2</sub>O)B, respectively) (Harrison 2018). Following this, the activated AP C3 convertase (C3bBb or C3(H<sub>2</sub>O)Bb) is able to cleave further C3 molecules, and the resulting cleavage product (C3b) may be deposited to nearby surfaces, thus providing basis for the amplification of complement activity. Moreover, C3 convertase activity of

the CP and LP also results in the generation of surface-bound C3b that may engage additional activated FB molecules and hence induce the assembly of further AP C3 convertases (Bajic et al. 2015). The AP C3 convertase however quickly dissociates upon activation with a half-life of approximately 90 seconds at 37°C, unless it is stabilized by properdin (Bajic et al. 2015). Properdin is the only positive regulator of the AP. It can stabilize the AP C3 convertase up to 60 minutes (Fearon et al. 1975) and it may direct fluid phase C3b or C3(H<sub>2</sub>O) to complement activator surfaces such as activated platelets, apoptotic or necrotic cells and invading pathogens (Kemper et al. 2008, Cortes et al. 2012, Saggu et al. 2013).

C3b is the major opsonin of the complement system, which similarly to C4b, carries a thioester bond that allows for the covalent attachment to hydroxyl groups of carbohydrates, present on all biological surfaces (Law et al. 1997). Although C3b may be deposited to host and non-host tissues alike, surface binding only occurs in the immediate vicinity of complement activation, provided by the rapid hydrolysis of its thioester bond following C3 cleavage (Law et al. 1997). Opsonization by C3b stimulates phagocytosis of the complement activator including invading pathogens as well as DAMPs generated by endogenous tissues, hence providing a fundamental tool for the homeostatic elimination of cellular debris.

Upon the assembly of a sufficient number of surface-bound C3b, the C3 convertase, regardless of its origin, may gather an additional C3b molecule, thus creating the C5 convertase (C3bBb3b or C4b2a3b, respectively). Through cleavage of C5, the C5 convertase initiates the downstream activation of the terminal pathway. This implies the formation of the membrane attack complex (MAC) via the assembly of C6, C7, C8 and multiple C9 molecules on target membranes of complement attack (Bajic et al. 2015). Contingent on the intensity of terminal pathway activation, the assembly of MACs may either lead to the lysis of the target cell or modify downstream cell signaling pathways through the assembly of a sublytic number of MACs (Tegla et al. 2011, Laudisi et al. 2013, Triantafilou et al. 2013, Bajic et al. 2015).

In addition to the launch of the succeeding step in the chain reaction of complement activation, cleavage of the complement factors C3 and C5 yields the powerful anaphylatoxins C3a and C5a. C3aR and C5aR1 receptor binding of these cytokines intensifies the pro-inflammatory response via evoking oxidative burst, synthesis and

release of inflammatory mediators and induction of chemotaxis (Klos et al. 2009, Laursen et al. 2012).

PAMPs and DAMPs alike may create an activation trigger of the complement cascade. However, following the initial activation of the complement pathways, the intensity and amplifying capacity of the AP determines, whether the elicited response will recoil by opsonization and tagging of the elimination target or will induce the full-blown activation of the terminal pathway with the concomitant induction of an inflammatory response (Harboe et al. 2004). The significance of AP activation is corroborated by its quantitative role in the augmentation of complement activity. Irrespective of individual pathway initiation, the majority of surface-bound C3b is generated by the amplification loop of the AP (Harboe et al. 2004, Harboe et al. 2009). Since the produced opsonin may be deposited on all surfaces, including intact host tissues (Harboe et al. 2004, Harboe et al. 2009), their protection from complement attack requires the regulation of complement activity.

### **2.1.2. Regulation of the complement system**

Under physiological conditions sufficient restraint of excessive complement activation is provided by specific fluid phase and surface-bound regulators of the complement system that are capable of complement inhibition as well as discrimination of host tissues from foreign structures. Restrain of complement activity may take place via multiple mechanisms. These include direct inhibition of the activator proteases, substrate-limited restraint of pathway initiation, decay acceleration of the formed C3 convertases together with cofactor-mediated cleavage of complement activator proteins and inhibition of the terminal pathway or inactivation of the released anaphylatoxins.

The initial activation steps of the CP and LP are kept under control by the shared fluid phase regulators C4b binding protein (C4BP) and C1-inhibitor (C1-INH). C1-INH is responsible for the irreversible blockade of both the C1-complex and MASPs via binding C1r and C1s, as well as MASP-1 and MASP-2 (Bajic et al. 2015). On the other hand, C4BP fills a dual function, aiding the dissociation of C2a from the CP C3 convertase (C4bC2a) and acting as a cofactor in the Factor I (FI)-mediated breakdown of surface-bound C4b to iC4b and further to C4c and C4d. Furthermore, two additional members of the MASP protein family (MAp44 and MAp19) (Takahashi et al. 1999,

Degn et al. 2009) may participate in the regulation of the LP by inhibiting the formation of the MBL-MASP co-complexes and thus limiting the transactivation of MASPs (MAp44) (Garred et al. 2016) or via competition with MASP-2 in MBL binding (MAp19) (Iwaki et al. 2006), however the physiological relevance of the latter mechanism is challenged by the reportedly low affinity of MAp19 to MBL (Degn et al. 2011).

Regulation of the assembled C3 convertases (C4bC2a and C3bBb) determines the amplification capacity of complement. Deactivation of C4b and C3b (either tissue-bound or as part of the activated C3 convertases) is regulated via two processes. Decay acceleration aids the dissociation of C2a or Bb from the preformed complexes, whereas surface-attached C3b and C4b are inactivated through FI-mediated cleavage that is substantiated by cofactors (Liszewski et al. 1991). Complement regulator proteins encoded in the regulators of complement activation (RCA) gene cluster may inhibit complement activation through both decay accelerating and cofactor activities. These proteins are encoded on chromosome 1 at q3.2 and consist of amino acid repeating motifs termed short consensus repeats (SCR) (Reid et al. 1986, Mayilyan 2012). The RCA family consists of both fluid phase (C4BP, Factor H (FH)) and host-cell expressed regulators (membrane cofactor protein (MCP), decay accelerating factor (DAF), complement receptor 1 (CR1), complement receptor 2 (CR2)) that are capable of the binding of C3b and C4b (Liszewski et al. 1991).

Effector functions of the terminal pathway are kept under control by soluble as well as membrane-associated complement regulators. The former ones include clusterin that hinders the assembly of the MAC through the prevention of C9 binding to C5b-8 (Jenne et al. 1989, Tschopp et al. 1994) and vitronectin, which inhibits the membrane attachment of the C5b-7 complex, thus creating a soluble form of MAC (Podack et al. 1978, Bajic et al. 2015), while the membrane expressed analogue of clusterin, CD59 blocks the binding of C9 to C5b-8 and thus inhibits the polymerization of the C5b-9 complex (Meri et al. 1990).

Cytokines of the complement system are inactivated by carboxypeptidases that remove the C-terminal arginine residue of C3a and C5a, thus creating C3a-desArg and C5adesArg (Bokisch et al. 1970). Whereas C3a-desArg loses its signaling potential through the C3aR, C5adesArg maintains approximately 10% of the C5a activity.

Therefore, control of C5a activity is also sustained by the decoy receptor C5aR2 that aids the removal of C5a from the circulation, and as recently suggested, may induce the retrieval of helper T-cells by the inhibition of the NACHT, LRR and PYD domains-containing protein 3 (NLRP3) inflammasome (Arbore et al. 2016).

### **2.1.3 Regulation of the alternative pathway**

Restriction of unwanted complement activation on intact host tissues requires tight regulation of the AP, given its significance in the amplification of complement activity. The rate limiting step of AP activation is the Factor D-mediated cleavage of FB, which is inevitable for the activation of the preformed AP C3 pro-convertase (C3bB or C3(H<sub>2</sub>O)B, respectively) (Harrison 2018). Since Factor D is present mainly in its active form in the human blood, provided by the physiological activation of its zymogen (pro-Factor D) by MASP-3 (Yamauchi et al. 1994, Iwaki et al. 2011, Oroszlan et al. 2016) or additional serine proteases upon activation of the coagulation and complement cascades, (Yamauchi et al. 1994), pro-convertase activation via Factor D is limited by the accessibility of its substrate, FB. However, the CP regulator C1-IHN may limit the AP convertase assembly via interfering with the binding of FB to C3b, too (Jiang et al. 2001).

Nevertheless, once the AP C3 convertase is activated, its strict regulation is a key component in the restraint of complement amplification. Deactivation of the C3bBb complex occurs via both direct cleavage of C3b and decay acceleration of the active complex. During the initiation of the AP, cleavage of C3 exposes a reactive thioester domain necessary for the covalent attachment of C3b to nearby surfaces and also exposes the molecular regions involved in FB binding. However, repositioning of the thioester domain simultaneously generates an extended surface on C3b that allows for the binding of complement regulators. Thus, attachment of complement regulator proteins to C3b may interfere with FB binding and accelerate the decay of the AP C3 convertase (Alcorlo et al. 2015). Furthermore, it allows the attached regulator to exert its cofactor activity in the FI-mediated degradation of C3b (Alcorlo et al. 2015) (Delvaeye et al. 2009, Kavanagh et al. 2013, Baines et al. 2017). Soluble or membrane-bound cofactors of FI that participate in the regulation of the AP C3 convertase include FH, Factor H-like protein 1 (FHL-1), MCP, CR1, CR2 and THBD. FH and FHL-1

(discussed in detail below) are plasma proteins that may simultaneously bind C3b and host surface elements, and exert both cofactor as well as decay accelerating activities. MCP is a regulator ubiquitously expressed on all nucleus-bearing cells of the human body (Andrews et al. 1985). It is a glycosylphosphatidylinositol (GPI)-anchored protein that may bind both C3b and C4b. Complement receptors CR1 and CR2 are immune adherence receptors expressed mainly on erythrocytes and leukocytes. Whereas both proteins exert cofactor activities via binding of C3b and C4b or C3dg, respectively (Liszewski et al. 1991), CR1 also accelerates the decay of the active C3 and C5 convertases (Baines et al. 2017). THBD, the major inhibitor of thrombin generation on endothelial cells, has been shown to enhance C3b cleavage via both FH-dependent and independent mechanisms (Heurich et al. 2016, Tateishi et al. 2016). Additionally, DAF inhibits the downstream assembly of both C3 and C5 convertases on the cellular-surface through its binding to surface-attached C3b and C4b (Medof et al. 1984) and substantiates the disassembly of the C3 convertases (Liszewski et al. 1991). Altogether, cleavage of C3b and acceleration of the decay of the preformed AP C3 convertases abrogates complement amplification and reduces the downstream activation of the C5 convertase and the terminal pathway of complement.

Beside the restraint of complex assembly, AP regulation is substantiated by the removal of the activator structures through the enhancement of phagocytosis. Following multiple cleavages of C3b, the emergence of more and more potent C3 degradation products (iC3b, C3c, C3dg) exposes binding sites for complement receptors (CR1-4 and CR1g) of immune effector cells, and stimulates macrophage phagocytic activity together with the initiation of adaptive immune responses (van Lookeren Campagne et al. 2007, Bajic et al. 2015).

#### **2.1.4. Significance of Factor H in the regulation of the alternative pathway**

Albeit regulation of the complement AP takes place through the joint interplay of complement regulator proteins, the most potent and abundant regulator of the AP is FH, a 155 kDa glycoprotein (Bajic et al. 2015, Sanchez-Corral et al. 2018), the systemic level of which ranges between 250-880 mg/L (Sanchez-Corral et al. 2018). FH is built up from 20 SCR domains that share a various degree of homology to one another and to SCR domains of other members of the FH protein family (Ripoche et al. 1988, Jozsi et

al. 2014). The N-terminal four SCR domains are responsible for the regulatory activities of FH (Gordon et al. 1995), whereas the C-terminal SCRs 19-20 and the middle SCRs 6-8 are in charge of ligand binding by the regulator (Schmidt et al. 2008, Kopp et al. 2012). These structural characteristics allow for the simultaneous binding of multiple ligands, while maintaining the AP regulatory activity both in the fluid and solid phases. FH regulates the AP via multiple mechanisms and targets complement activity through ligand-based discrimination of complement activator surfaces from intact host tissues (Meri 2016).

FH may compete with FB in C3b binding, thus inhibiting the assembly of C3 as well as C5 convertases, both in the plasma and on cellular membranes. Furthermore, FH accelerates the decay of the preformed convertases by acting as a cofactor in the FI-mediated degradation of C3b, while it may also anchor to the cell-bound degradation products C3c and C3d (Sanchez-Corral et al. 2018).

FH has specific ligands on intact host tissues and dead or damaged cells targeted for elimination (Sanchez-Corral et al. 2018). Binding of glycosaminoglycans (GAGs), sialic acid and heparin anchors functionally active FH to the extracellular matrix or directly to the cell membrane of intact host tissues, thus preventing further complement activation on non-activator surfaces. In contrast, ligand binding on apoptotic and necrotic tissues including direct attachment to e.g. Annexin-II, deoxyribonucleic acid, histones and malondialdehyde epitopes or recruitment to damaged cells by ligand bound pentraxins (Bottazzi et al. 2010, Leffler et al. 2010, Weismann et al. 2011, Daigo et al. 2016) compensates for the downregulation of membrane-expressed complement regulators and aids the silent elimination of these targets via opsonophagocytosis (Trouw et al. 2007).

Divergence and overlaps in the epitopes of the distinct FH ligands play an important role in the context specific recruitment and correct positioning of FH, thereby localizing the complement inhibitory activity. For instance binding of C3b and its degradation products through both SCR domains 1-4 and 19-20 (Schmidt et al. 2008) provides structural basis for the recognition of C3b on host tissues via the simultaneous binding of host-specific GAGs and sialic acids (Morgan et al. 2011), and also permits the binding of additional C3b molecules attached to the same surface (Schmidt et al. 2008). Binding of fluid phase PRMs such as pentraxins likewise leaves room for C3b binding,

since the former involves both SCR domains 6-8 and 19-20 (Jarva et al. 1999, Deban et al. 2008, Kopp et al. 2012). In the meantime, the regulator maintains its functional activity exerted through the N-terminal four SCRs, both in the fluid phase and when attached to cellular surfaces (Sanchez-Corral et al. 2018).

These structural and functional characteristics of FH significantly contribute to a targeted but at the same time restricted complement activation and an optimal degree of phagocytosis avoiding the unnecessary amplification of the complement cascade on intact host tissues during homeostatic clearance of cellular debris and also in the context of an infection. It is noteworthy however, that recruitment of functionally active FH is a well-evolved escape mechanism of tumor cells and pathogens from complement mediated killing. The FH binding site of such pathogens is also located on SRC domains 6-7 and 19-20, binding of which may provide molecular mimicry and also interfere with the physiological, host-cell associated functions of FH (Lambris et al. 2008, Jozsi 2017). Since FH is the most important regulator of the continuously active AP, any dysfunction of the regulator or siege of its ligand binding sites may destroy the above described peculiar mechanisms of host-cell defense and lead to the dysregulation of the complement AP with destructive consequences (Kopp et al. 2012).

In summary, the activation status of the AP and therefore that of the whole complement cascade is determined by the combined effect of activator and regulator mechanisms. Following the local initiation of the distinct complement pathways, the fate of the generated C3b depends on the strictly regulated balance of amplification and regulator factors (Harrison 2018), the dysfunction of which may lead to the dysregulation of the AP and the manifestation of complement-mediated diseases (Baines et al. 2017).

### **2.1.5. Role of pentraxins in complement regulation**

Pentraxin-3 (PTX3) and C-reactive protein (CRP) are fluid PRMs that participate in the innate immune response via the recognition and binding of pathogenic moieties, opsonization of target cells and activation as well as regulation of the complement system (Figure 1) (Daigo et al. 2016). Members of the pentraxin family include short and long pentraxins that share a conserved pentraxin-like domain on their C-terminal ends (Daigo et al. 2016). The primary sequence of the long pentraxin PTX3 is also highly conserved among different animal species, and encodes a 381 amino acid long

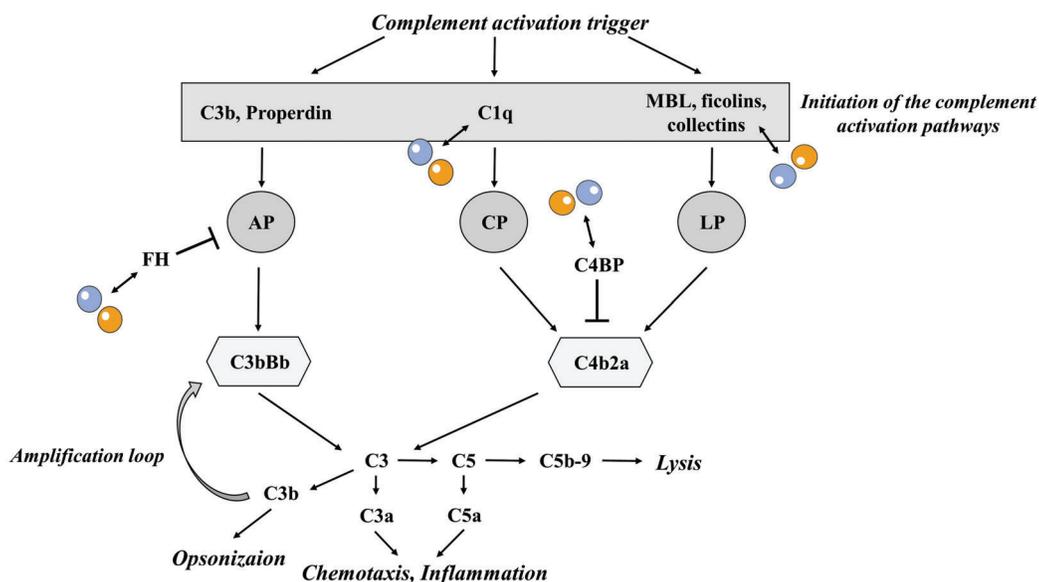
protein with a unique N-terminal domain (Inforzato et al. 2013), not related to any known protein structure (Daigo et al. 2016). PTX3 is generated in response to inflammatory stimuli via gene expression induction in innate immune cells, endothelial cells and fibroblasts (Kunes et al. 2012). However, prompt release of PTX3 originates from neutrophil granulocytes (Kunes et al. 2012), where constitutively produced, glycosylated PTX3 monomers are stored in lactoferrin<sup>+</sup> intracellular granules (Daigo et al. 2016). Upon neutrophil cell activation and neutrophil extracellular trap (NET) release, PTX3 is secreted from neutrophils and its monomers form octamers through inter-chain disulfide bonds (Daigo et al. 2016).

CRP on the other hand is a member of the short pentraxin protein family (Daigo et al. 2016). Its native, pentameric form is produced and stored in the endoplasmic reticulum of resting hepatocytes (Sproston et al. 2018). Upon inflammatory stimuli, CRP is secreted into the circulation and following the recognition of target cell membranes, its attachment to phosphocholine induces the disassembly of the pentameric structure to CRP monomers in a calcium-dependent fashion (Sproston et al. 2018). CRP is a routinely used biomarker of systemic inflammation in clinical setting, whereas PTX3 was reported to be a prognostic marker of disease activity and severity in an increasing number of diseases (Maekawa et al. 2011, Lech et al. 2013, Speeckaert et al. 2013, Sjoberg et al. 2014, Krzanowski et al. 2017).

Both molecules have been shown to interact with the complement system on multiple levels. PTX3 may initiate the activation of the CP and LP via binding to surface-associated MBL (Ma et al. 2011), ficolins, collectins (Ma et al. 2009) and C1q (Nauta et al. 2003). MBL-bound PTX3 may even recruit C1q molecules to the activator surface thus providing a link between the activation of the LP and CP, whereas the interaction of PTX3 with C1q in the fluid phase inhibits CP activity. Hence, PTX3 restricts unwanted complement activation in the circulation (Nauta et al. 2003, Kunes et al. 2012). Furthermore, ligand binding of PTX3 may prevent AP amplification via the recruitment of the complement regulators FH (Deban et al. 2008) and C4BP (Braunschweig et al. 2011) to the surface of apoptotic cells, thus facilitating phagocytosis of pathogens and clearance of cellular debris without the unnecessary activation of the terminal pathway (Inforzato et al. 2013). CRP also has the ability to activate the complement system. The native form of CRP however, may only attach to

membrane-bound C1q (Biro et al. 2007) with concomitant restraint of the terminal pathway (Thiele et al. 2014). Nevertheless, localized dissociation of pentameric CRP, induced by binding to cell-membrane phosphatidylcholine, allows for the excessive activation of the CP both *in vitro* and *in vivo* (Biro et al. 2007, Thiele et al. 2014). Parallel to complement activation, CRP may also regulate the CP and AP via recruitment of C4BP, FH, but also properdin (Sjoberg et al. 2006, Biro et al. 2007, O'Flynn et al. 2016).

Even though there are differences in their production and specific activities, the expression of both pentraxins is induced in inflammatory conditions. Ligand-bound pentraxins were shown to initiate as well as regulate all three pathways of complement *in vitro*, while their ligand binding provides basis for cross-talk between the individual pathways (Garred et al. 2016). However, *in vivo* studies reported inconclusive data on the overall impact of PTX3 on tissue injury and recovery (Souza et al. 2002, Salio et al. 2008, Souza et al. 2009, Lech et al. 2013) and no study has been designed so far to explore changes in the systemic level of pentraxins in relation to complement overactivation *in vivo*.



**Figure 1. Interaction of pentraxins with the complement system**

A sketch of the complement activation pathways and the interaction of pentraxin-3 (yellow sphere) and C-reactive protein (blue sphere) with complement activator and regulator proteins is displayed based on the publication of Daigo et al. 2016 with modifications. (AP= alternative pathway, CP= classical pathway, LP= lectin pathway, MBL= mannose-binding lectin)

### **2.1.6. Dysregulation of the complement alternative pathway**

The proper control of AP activation requires a delicate regulatory mechanism, impairment of which can easily lead to complement dysregulation and disease pathogenesis. Dysregulation of the AP may originate from either the drop-out of restrain mechanisms, or from the hyperfunction of complement activators. Reduction of complement regulatory mechanisms may be caused by the dysfunction of the complement regulator proteins or may be attributed to factor consumption secondary to overactivation of the complement system.

Primary mechanisms that lead to the dysregulation of the complement AP include impaired protease activity of FI with or without cofactor dysfunction, reduction of decay mechanisms or increased stability and half-life of the AP C3 convertase. These impairments may be attributed to either genetic mutations in the complement genes causing a quantitative or functional deficiency (or excessive activity) of the expressed protein, or complement factor blockade and complex stabilization by autoantibodies, all of which may induce the manifestation of complement-mediated diseases (Baines et al. 2017). In recent years, deficiencies in complement regulatory processes associated to alterations of the complement genes or autoimmune mechanisms have been described in an increasing number of conditions (Wong et al. 2018).

Dysfunction of FI leads to reduced C3b and C4b cleavage and thus prolongs the inactivation of both the AP and CP C3 convertases, leading to the dysregulation of the AP and CP in the fluid as well as in the solid phases. Whereas genetic mutations in the *CFI* gene have been linked to disease pathogenesis in atypical hemolytic uremic syndrome (aHUS), autoantibodies against FI were only demonstrated to have a minor contribution to the dysregulation of fluid and solid phase complement regulation, suggesting that their detection is rather an epiphenomenon than a pathogenic factor of disease manifestation (Kavanagh et al. 2012, Kavanagh et al. 2013).

Mutations may also impair the cofactor activity of multiple complement regulator proteins, thus playing a major role in the pathogenesis of complement-mediated diseases. Pathogenic mutations identified in *CFH* (the most frequently detected genetic alteration in aHUS patients) (Noris et al. 2010, Fremeaux-Bacchi et al. 2013) are usually heterozygous point mutations that cluster at the C-terminal end of the protein. Hence, they interfere with the membrane-associated regulatory mechanisms and host-

surface discrimination of FH without a quantitative deficiency in the circulatory levels of the protein (Kavanagh et al. 2013). Functional assays have shown that expression of the mutant protein causes a varying degree of impairment in the recognition and binding of heparin and C3b on e.g. endothelial cells and platelets, with subsequent cellular damage and the activation of thrombocytes (Vaziri-Sani et al. 2006, Abarategui-Garrido et al. 2008, Stahl et al. 2008, Ferreira et al. 2009). Mutations located at the N-terminal regulatory regions of FH, even though are detected less frequently, cause a deficiency of AP regulation both in the fluid and solid phases (Pechtl et al. 2011). In addition to pathogenic point mutations, the regulatory functions of FH may also be altered due to genomic rearrangements in the RCA gene cluster that may yield hybrid genes of *CFH* and *CFHRs*, the genes encoding its related proteins (Factor H-related proteins 1-5, FHRs, respectively). Albeit the exact role of FHRs in complement dysregulation remains to date uncertain, mutations in *CFHR5* have been associated with disease manifestation in multiple independent cohorts of aHUS and C3 glomerulopathy (C3G) patients (Monteferrante et al. 2007, Maga et al. 2010, Westra et al. 2012, Wong et al. 2018). Autoantibodies against FH have also been described in 4-14% of aHUS patients and 11% of C3G patients, and functional characterization of these antibodies has proven their causative role AP dysregulation and disease pathogenesis, discussed in detail below (Goodship et al. 2012, Zhang et al. 2012, Kavanagh et al. 2013)

Similarly to *CFI*, mutations affecting *CD46*, the gene encoding MCP contribute to the dysregulation of both the AP and CP, since under physiological conditions MCP may engage both C3b and C4b. Unlike most of the *CFH* mutations however, about 75% of the alterations found in *CD46* cause a reduced expression of the protein, thus contributing to altered regulation of complement (Kavanagh et al. 2013). In the presence of mutations in the *THBD* gene, THBD also loses its cofactor activity in the FI-mediated cleavage of C3b, which has been linked to the manifestation of aHUS (Wong et al. 2018).

Mutations the *C3* and *CFB* genes on the other hand belong to the activating mutations of complement. These mutations were reported to either cause altered binding of regulators (e.g. MCP) or an enhanced formation of the AP C3 convertase that is more resistant to decay due to a stronger bond between FB and C3b (Kavanagh et al. 2013). This leads to increased complement deposition on cellular surfaces and the disruption of

the balance between amplification and regulation (Harrison 2018). However, mutations of *C3* have also been described to cause reduced secretion of the protein, the role of which in AP dysregulation and disease pathogenesis remains to date unrevealed (Kavanagh et al. 2013). Increased convertase stability and activity have also been associated with the presence of anti-C3b and anti-FB antibodies (Strobel et al. 2010, Chen et al. 2011, Marinozzi et al. 2017) as well as the production of a C3bBb stabilizing IgG, the C3 Nephritic Factor (C3Nef) (Daha et al. 1976, Daha et al. 1977).

In addition to germline genetic mutations, enhanced AP C3 convertase activity and MAC formation may also be attributed to somatic mutations in the *phosphatidylinositol glycan anchor biosynthesis class A (PIGA)* gene. The *PIGA* gene encodes a glycosyl transferase, alterations of which cause clonal deficiency of GPI-anchored proteins including DAF and CD59. Their dysfunction causes dysregulation of complement activity on the surface of red blood cells, clinically manifesting in paroxysmal nocturnal hemoglobinuria, characterized by chronic hemolysis with acute paroxysms and an increased risk of thrombus formation (Baines et al. 2017).

Despite adequate functional evidence on the contribution of the above genetic alterations and autoimmune mechanisms to complement dysregulation, fundamental questions regarding the clinical manifestation of complement mediated diseases remain to date unrevealed. Multiple facts e.g. incomplete penetrance of the genetic alterations, presence of autoantibodies in disease remission and disease manifestation in adulthood suggest that an additional, yet undiscovered trigger event is required to set off the self-amplifying mechanism of AP dysregulation. Furthermore, despite the accumulated knowledge on the structure-function relationships of genetic alterations, to date we still have no definitive explanation for the association of certain mutations to one clinical condition rather than another (Pechtl et al. 2011). Therefore further molecular as well as clinical investigations are required to challenge these controversies and to fill the gaps in our ever increasing knowledge on the pathogenesis of complement mediated diseases.

## **2.2. Thrombotic microangiopathies**

### **2.2.1. Classification and pathogenesis of thrombotic microangiopathies**

Thrombotic microangiopathies (TMA) are rare but life-threatening disorders characterized by microangiopathic hemolytic anemia and acute thrombocytopenia with or without organ impairment (e.g. signs of neurological or kidney injury). The bed-side diagnosis of TMAs is based on this triad of clinical symptoms and routine laboratory results, however, intensive research of the recent decades revealed that TMAs cover distinct subgroups of diseases. Their etiology-based classification discriminates between acquired or inherited primary TMAs with known molecular etiology, secondary TMAs as complication of underlying diseases or conditions, and specific infection-associated TMAs. TMAs are severe conditions that require immediate attention and diagnostic workup for complement abnormalities. Their expeditious differential diagnostics is especially important, since the identification of the exact subgroup of TMA has an impact on the determination of the optimal therapeutic choice immediately from the onset of the acute phase.

### **2.2.2. Thrombotic thrombocytopenic purpura**

Thrombotic thrombocytopenic purpura (TTP), covers a TMA subgroups, where beyond the clinical and laboratory triad of TMA, patients usually present with critical thrombocytopenia (as low as 4-5G/L) and neurological symptoms, whereas signs of kidney injury are less frequent clinical features of disease. This form of TMA is caused by the deficiency of a disintegrin and metalloproteinase with a thrombospondin type 1 motif member 13 (ADAMTS13), either due to its blockade by antibodies or mutations in the *ADAMTS13* gene. Under physiological conditions this enzyme cleaves the ultra-large form of von Willebrand Factor (ULVWF), which is secreted by activated endothelial cells, and can spontaneously bind and activate platelets, thus contributing to thrombus formation at the site of activation. Although ADAMTS13 deficiency is accountable for TTP, it may also be present in disease remission, which supports the hypothesis that the onset of TTP requires a trigger event (such as pregnancy or infections), which leads to endothelial activation and therefore an enhanced expression of ULVWF, hence causing the imbalance of pro- and anti-thrombotic factors in the microvasculature.

### **2.2.3. Typical hemolytic uremic syndrome**

Hemolytic uremic syndrome (HUS) on the other hand covers distinct forms of primary TMAs, which usually present with the triad of hemolytic anemia, thrombocytopenia and acute renal failure, while neurologic symptoms have only been reported in a minority of patients. In about 90% of the cases, HUS is caused by Shiga toxin-producing *Escherichia coli* (STEC), defined as typical or STEC-HUS (Wong et al. 2018) that represents the most frequent cause of acute kidney failure in children. It is induced by a gastrointestinal infection, where Shiga-toxin (Stx) is released to the circulation from mucus-adhered bacteria and causes microangiopathy through the blockade of protein synthesis and subsequent activation of the endothelial cells, red blood cells and platelets (Karpman et al. 2006, Orth-Holler et al. 2014, Arvidsson et al. 2015, Jokiranta 2017).

### **2.2.4. Atypical hemolytic uremic syndrome**

Atypical HUS on the other hand represents a rare form of TMA (Fakhouri et al. 2017) with an incidence rate of approximately 0.23-0.42 per 10<sup>6</sup> per year (Fakhouri et al. 2017). Atypical HUS is mediated by the dysregulation of the AP, evoked either by pathogenic mutations in the complement genes or by the presence of autoantibodies directed against the complement regulator FH (discussed in detail below). Mutation associated forms of aHUS usually manifest in either very early childhood, often in infants younger than 1 year of age, or in women at the postpartum period, whereas autoimmune aHUS is a disease of adolescence. Atypical HUS has a relapsing-remitting disease course and therefore requires long-term patient management. Just like in the case of STEC-HUS, prodromal symptoms of aHUS may include gastrointestinal complaints and signs that could be misleading at the initial presentation. However, since the endothelial injury in aHUS is caused by the dysregulation of the AP, detection of laboratory signs of complement AP consumption may aid the differential diagnostics of aHUS together with the lack of proof for an STEC infection and clinical improvement of patients. Definite diagnosis of aHUS however, requires the functional and genetic testing of the patients' complement profile that reveals the underlying molecular mechanism of disease pathogenesis in nearly two third of the cases. Likely pathogenic mutations are identified by 50-60% of aHUS patients, with a combination of mutations identified in 10-25% of the cases (Kavanagh et al. 2013). Approximately 25% of the

pathogenic mutations are found in the *CFH* gene (Noris et al. 2010, Fremeaux-Bacchi et al. 2013), whereas in addition to mutations in the complement genes, genetic alterations of *THBD* and *diacylglycerol kinase epsilon (DGKE)* have been associated with aHUS, too (Delvaeye et al. 2009, Lemaire et al. 2013, Azukaitis et al. 2017). Along likely pathogenic mutations that have established functional consequences, haplotypes and a multitude of variations with an unknown significance have been linked to an increased risk of aHUS development. Even though these have not been shown to directly contribute to disease pathogenesis, in combination with other trigger mechanisms they might induce the manifestation of disease.

Unlike the genetically determined forms, autoimmune aHUS usually manifests in older children and adolescents following a preceding minor infection of the respiratory or gastrointestinal tracts (Dragon-Durey et al. 2010). It accounts for 4-14% of all aHUS cases (Skerka et al. 2009, Hofer et al. 2013, Kavanagh et al. 2013, Nester et al. 2015), although an Indian cohort reported an incidence rate of 56% with an unexplained reason behind the high frequency of autoantibody positivity (Sinha et al. 2014). Autoimmune aHUS has a high relapse rate with the risk of developing end stage renal disease (Noris et al. 2009, Dragon-Durey et al. 2010, Dragon-Durey et al. 2010, Noris et al. 2015). Laboratory diagnostics of autoimmune aHUS involve the detection of anti-Factor H (anti-FH) antibodies that often goes together with low levels of FH due to immune complex formation (Blanc et al. 2012), the levels of which were shown to correlate with disease severity (Blanc et al. 2012). Further characteristic laboratory findings in autoimmune aHUS are low C3 and FB levels indicative of the overactivation and subsequent factor consumption of the AP (Blanc et al. 2012), frequently accompanied by a decreased activity or deficiency in the whole complement AP activity. The genetic linkage of autoimmune aHUS to deficiency of *CFHR1* justifies testing for copy number variations in the *CFHR* genes via multiplex ligation-dependent probe amplification (MLPA) analysis. However, a negative finding in this regard does not exclude the presence of autoimmune aHUS, since the lack of *CFHR1* is not a prerequisite for the development of autoantibodies, indicated by a considerable proportion of autoimmune aHUS patients with no genetic alterations in the *CFHR* genes (Dragon-Durey et al. 2009, Moore et al. 2010, Strobel et al. 2011, Geerdink et al. 2012).

### **2.2.5. Secondary thrombotic microangiopathies**

Secondary forms of TMA encompass a heterogeneous group of disorders all of which are evoked by the progression of a preexisting condition. Secondary TMA may be associated among others with glomerular diseases, severe infections and septic conditions, allogenic hematopoietic stem cell or solid organ transplantation, systemic autoimmune diseases, pregnancy, tumor progression and antitumor therapy as well as malignant hypertension. Despite their distinct etiologies, shared features of secondary TMAs include decreased ADAMTS13 activity and overactivation of multiple complement pathways, leading to laboratory signs of complement consumption.

### **2.2.6. Pathological complement activation in thrombotic microangiopathies**

Endothelial damage and subsequent microvascular thrombosis are key pathogenic factors in TMA. Albeit traditionally aHUS was recognized as a TMA form mediated by complement dysregulation, by now vascular injury has been associated with complement overactivation in all etiological forms of this disease (Table 1) (Noris et al. 2012, Reti et al. 2012, Baines et al. 2017, Farkas et al. 2017).

A shared characteristic of the coagulation and complement cascades is their initial activation at local sites of infection or tissue injury (Baines et al. 2017). There is a considerable interaction between the activator mechanisms of both cascades (Oikonomopoulou et al. 2012, Kenawy et al. 2015, Foley 2016), provided by the diverse ligand binding capacity of certain PRMs and activation enzymes of complement (Bossi et al. 2011, Takahashi et al. 2011, La Bonte et al. 2012), augmented by complement activation through thrombin and additional coagulation factors (Huber-Lang et al. 2006, Amara et al. 2010, Krisinger et al. 2012, Berends et al. 2014). Components of each system may cleave and activate one another (Baines et al. 2017), thus providing molecular basis for the joint manifestation of pathological thrombosis and complement overactivation in distinct etiological forms of TMA (Noris et al. 2012, Reti et al. 2012, Baines et al. 2017, Farkas et al. 2017).

Atypical HUS is a prototypic disease of AP dysregulation attributed to the above discussed genetic alterations of complement components and autoimmune mechanisms (Wong et al. 2018). STEC-HUS on the other hand is primarily not a complement-mediated disorder. Nevertheless, increased levels of complement degradation products

(C3a(desArg) and C3d), Bb and the properdin-stabilized AP C3 convertase (C3bBbP) together with the elevation of sC5b-9 have been detected in the circulation of STEC-HUS patients (Thurman et al. 2009, Stahl et al. 2011, Westra et al. 2017), indicative of complement overactivation in the acute phase of disease. Intensified complement activity in STEC-HUS is supposedly due to the combined effect of Stx and bacterial lipopolysaccharide (LPS) on complement activation and coagulation (Stahl et al. 2011, Arvidsson et al. 2015), a hypothesis corroborated by independent *in vitro* and *in vivo* observations. Inter alia, Stx was shown to activate the AP in the fluid phase and to interfere with the regulatory activity of FH on cellular surfaces (Orth et al. 2009), together with the upregulation of P-selectin on microvascular endothelium, thus promoting C3b deposition and thrombus formation (Morigi et al. 2011). Stx together with LPS was also reported to induce the formation of platelet-leukocyte aggregates *in vitro* (Stahl et al. 2011, Arvidsson et al. 2015), whereas the combined thrombotic effects of Stx and LPS could be diminished by the admission of a C3a receptor antagonist or by the application of FB-deficient mice in animal models of STEC-HUS *in vivo* (Morigi et al. 2011).

Increased complement activation product levels together with an elevated cytokine response have also been reported in TTP (Reti et al. 2012, Westwood et al. 2014). Increased complement activity has been detected at both the acute presentation and in remission of TTP, the extent of which was reported to be associated with the level of anti-ADAMTS13 antibodies in the autoimmune form of this disease (Reti et al. 2012, Westwood et al. 2014). The underlying mechanism of the sustained complement response in TTP could be attributed to multiple mechanisms participating in disease pathogenesis. On the one hand, increased expression of P-selectin on activated platelets and endothelial cells may lead to an enhanced C3b deposition and thus substantiate complement activation on the tagged endothelial surface (Del Conde et al. 2005). On the other hand, ULVWF may also initiate the AP and the terminal pathway of complement via direct binding of complement factors (Bettoni et al. 2017). Enhanced generation of the complement activation products C3a, C5a, and sC5b-9 may in turn lead to further activation of endothelial cells with inherently increased ULVWF and P-selectin expression as well as decreased THBD expression, ultimately causing the

uncontrolled activation of coagulation and the dysregulation of complement (Bettoni et al. 2017).

Despite the many different etiologies of secondary TMA, overactivation and subsequent consumption of both the CP and AP have been described in this condition, too (Farkas et al. 2017). Elevated systemic levels of sC5b-9 and C3a have been associated with poor long-term outcome in independent cohorts of secondary TMA patients (Jodele et al. 2013, Farkas et al. 2017, Wong et al. 2018). Although dysregulation of the AP is not a distinguished mediator of disease pathogenesis in secondary TMA, its involvement has been recently suggested in the development of hematopoietic stem cell transplantation-associated TMA, that could be attributed to chemotherapy, radiation, and infection-induced endothelial injury and neutrophil cell activation (Jodele et al. 2013, Yuen et al. 2016, Gloude et al. 2017, Wong et al. 2018).

In conclusion, the acute phase of TMAs are accompanied by complement dysregulation or consumption, and an overdrawn inflammatory response. This may induce among others the production of inflammatory proteins, such as CRP and PTX3. Based on the reported complement regulatory activity of pentraxins, the released proteins could interfere with local complement activity at the site of vascular injury (Jaillon et al. 2007). However, so far only *in vitro* studies have been published in this regard, whereas no investigation involving human subjects has explored the systemic levels of pentraxins in the acute phase of TMA or the *in vivo* relation of PTX3 and CRP to complement activity.

### **Table 1. Role of complement in thrombotic microangiopathies**

Distinct forms of thrombotic microangiopathies (TMA) are displayed, grouped based on the most important, common pathogenic factors and the role of complement in each subgroup of disease. (ADAMTS13 = a disintegrin and metalloproteinase with a thrombospondin type 1 motif member 13, *ADAMTS13* = the gene encoding ADAMTS13, aHUS = atypical hemolytic uremic syndrome, AP = alternative pathway, CP = classical pathway, DGKE = diacylglycerol kinase epsilon, IgM = Immunoglobulin M, MMACHC-TMA = thrombotic microangiopathy associated to methylmalonic aciduria and homocystinuria, cblC complementation type, *MMACHC* = the gene encoding methylmalonic aciduria and homocystinuria type C protein, STEC-HUS =

Shiga toxin-producing *Escherichia coli*-associated hemolytic uremic syndrome, Stx = Shiga-toxin, TTP = thrombotic thrombocytopenic purpura)

TMA subgroup	Key pathogenic factors	Role of complement in the pathogenesis
<b>Inherited primary TMA</b>		
Complement-mediated aHUS	-Endothelial damage due to severe dysregulation of the complement AP	-Genetic alteration in the complement genes and subsequently altered expression or function of their encoded proteins
DGKE-aHUS	-Prothrombotic state on the endothelium due to the loss of DGKE function	-Enhanced terminal pathway activation and therefore increased sC5b-9 levels that may alter podocyte metabolic pathways, the structure and function of the extracellular matrix, membrane lipids and key proteins of the cytoskeleton and the slit-diaphragm
MMACHC-TMA	-Impaired intracellular vitamin B12 metabolism due to mutations in the <i>MMACHC</i> gene	-Not known
Inherited TTP (Upshaw-Schulman Syndrome)	-Congenital deficiency of the ADATMS13 metalloproteinase due to mutations in the <i>ADATMS13</i> gene	-C3b binding and complement activation through activated endothelial cells and platelets -Direct activation of the complement AP by the ULVWF
<b>Acquired primary TMA</b>		
Autoimmune aHUS	-Endothelial damage due to severe dysregulation of the complement AP	-Dysfunction of the complement regulator Factor H due to autoantibodies
Acquired TTP	-Inhibitory antibodies that block the ADATMS13 metalloproteinase	-C3b binding and complement activation through activated endothelial cells and platelets -Direct activation of the complement AP by the ULVWF
<b>Infection-associated TMA</b>		
STEC-HUS	-Endothelial damage caused by the binding and internalization of Stx	-Complement activation through Stx and LPS -Increased C3b binding to the endothelium due to high P-selectin expression induced by Stx
<i>Streptococcus pneumoniae</i> /Influenza induced HUS	-Neuraminidase production and cleavage of N-acetylneuraminic acid from glycoproteins on the cell membrane of erythrocytes, platelets, glomeruli and hepatocytes, exposure of the Thomsen–Friedenreich antigen	-Interaction of the Thomsen–Friedenreich antigen with preformed IgM initiates excessive complement activation of both the CP and AP
<b>Secondary forms of TMA</b>		
Secondary TMA	-Worsening of a known preexisting condition and subsequent coagulopathy with tissue or organ damage involving the endothelium	-Dysregulation of both the CP and AP with severe consumption of the individual complement factors

### **2.2.7. Alternative pathway dysregulation associated with anti-Factor H antibodies in atypical hemolytic uremic syndrome**

Anti-FH antibodies in aHUS have first been reported in a French patient cohort in 2005 (Dragon-Durey et al. 2005), and since then multiple independent investigations have shown their functional relevance in disease pathogenesis. Upon binding to FH, autoantibodies interfere with the complement regulatory activity on the surface of host cells that leads to overactivation of the complement AP and subsequent complement-mediated tissue damage. The majority of the anti-FH antibodies belongs to the IgG subclass, although some patients have been reported to develop IgA antibodies, too (Strobel et al. 2011). Albeit the antibodies may recognize multiple domains on FH (Moore et al. 2010, Blanc et al. 2012), epitope mapping using recombinant FH fragments have shown that most of the antibody binding epitopes cluster on the C-terminal SCRs of FH (Jozsi et al. 2007, Jozsi et al. 2008, Moore et al. 2010, Bhattacharjee et al. 2015). The antibody binding site was reported to overlap with that of C3b and cellular ligands, which explains why anti-FH antibodies inhibit the binding of C3b and its degradation products thus reducing the cofactor and decay accelerating activities of FH. Furthermore, antibody binding perturbs the FH-mediated cell-protection, shown by increased hemolysis of red blood cells in the presence of patient IgG (Jozsi et al. 2007, Blanc et al. 2012). Anti-FH antibodies have also been described in C3G, however their functional characteristics were reported to be substantially different from those identified in aHUS patients (Blanc et al. 2015). Whereas the functional consequence of the antibody binding to FH is widely studied and comprehensive knowledge has been accumulated in regard to the structural exploration of antibody binding to the folded FH domains, detailed structural analysis of where the aHUS-associated FH epitopes are localized at the amino acid level are still lacking. Furthermore, the immediate trigger that would evoke autoantibody production against FH has not yet been identified. Nevertheless, since a mild infection is often recorded prior to the manifestation of aHUS, the higher prevalence of gastrointestinal pathogens (Togarsimalemath et al. 2018) together with the genetic linkage of the disease (e.g. deficiency of FHR-1) have been proposed to contribute to the pathological activation of B lymphocytes and trigger the production of anti-FH antibodies (Bhattacharjee et al. 2015).

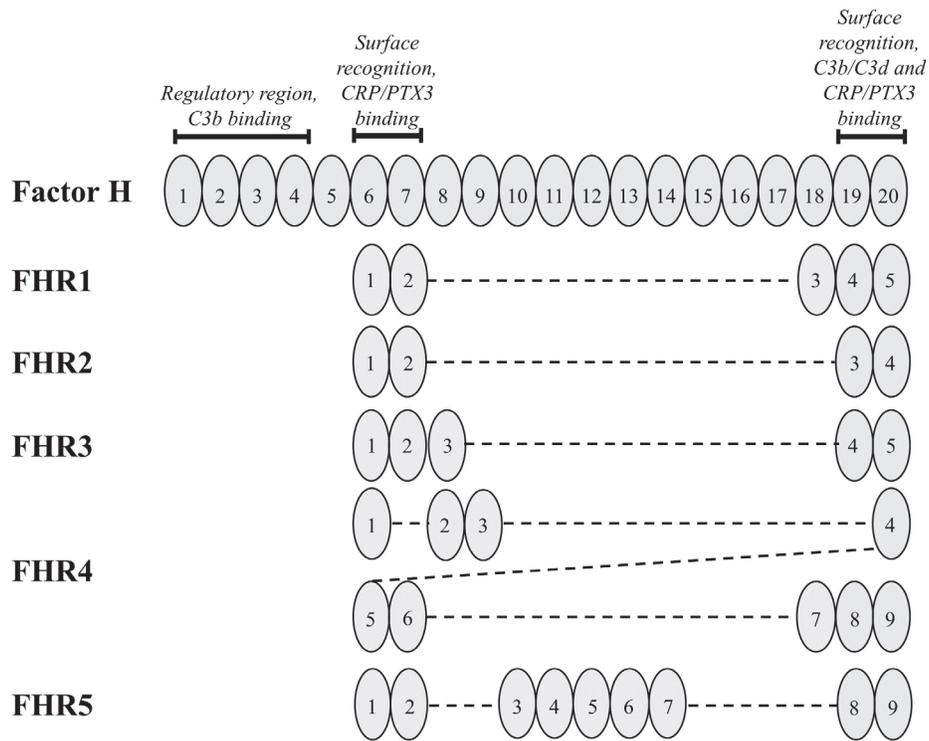
### **2.2.8. Factor H-related proteins and their genetic-linkage to anti-Factor H antibodies**

Alongside with FH, there are six additional members of the FH protein family: FHL-1 and the FHRs 1-5 (Figure 2). FHL-1 is a splice variant derived from the *CFH* gene, whereas FHRs evolved from *FH* via non-allelic homologous recombination (Jozsi et al. 2014). As a result of this, all members of the FH protein family are composed of SCR domains that share a high sequence homology to each other. Corresponding to their structural similarities, FHL-1 and FHRs were reported to participate in the regulation or sometimes de-regulation of complement activity through multiple mechanisms. FHL-1 carries a unique four amino acid long C-terminus, a so called SFTL tail corresponding to its components: serine, phenylalanine, threonine and leucine (Perez-Caballero et al. 2000), but the rest of the protein is identical to the first 7 SCR domains of FH (Schwaeble et al. 1987). Therefore, FHL-1 shares functional similarities to FH including complement inhibition through C3b binding (Kuhn et al. 1995) and the recognition of common ligands such as pentraxins and malondialdehyde epitopes (Sanchez-Corral et al. 2018). However, apparent contrasts in the conformation of the two proteins and the presence of the C-terminal tail on FHL-1 contribute to considerable differences in the ligand interactions of the two proteins (Sanchez-Corral et al. 2018). FHRs on the other hand do not carry all the four N-terminal regulatory domains of FH, therefore they have no or only residual cofactor and decay accelerating activities in the regulation of the complement AP. However, their reported binding of C3b and other deposited fragments of C3 (iC3b and C3d) (Goicoechea de Jorge et al. 2013) via their C-terminal domains may induce competition with FH for the C3 binding site, a process referred to as complement de-regulation (Sanchez-Corral et al. 2018). Additional ligand binding similarities include the recognition of host surface ligands and ECM components (Blackmore et al. 1998, Hellwage et al. 1999, McRae et al. 2005, Heinen et al. 2009, Strobel et al. 2011, Csincsi et al. 2015, Rudnick et al. 2018) as well as recruitment of FHRs to apoptotic and necrotic cells via surface-bound PRMs (e.g. pentraxins), where in contrast to FH some FHRs have been reported to enhance the activation of not only the alternative but also the classical pathways of complement (Mihlan et al. 2009, Hebecker et al. 2010, Hebecker et al. 2012, Csincsi et al. 2015, Csincsi et al. 2017, Rudnick et al. 2018). However, given the relatively low

concentration of FHRs and their lower avidity for C3b binding compared to that of FH (Sanchez-Corral et al. 2018), the complement activator effect of FHRs may be insignificant in the physiological regulation of the AP.

Under pathological AP activation however, locally increased levels and activity of FHRs may contribute to disease pathogenesis. Numerous reports have described the association of genetic alterations in *CFHRs* to complement-mediated diseases (Hughes et al. 2006, Gharavi et al. 2011, Sanchez-Corral et al. 2018). The autoimmune form of aHUS is linked to the deficiency of *CFHR1* and -3 that covers the homozygous deletion of an 80 kb-long genomic region in 82-88% of patients with FH autoantibodies (Zipfel et al. 2007, Jozsi et al. 2008, Dragon-Durey et al. 2010, Geerdink et al. 2012, Hofer et al. 2013). Conversely, the frequency of the heterozygous deletion of *CFHR1* is similar in healthy individuals and aHUS patients.

FHR1 shares a high sequence homology with FH. The C-terminal SCRs of the two proteins differ in only two amino acids, namely FH carries a serine at amino acid position 1191 and a valine at position 1197, while FHR1 contains a leucine and an alanine at the corresponding positions (residue 290 and 296), whereas FHR1 domain 4 and FH domain 19 are identical to one another (Abarategui-Garrido et al. 2009). FHR1 was shown not only to compete with FH in C3b binding (Goicoechea de Jorge et al. 2013), but also to cross react with anti-FH antibodies and to neutralize the antibody-associated enhanced red blood cell lysis *in vitro* (Strobel et al. 2011, Bhattacharjee et al. 2015). These functional studies together with the high sequence homology of the C-terminal domains of FH and FHR1 suggest an overlap of the antibody binding sites on the two proteins. Genetic and structural analyses have pointed out *CFHR1* deficiency as a potential predisposing factor for the development of autoimmune aHUS (Kavanagh et al. 2013, Bhattacharjee et al. 2015) and anti-FH binding of the folded FH and FHR1 C-terminal domains have recently been compared (Bhattacharjee et al. 2015), however no investigation aimed to date for the localization of the antibody binding epitopes on FH as well as FHR1 on the amino acid level, which however, could provide a direct explanation for the reported interference of autoantibody binding with FH function.



**Figure 2. Homology between Factor H and the Factor H related proteins**

Illustration of Factor H (FH) and its related proteins (FHRs) based on the publication of Sanchez-Corral et al. 2018 with modifications. The proteins are displayed with the sketch of their short consensus repeat (SCR) domains indicated by numbers. SCR domains of the FHRs are aligned vertically to their homologous domains in FH. Ligand binding sites and functional characteristics of FH are indicated above the schematic pictures of the FH domains. (C3b = complement component 3b, C3d = complement component 3d, CRP = C-reactive protein, FHR1-5 = Factor H-related proteins 1-5, PTX3 = pentraxin-3)

### **3. SPECIFIC AIMS**

#### **3.1. Analysis of the association between systemic pentraxin levels and laboratory signs of disease activity in thrombotic microangiopathies**

Albeit both PTX3 and CRP have been described to regulate complement activation *in vitro* (Csincsi et al. 2015), no study has been designed so far to explore changes in the systemic level of pentraxins in complement mediated diseases, such as TMAs, *in vivo*. Therefore we performed a case control study to determine the systemic levels of PTX3 and CRP in patients at the acute phase as well as remission of TMA. We analyzed the relationship between the systemic level of pentraxins and TMA etiology, the clinical outcome of patients and classical laboratory markers of TMA.

#### **3.2. Investigation of the relation of complement overactivation and systemic pentraxin levels in distinct forms of thrombotic microangiopathies**

In distinct etiological groups of TMAs including aHUS, STEC-HUS, secondary TMA and TTP we further investigated the relation of systemic pentraxin levels to laboratory signs of complement overactivation and consumption in the acute phase of disease. We determined the patients' complement factor levels, the whole complement CP and AP activities and the systemic levels of complement activation products to reveal any potential association between complement overactivation and the systemic levels of PTX3 and CRP in the acute phase and remission of TMA, as well as in the distinct subgroups of disease. Furthermore we determined the effect of excess PTX3 on the AP activity *in vitro* to explore the role of PTX3 release in the pathogenesis of TMA.

#### **3.3. Epitope analysis of anti-Factor H antibodies in atypical hemolytic uremic syndrome**

Antibody binding to FH induces loss of host cell recognition and dysregulation of the complement AP (Kavanagh et al. 2013). The functional consequence of the FH-antibody interaction has been widely studied, however little is known about the structural basis of FH-blockade and the role of FHR proteins in disease pathogenesis. Based on recent observations (Bhattacharjee et al. 2015, Nozal et al. 2016) we hypothesized that the antibody binding site is located at the C-terminal domains of FH,

therefore we aimed for the structural characterization of antibody binding to FH SCR domains 19-20. To localize the aHUS-associated FH epitopes on the amino acid level, we performed fine epitope mapping of the complement regulator using point-mutated FH domains and linear epitope mapping with overlapping synthetic peptides. We further investigated whether aHUS specific linear epitopes are present on FHR1, based on its homology to FH and its described cross-reactivity with the anti-FH antibodies (Strobel et al. 2011, Bhattacharjee et al. 2015).

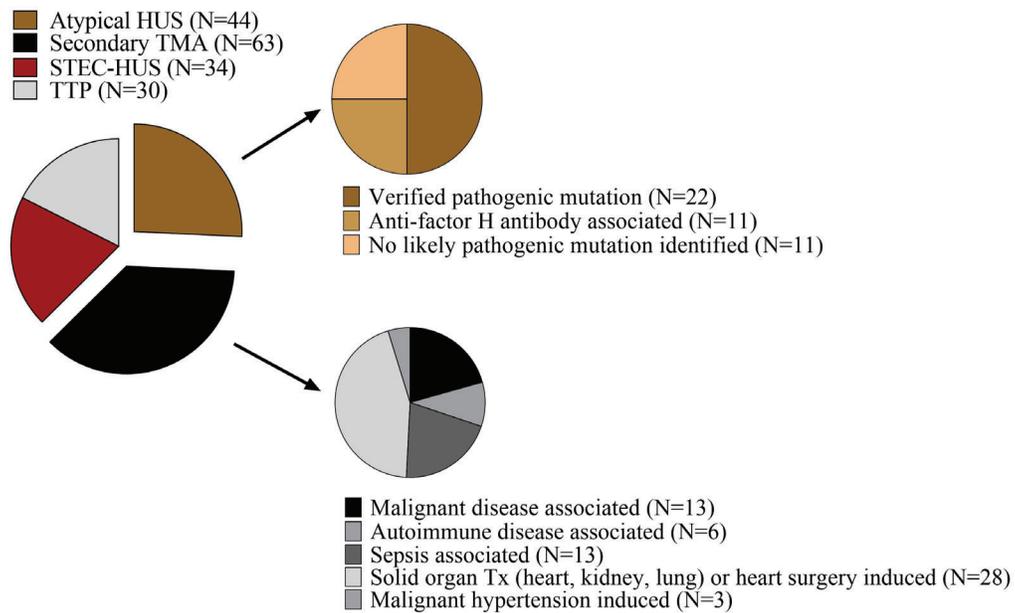
## 4. METHODS

### 4.1. Patient selection, sample collection and study design

#### 4.1.1. Sample collection from patients at the acute phase of thrombotic microangiopathy

171 TMA patients with acute disease flare were enrolled in the case-control study for the analysis of systemic pentraxin levels and complement consumption, whose blood samples were sent to our laboratory for differential diagnostic evaluation between November 2007 and October 2017. Acute phase serum and plasma samples from all enrolled subjects were collected prior to the start of plasma exchange therapy, however in some cases (N=16) fresh frozen plasma (FFP) had been administered to the patients prior to sampling. Diagnosis of TMA was established based on laboratory signs of microangiopathic hemolytic anemia, thrombocytopenia (<150 G/L) and clinical or laboratory signs of organ damage. Patients were included in the study only if all of the above criteria were met. For stratification of TMA patients by disease etiology the following groups were formed (Figure 3): (1) STEC-HUS (N=34): acute gastroenteritis and signs of acute kidney injury with proof of Shiga like-toxin producing *Escherichia coli* infection, (2) TTP (N=30): ADAMTS13 deficiency (activity below 10%) with the presence of ADAMTS13 inhibitors, (3) aHUS (N=44): HUS with presence of anti-FH autoantibodies, or HUS with identified pathogenic or likely pathogenic variations in the complement genes (*CFH*, *CFHR5*, *CFI*, *CD46*, *C3*, *CFB*), *THBD* or *DGKE*, or HUS cases without identified likely pathogenic rare variations in these genes, (4) secondary TMA (N=63): evidence of coexisting disease including malignancy, autoimmune disease, sepsis, solid organ transplantation, open heart surgery or malignant hypertension. Exclusion criteria were ongoing plasma exchange or complement inhibitory therapy at the time of sample collection (during the first acute flare), or the lack of available blood sample. Genetic analysis was performed by direct DNA sequencing of polymerase chain reaction products amplified from total genomic DNA. Pathogenic and likely pathogenic mutations were defined based on published literature data and included mutations either described previously in aHUS patients or expected to cause aHUS based on functional analysis reported in literature. Data on the clinical course, blood count and chemistry were collected from the medical charts. Acute kidney

injury was defined as documented oligo- or anuria or creatinine and carbamide levels above the upper limit of the laboratory normal range. Patients were followed-up after hospital discharge and outcome including mortality was registered. Follow-up samples were available for aHUS (N=31, 15-month median time interval between sampling) and TTP (N=19, 8-month median time interval between sampling) patients in remission. Whole blood samples were immediately separated after collection (to yield serum, EDTA-anticoagulated plasma, and sodium-citrate-anticoagulated plasma) and stored in aliquots at  $-70^{\circ}\text{C}$  until further analysis. For appropriate comparison age- and sex-matched healthy individuals were selected, none of whom showed clinical or laboratory signs of TMA or an acute phase reaction that could have influenced the measured laboratory parameters. This study was carried out in conformity with the Helsinki Declaration. Written informed consent was obtained from all participants, and the study was approved by the Ethics Committee on Human Clinical Research in Budapest (8361-1/2011-EKU).

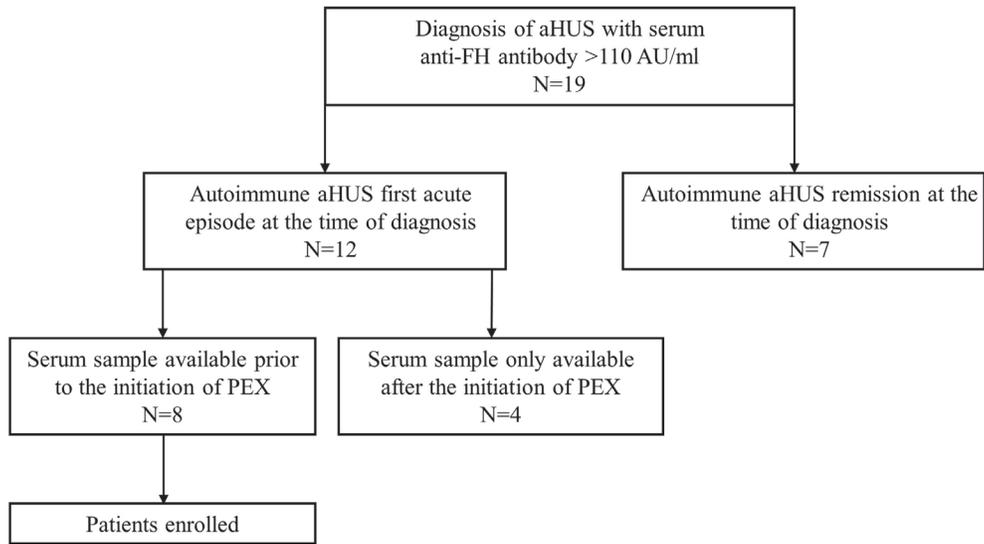


**Figure 3. Stratification of TMA patients based on disease etiology**

Confirmed disease etiology of the TMA patients enrolled in the case-control study for the analysis of the systemic pentraxin levels in the acute phase of TMA. Number of participants (N) is shown as proportion of a whole. (HUS=hemolytic uremic syndrome, STEC-HUS=Shiga-like toxin-associated hemolytic uremic syndrome, TMA=thrombotic microangiopathy, TTP=thrombotic thrombocytopenic purpura, Tx=transplantation)

#### **4.1.2. Sample collection from autoimmune atypical hemolytic uremic syndrome patients**

For linear epitope mapping sera of children in the acute phase of aHUS were used, from whom a sample was obtained prior to the start of plasmapheresis. Inclusion criteria were as follows: diagnosis of HUS based on the presence of microangiopathic hemolytic anemia, thrombocytopenia (<150G/L) and renal injury, with an anti-FH antibody level over 110 AU/mL (Dragon-Durey et al. 2010). Exclusion criteria included aHUS in remission or active therapy with either plasmapheresis, corticosteroids, cyclophosphamide or rituximab as well as the lack of available serum sample. Altogether eight patients were enrolled in this investigation, seven of which had an available remission phase serum sample (Figure 4). Remission phase sample collection was performed 6-12 months after the termination of any specific treatment of the subjects. Patient enrollment was closed in September 2016. Control sera samples were obtained from leftover serum specimens from children (median age of 9 years) admitted to the 1st Department of Pediatrics at Semmelweis University upon distinct indications, by whom a detailed laboratory analysis (including inflammatory markers) did not reveal any pathological findings. All control children were negative (below cutoff) for anti-FH antibodies. This study was carried out in accordance with the Helsinki Declaration. The study was approved by the Ethics Committee on Human Research in Budapest (8361-1/2011-EKU), and written informed consent was obtained from each subject.



**Figure 4. Patient selection flow chart for the epitope analysis studies**

Nineteen patients with the diagnosis of atypical HUS (aHUS) who had a positive serum anti-Factor H antibody test result (>110 AU/ml) were identified in our laboratory during the study period. Patients with the first acute phase of aHUS, who had available sera samples taken prior to the initiation of plasma exchange therapy were included in our investigation. (aHUS = atypical hemolytic uremic syndrome, anti-FH antibody = anti-Factor H antibody, PEX = plasma exchange therapy)

#### **4.2. Determination of laboratory parameters in patients at the acute phase of thrombotic microangiopathy**

Complement activity-, component-, regulator-, and activation product determinations, CRP and PTX3 measurements were performed in our laboratory from the blood samples of acute phase-TMA patients. The AP activity was determined with the commercially available WIESLAB Alternative pathway ELISA kit (EuroDiagnostica, Malmö, Sweden) (Seelen et al. 2005), whereas total complement CP activity was assessed using the sheep-erythrocyte hemolytic titration test. CRP, C3 and C4 levels were measured by turbidimetry (Beckman Coulter, Brea, CA), while FB and FI were determined by radial immunodiffusion assay. The level of the complement regulators C1q and FH as well as the titer of the anti-FH antibody were measured using in house ELISA techniques, described in detail in our earlier publications or elsewhere (Delamarche et al. 1988, Reti et al. 2012). ADAMTS13 activity was evaluated by the application of the fluorogenic substrate FRET-S-VWF73 (Reti et al. 2012). Commercially available kits were used to assess the levels of the complement activation products sC5b-9 and C3a (C3a-desArg) (Quidel, San Diego, CA, USA) and for the measurement of PTX3 (R&D systems Minneapolis, MN, USA).

#### **4.3. *In vitro* assessment of pentraxin-3 effect on alternative pathway activation**

*In vitro* effect of recombinant human PTX3 on AP activation was assessed using two assays. The AP hemolytic activity in the presence of excess PTX3 was determined with a modified C3Nef hemolytic assay, and the AP activity on LPS-coated ELISA plates was measured by the WIESLAB Alternative pathway ELISA kit (EuroDiagnostica, Malmö, Sweden). The C3Nef assay was performed on washed sheep erythrocytes, where patients' samples were replaced by normal human serum (NHS) spiked with recombinant human PTX3 (R&D systems Minneapolis, MN, USA) in gradually decreasing concentrations. After a 20 minute incubation of PTX3 with NHS, the solution was added to sheep erythrocytes. The formation of the C3 convertase was allowed within a 10 minute incubation time at 30°C, and assembly of the terminal pathway MAC was achieved by the addition of undiluted rat serum to the cells and incubation at 37°C for 60 minutes. The extent of hemolysis was detected by reading the OD at 412 nm. The effect of PTX3 on the assembly of C5b-9 on a plastic surface was

assessed with the WIESLAB Alternative pathway ELISA. As in the hemolytic assay, patients' sera were replaced by PTX3 spiked NHS, otherwise the assay was performed according to the manufacturer's instructions. To allow comparison of data, the AP activities in each experiment were expressed as ratio of the reference (optical density of NHS with buffer control) in percentage.

#### **4.4. Determination of the anti-Factor H antibody levels**

Serum anti-FH IgG level was determined by an enzyme-linked immunosorbent assays (ELISA) as described previously (Dragon-Durey et al. 2005), with the following modifications applied: Nunc MaxiSorp plates (Nunc, Roskilde, Denmark) were coated with 1 µg/ml purified human FH (Calbiochem, San Diego, CA, USA) overnight. Following blocking with 1% bovine serum albumin in phosphate-buffered saline (PBS), serum samples were added at a dilution of 1:200. As secondary antibody a rabbit anti-human IgG-HRP was used (Dako, Glostrup, Denmark) and detection of bound IgG was performed by 3,3',5,5'-tetramethylbenzidine (TMB). Optical density (OD) was read at 450 nm (reference at 620 nm). Calibration of the assay was completed with a sample obtained as a kind gift from Dr. Dragon-Durey, and the cutoff value (>110 AU/ml) was determined according to the mean + 2 times the standard deviation of the values of 80 healthy individuals.

#### **4.5. Peptide synthesis for epitope mapping**

Fifteen amino acid-long peptides of FH SCR domains 19–20 and the FHR1 region homolog to the amino acid sequence 1177–1211 on FH (amino acids 276–310 of FHR1) were synthesized by Dr. Katalin Uray at Eötvös Loránd University Budapest, according to Geysen's method (Geysen et al. 1984) with modification applied as described previously (Uray et al. 2003). Peptides overlapping by five amino acid residues were synthesized in duplicates on β-alanyl-glycine functionalized polyethylene pins (Mimotopes NCP gears (Clayton, VIC, Australia)) with Fmoc/tBu chemistry. We used <sup>t</sup>Bu (Thr, Ser, Tyr), <sup>O</sup>Bu (Asp, Glu), Acm (Cys), Pmc (Arg) and Boc (His, Lys) as side-chain-protecting groups. The Nα-Fmoc α-amino-protecting group was removed with 2% piperidine/ 2% 1,8-diazabicyclo(5.4.0)undec-7-ene in DMF, and the protected amino acid residues were coupled with N,N'-diisopropylcarbodiimide/1-hydroxybenzotriazol. The side chain protecting groups were the following: aspartate,

glutamate: tert-butyl ether; threonine, serine, tyrosine: tert-butyl ether; arginine: 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Pbf); histidine, asparagine, glutamine: triphenylmethyl; cysteine: acetamidomethyl. The peptides were acetylated at the N-terminus and the protecting groups (with the exception of acetamidomethyl) were removed by a solution of trifluoroacetic acid–water–phenol–thioanisole–1,2 ethanedithiol 90:5:7,5:5:2,5 (V/V/m/V/V%), while the unprotected peptides remained covalently attached to the pins. The only remainder protecting group, acetamidomethyl on cysteine was masking the thiol-group of cysteine, which forms a disulfide bond in the folded protein. A negative control peptide (heat shock protein 60 (HSP60\_480-489: 480AKNAGVEGSL<sub>489</sub>)) was synthesized for standardized comparison of antibody binding. For verification of peptide synthesis three peptides were subjected to amino acid analysis, and their precise amino acid composition was verified. The amino acid sequence of each synthetic peptide, as well as further details of the peptide synthesis are listed in Table 2 A-B.

**Table 2A. Amino acid sequence of the synthesized peptides of Factor H domain 19**

Amino acid sequence of the synthesized peptides on Factor H short consensus repeat domain 19. Numbers indicate the position of the initial and last amino acid of each synthesized peptide on Factor H, capitals refer to the single letter amino acid codes.

FH DOMAIN 19	
1107GKCGPPPIDNGDIT <sub>1121</sub>	1137QCQONLYQLEGNKRIT <sub>1151</sub>
1112PPPIDNGDITSFPLS <sub>1126</sub>	1142YQLEGNKRITCRNGQ <sub>1156</sub>
1117NGDITSFPLSVYAPA <sub>1131</sub>	1147NKRITCRNGQWSEPP <sub>1161</sub>
1122SFPLSVYAPASSVEY <sub>1136</sub>	1152CRNGQWSEPPKCLHP <sub>1166</sub>
1127VYAPASSVEYQCQNL <sub>1141</sub>	1157WSEPPKCLHPCVISR <sub>1171</sub>
1132SSVEYQCQONLYQLEG <sub>1146</sub>	1162KCLHPCVISREIMEN <sub>1176</sub>

**Table 2B. Amino acid sequence of the synthesized peptides of Factor H domain 20 and Factor H-related protein 1**

Amino acid sequence of the synthesized peptides on Factor H short consensus repeat domain 20 and part of its homolog region on Factor H-related protein 1. Numbers indicate the position of the initial and last amino acid of each synthesized peptide on the

respective protein, capitals refer to the single letter amino acid codes. The two amino acid difference (serine→leucine (S→L); valine→alanine (V→A) between FH and FHR1 is highlighted in bold.

FH DOMAIN 20	FHR1
1167CVISREIMENYNIAL <sub>1181</sub>	
1172EIMENYNIALRWTAK <sub>1186</sub>	
1177YNIALRWTAKQKLYS <sub>1191</sub>	276YNIALRWTAKQKLYL <sub>290</sub>
1182RWTAKQKLYSRTGES <sub>1196</sub>	281RWTAKQKLYLRTGES <sub>295</sub>
1184TAKQKLYSRTGESVE <sub>1198</sub>	283TAKQKLYLRTGESAE <sub>297</sub>
1187QKLYSRTGESVEFVC <sub>1201</sub>	286QKLYLRTGESAEFVC <sub>300</sub>
1192RTGESVEFVCKRGYR <sub>1206</sub>	291RTGESAEFVCKRGYR <sub>305</sub>
1197VEFVCKRGYRLSSRS <sub>1211</sub>	296AEFVCKRGYRLSSRS <sub>310</sub>
1202KRGYRLSSRSHLRT <sub>1216</sub>	
1207LSSRSHLRTTCWDG <sub>1221</sub>	
1212HTLRTTCWDGKLEYP <sub>1226</sub>	
1217TCWDGKLEYP <sub>1231</sub>	

#### 4.6. Antibody binding to the immobilized synthetic peptides of Factor H and Factor H-related protein 1

Serum antibody binding to the overlapping synthetic peptides was determined by a modified ELISA, as previously described (Fust et al. 2012). In the current experiments, after blocking of the non-specific binding sites with 0.5% gelatin in PBS, pins were incubated with 150µL of 1:1000 diluted sera in 0.5% gelatin and 0.05% Tween 20 in PBS for 1h at room temperature. Binding of anti-FH antibodies to the immobilized peptides was determined using a rabbit anti-human IgG peroxidase-labeled antibody (Dako, Agilent Technologies, Santa Clara, CA, United States) in a dilution of 1:5000 and the TMB detection system with OD read at 450 nm (reference at 620 nm). Pins were used repeatedly after thorough washing by sonication in disruption buffer (1% sodium dodecyl sulfate and 0.1% 2-mercaptoethanol in PBS). As a negative control peptide, the HSP60 fragment from amino acids 480–489 was applied, based on our earlier observations, where this peptide showed the lowest binding with human sera

(Fust et al. 2012). Data were normalized by the following formula:  $OD_{\text{sample}}/OD_{\text{min}}$ , where  $OD_{\text{sample}}$  equaled the mean of duplicate OD values of the test samples and  $OD_{\text{min}}$  the mean binding to the negative control HSP60 peptide. This ratio represents the fold change of antibody binding over the background to each peptide. Epitope specific autoantibody binding in the acute phase and in remission serum samples are both presented according to the above formula.

#### **4.7. Synthesis and antibody binding of recombinant Factor H 19-20 mutants**

Determination of serum antibody binding to the recombinant FH domains 19–20 displaying various single amino acid changes was performed with an ELISA-based method by Dr. Mihály Józsi and his colleagues at Eötvös Loránd University Budapest, as previously described (Bhattacharjee et al. 2015, Nozal et al. 2016). Cloning, expression, and purification of FH 19–20 wild type and mutant protein fragments was performed according to well-established, published protocols of mutagenesis, expression and purification (Jokiranta et al. 2006, Lehtinen et al. 2009, Bhattacharjee et al. 2010) using the QuikChange® Multi Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) and the *Pichia*-expression system followed by heparin affinity chromatography (HiTrap Heparin, Amersham Bioscience). Antibody binding to each protein fragment was determined by a microtiter plate assay, where Nunc MaxiSorp plates (Thermo Fisher Scientific, Waltham, MA USA) were coated by the protein fragments in a concentration of 5µg/mL and patients' sera was applied according to the antibody titer (1:50–1:200). Antibody binding was visualized by the application of peroxidase-conjugated anti-human IgG and the TMB detection system. For standardized comparison of data, the relative binding to the FH mutant peptides compared to wild type FH 19-20 is expressed in percentage, where binding to the wild type peptide is 100%.

#### **4.8. Visualization of the linear epitopes and point mutations on the folded Factor H structure**

We analyzed the localization of the discovered epitopes on the crystal structure of FH domains 19–20 [DOI:10.2210/pdb2g7i/pdb (Jokiranta et al. 2006)] using the SWISS-PDB Viewer software [(Guex et al. 1997), <http://www.expasy.org/spdbv/>].

#### **4.9. Statistical analysis**

Data analysis in all our investigations was performed using the GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla, CA, USA, [www.graphpad.com](http://www.graphpad.com)). Since continuous variables of the above studies failed the Shapiro-Wilk's normality test, non-parametric tests were carried out for group comparisons with two-tailed p values calculated, and the significance level set at 0.05. For descriptive purposes, continuous variables are displayed as median with interquartile range, while categorical variables are indicated with numbers (expressed in percentage). The respective statistical method, applied for comparison of datasets is indicated in each figure legend. In addition to the correlation analysis, multivariate statistics was carried out for the evaluation of the association of pentraxin levels with laboratory markers and clinical status of acute phase-TMA patients. Multiple linear regression analysis (on log-transformed variables) was used to explore the relationship between covariates of PTX3 and CRP levels, whereas logistic regression analysis with one-by-one adjustment for covariates was used to test dichotomized pentraxin levels as predictors of mortality.

## **5. RESULTS**

### **5.1. Analysis of the association between systemic pentraxin levels and laboratory signs of disease activity in thrombotic microangiopathies**

#### **5.1.1. Characteristics of patients with acute phase thrombotic microangiopathy selected for the determination of the systemic pentraxin levels**

The pentraxin superfamily consist of the long pentraxin PTX3 and the short pentraxins CRP and serum amyloid P, all of which are pattern recognition molecules that share a conserved pentraxin domain on their C-terminal ends (Daigo et al. 2016). Both PTX3 and CRP have been reported to interact with activators and regulators of the complement system *in vitro* (Inforzato et al. 2013, Daigo et al. 2016), however their role in pathological complement activation *in vivo* remains to date unexplained. To explore the association of circulatory pentraxin levels with complement consumption in TMA, a disease often accompanied by overactivation of the complement cascade, we collected blood samples from 171 TMA patients in the acute disease flare and measured the systemic CRP and PTX3 levels together with complement activity, factor levels and complement activation products. We analyzed our findings in regard to the clinical outcome and basic laboratory characteristics of the patients and selected 69 age- and sex-matched healthy individuals for comparison, none of whom showed clinical or laboratory signs of TMA or an acute inflammatory reaction that could have modified the pentraxin levels. Examined clinical and laboratory characteristics of the patients and the controls are summarized in Table 3. All enrolled TMA patients presented with hemolysis and thrombocytopenia (<150 G/L) at the acute disease flare regardless of disease etiology, with the lowest median platelet count (i.e. 16 G/L) in the TTP subgroup. ADAMTS13 activity was decreased or deficient in 79% of the patients, and by definition it was deficient (i.e. activity below 10%) in all of the TTP patients. Organ involvement was present in the form of kidney damage by most of the TMA patients, although some of the TTP patients presented with neurological symptoms as a sign of central nervous system involvement with no laboratory signs of impaired kidney function. Classical laboratory parameters indicative of ongoing TMA in each of the study groups are summarized in Table 4. Our TMA patient cohort included patients with the following etiologies: STEC-HUS (N=34), aHUS (N=44), secondary TMA

(N=63) and TTP (N=30) (Figure 3), with over 90% of the admitted patients presenting at the first acute episode of the disease. Blood samples were collected from all subjects enrolled prior to the start of plasma exchange or complement inhibitory therapy, although 16 patients received FFP before the time of sampling. However, until the time point of blood sample collection administration of FFP did not result in an improvement of the clinical status or the classical laboratory signs of TMA in any of the enlisted patients.

**Table 3. Characteristics of TMA patients and healthy controls**

Group characteristics and laboratory data of the enrolled patients and healthy controls. Categorical variables are expressed in percent and continuous variables are shown as medians and with interquartile range, whereas results of the statistical comparison are indicated with the respective p value of the Mann-Whitney test. (ADAMTS13= a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13, CRP= C-reactive protein, FH= Factor H, NA= not applicable, PTX3= pentraxin-3, sC5b-9= soluble C5b-9, TMA= thrombotic microangiopathy).

Characteristics analyzed	TMA	Healthy controls	Result of statistical comparison
Number of individuals enrolled	171	69	NA
Age	35.2 (7.7-56.9)	33.0 (18.7-41.0)	P=0.608
Sex (male/female in %)	43/57	48/52	NA
First acute episode (%)	93.6	NA	NA
31-day mortality (%)	11.7	0	NA
Complement C3 < 0.9 g/L (%)	49.7	0	NA
Complement FH < 250 mg/L (%)	25.7	0	NA
<b>Laboratory parameters indicative of ongoing TMA</b>			
Red blood cell count (10 <sup>9</sup> /L)	2.9 (2.6-3.4)	4.9 (4.6-5.2)	p<0.001
Hemoglobin (g/L)	85 (75-97)	141 (134-152)	p<0.001
Platelet count (10 <sup>9</sup> /L)	46 (22-75)	262 (235-309)	p<0.001
Lactate dehydrogenase (U/L)	1819 (893-3051)	Not done	NA
Creatinine (μmol/L)	188 (86-320)	71 (64-78)	p<0.001
Carbamide (mmol/L)	16.9 (10.9-25.9)	4.5 (3.8-5.6)	p<0.001
<b>Pentraxin levels and white blood cell profile</b>			
PTX3 level (μg/L)	5.19 (2.08-13.17)	1.08 (0.75-1.66)	p<0.001

CRP level (mg/L)	16.9 (4.3-72.0)	1.4 (0.8-2.0)	p<0.001
White blood cell count (G/L)	10.4 (7.1-15.3)	6.5 (5.4-7.9)	p<0.001
Absolute neutrophil count (G/L)	7.1 (4.8-12.4)	4.0 (3.0-4.7)	p<0.001
Absolute lymphocyte count (G/L)	1.4 (0.8-2.6)	2.0 (1.8-2.4)	p<0.001
<b>Complement parameters</b>			
ADAMTS13 activity (%) (Reference range: 67-147%)	38 (17-54)	Not done	NA
Classical pathway activity (CH50/ml)	57 (45-71)	70 (62-77)	p<0.001
Alternative pathway activity (%)	86 (56-101)	101 (78-117)	p<0.001
C3 level (g/L)	0.90 (0.68-1.15)	1.26 (1.18-1.47)	p<0.001
C4 level (g/L)	0.23 (0.14-0.32)	0.34 (0.27-0.40)	p<0.001
Factor H level (mg/L)	390 (245-513)	560 (462-692)	p<0.001
Factor I level (%)	98 (81-123)	102 (92-108)	p=0.192
Factor B level (%)	98 (73-116)	101 (91-113)	p=0.791
C1q level (mg/L)	87 (56-112)	100 (80-124)	p=0.020
sC5b-9 level (ng/mL) (Reference range: 110-252 ng/mL)	352 (265-517)	Not done	NA
C3a level (ng/mL) (Reference range: 70-270 ng/mL)	171 (120-259)	Not done	NA

**Table 4. Characteristic laboratory parameters of patients in the distinct TMA subgroups**

Laboratory parameters indicative of disease activity are shown in each TMA group. Data are expressed as median with interquartile range. Please note that blood count and chemistry data were not accessible by all the enrolled patients, therefore the analysis might include less than the total number of patients (N=171) in this study. (BUN= carbamide, Crea= creatinine, Hb= hemoglobin, aHUS= atypical hemolytic uremic syndrome, LDH= lactate dehydrogenase, PLT= platelet count, RBC= red blood cell count, STEC-HUS= Shiga-like toxin associated HUS, TMA=thrombotic microangiopathy, TTP= thrombotic thrombocytopenic purpura, WBC= White blood cell count, ANC= Absolute neutrophil count)

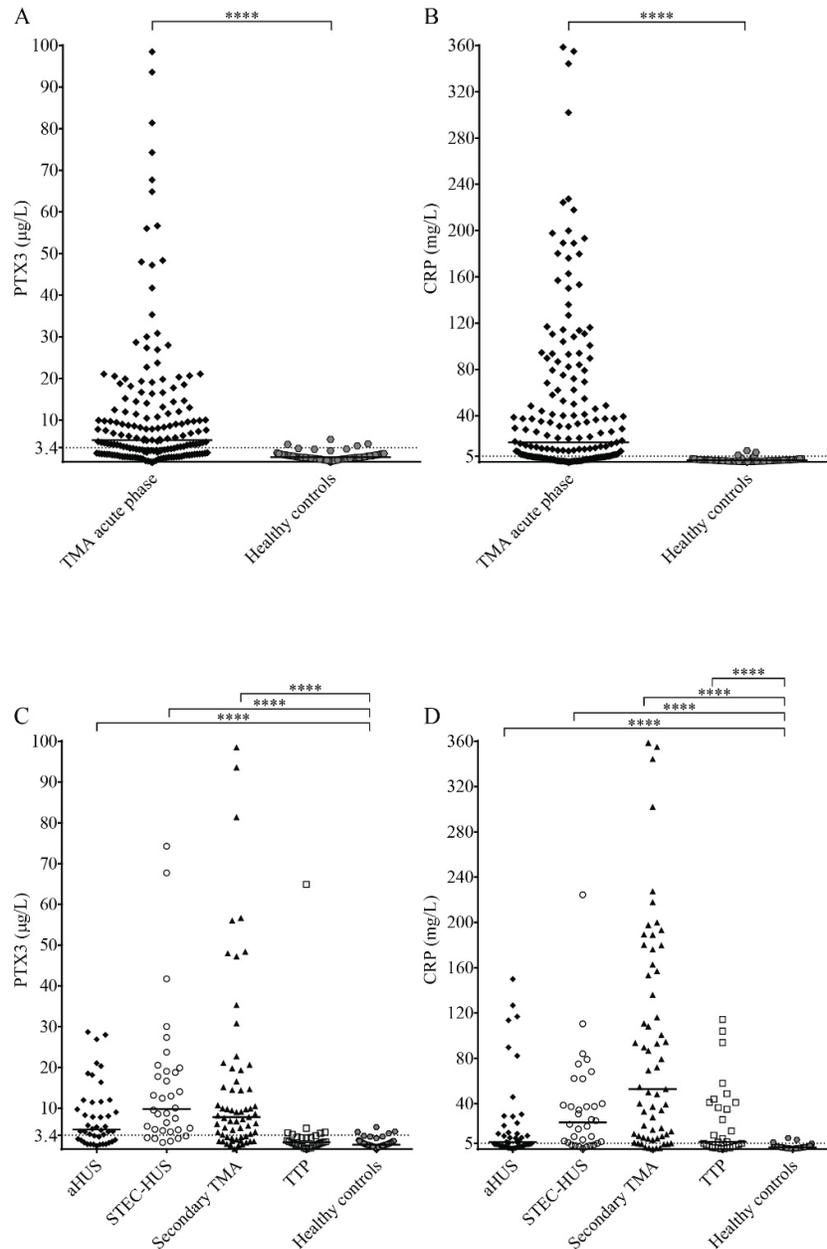
Laboratory parameters	aHUS	N	STEC-HUS	N	Secondary TMA	N	TTP	N
RBC (T/L)	2.8 (2.5-3.3)	17	3.3 (2.8-3.7)	31	2.9 (2.6-3.3)	49	2.7 (2.1-3.2)	24
Hb (g/L)	80 (67-95)	30	87 (76-101)	33	88 (79-95)	53	80 (67-99)	29
PLT (G/L)	53 (29-86)	32	51 (33-88)	33	50 (34-76)	54	16 (11-27)	29
LDH (U/L)	2777 (1806-4027)	24	3280 (2374-5157)	30	1005 (545-1634)	51	1444 (819-2370)	29
Crea (µmol/L)	269 (113-535)	37	292 (166-489)	32	188 (89-302)	58	79 (68-100)	29
BUN (mmol/L)	20.3 (13.0-25.7)	24	19.0 (14.2-40.1)	32	17.9 (11.7-28.0)	46	6.5 (5.0-9.0)	21

**5.1.2. Pentraxin levels in acute phase thrombotic microangiopathy and their relation to the laboratory markers of disease and clinical characteristics of patients**

The circulatory PTX3 and CRP levels were significantly elevated in acute phase-TMA compared to healthy controls. Elevated PTX3 and CRP levels could be detected in all etiological forms of TMA, although PTX3 elevation was exceptional in TTP, despite the elevated CRP level in 53% of the TTP patients (Figure 5 A-D). With further subdivision of the study groups, we found that the elevation of both pentraxins was independent of the molecular background in aHUS or the underlying condition in secondary TMA. The calculated cutoff of CRP levels (5.01 mg/L) was equivalent to the upper limit of normal range (5 mg/L) used in our laboratory in frames of diagnostics,

whereas the cutoff of PTX3 levels was determined based on the levels measured in the healthy control group and set as 3.40  $\mu\text{g/L}$ .

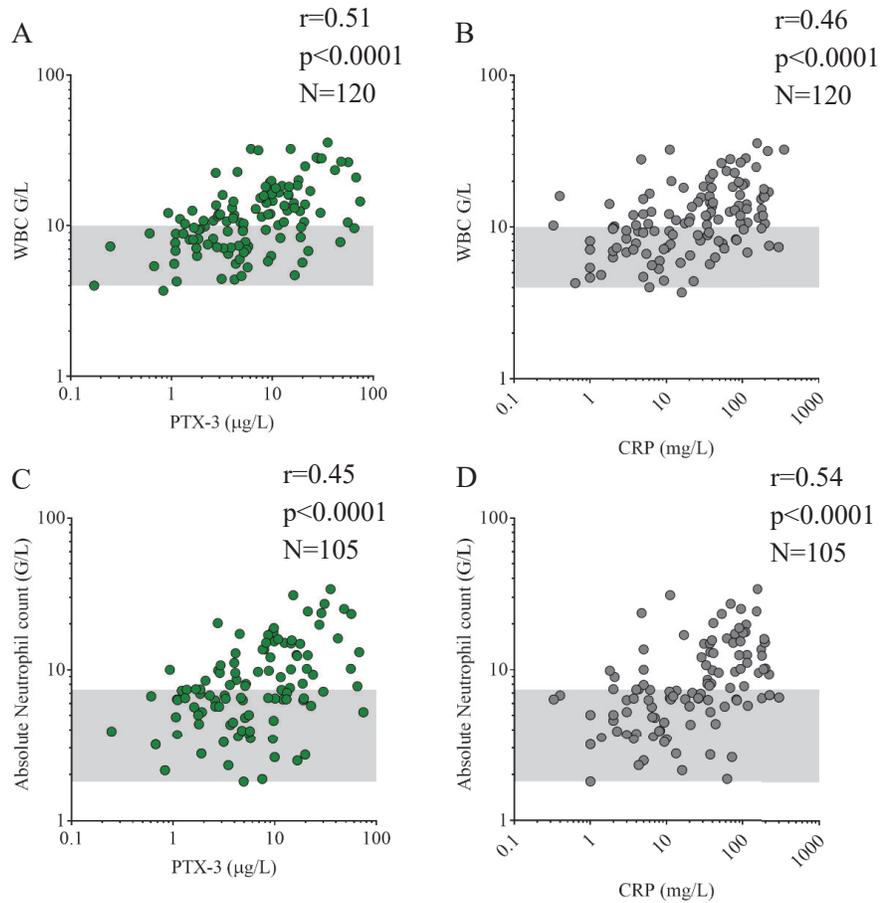
PTX3 levels were associated with markers of disease activity and organ damage. We observed a significant positive correlation between lactate dehydrogenase (LDH) and PTX3 levels, and a weaker yet significant correlation of the platelet count as well as laboratory signs of kidney damage to PTX3. By contrast, association between CRP and disease activity was not present, except a significant positive correlation to creatinine levels. Furthermore, systemic PTX3 and CRP levels showed a strong positive correlation to each other and to markers of systemic inflammation such as the white blood cell (WBC) and absolute neutrophil counts (ANC) of the patients (Figure 6 A-D). Low neutrophil counts may indicate both the disturbance of production with a subsequently low PTX3 level, or neutrophil activation and consumption, with a consequently high PTX3 level. To generate homogeneous patient groups during the assessment of pentraxin production in frames of the TMA-associated inflammatory response, we excluded patients with a WBC or ANC below the lower limit of the laboratory normal range (WBC < 4G/L, N=10; ANC < 1.8G/L, N=5) from the latter analysis. Since platelet count is a reliable marker of disease activity in TMA, we grouped the patients according to platelet counts at the time of admission. However, despite the positive correlation, we found that irrespective of the classification, PTX3 and CRP levels of all subgroups remained significantly elevated compared to healthy controls (Figure 7 A-B). In order to adjust for confounding variables, the laboratory parameters presented in Table 4 were also entered into two multiple regression models to explore the relationship between them and the determined pentraxin levels. LDH (standardized regression coefficient  $\beta=0.299$ ) turned out to be a significant predictor of PTX3 in the multivariable model, whereas platelet and kidney function measures did not. For CRP, significant predictors were hemoglobin ( $\beta=0.183$ ), platelet number ( $\beta=-0.179$ ) and creatinine ( $\beta=0.338$ ) levels.



**Figure 5. Systemic PTX3 and CRP levels in TMA patients and healthy controls**

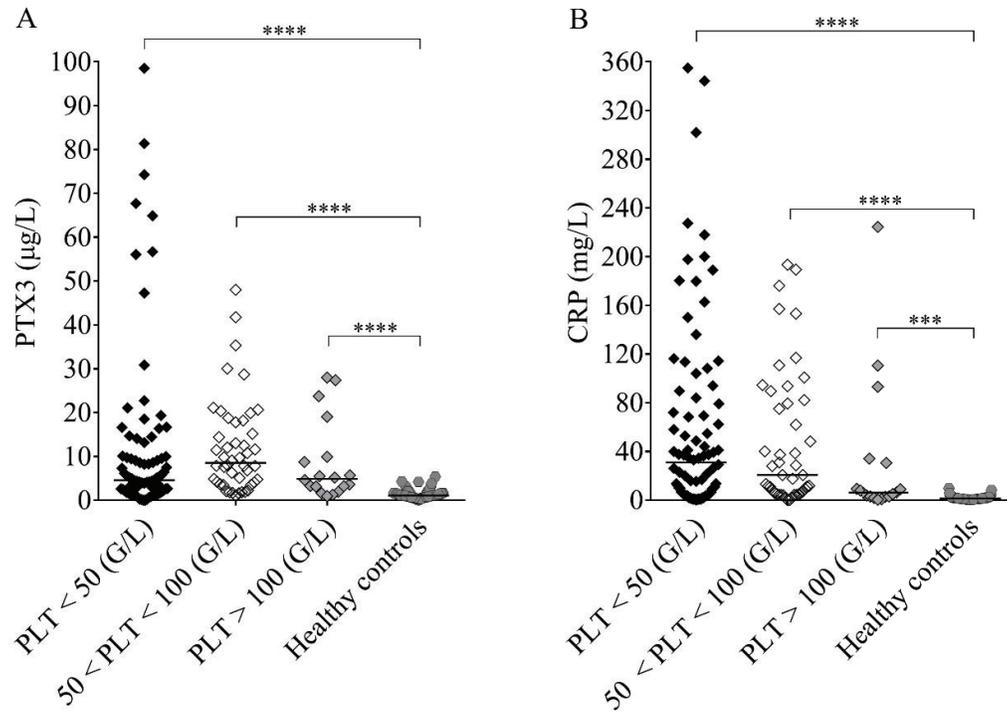
PTX3 and CRP levels of acute phase-TMA patients compared to those of healthy individuals are displayed on panels A-B, whereas pentraxin levels of TMA patients subdivided based on disease etiology are shown on panels C-D. Data points are expressed as mean of technical duplicates, the horizontal line indicates the median of each group and an intermittent line indicates the calculated cutoff of each pentraxin, respectively. Statistical analysis was performed with the Kruskal-Wallis test corrected

for multiple comparisons using the Dunn's post hoc test. Statistical significance is indicated by asterisks (\*\*\*\* $p < 0.0001$ ). (aHUS= atypical hemolytic uremic syndrome, CRP= C-reactive protein, PTX3= pentraxin-3, STEC-HUS= Shiga-like toxin associated HUS, TMA= thrombotic microangiopathy, TTP= thrombotic thrombocytopenic purpura)



**Figure 6. Correlation of the systemic pentraxin levels to inflammatory parameters**

Correlation of the PTX3 (green circles) and the CRP (grey circles) levels to the WBC (panels A-B) and absolute neutrophil counts (panels C-D) of the patients. Laboratory normal ranges are shown with grey shading. To generate homogeneous patient groups, patients with a WBC below 4 G/L (N=10) and those with an absolute neutrophil count below 1.8 G/L (N=5) were excluded from this analysis. Statistical analysis was performed with the Spearman correlation analysis. Respective r and p values are indicated in the upper right corner of each graph. Please note that blood count and chemistry data were not available by all the enrolled patients, therefore the correlation analyses might include less than the total number of patients included in this study (N=171). Patients with an undetectable pentraxin level (N=3) are not shown in the graph, however their data were included in the statistical analysis. (CRP=C-reactive protein, PTX3=pentraxin-3, WBC=white blood cell count)



**Figure 7. Systemic pentraxin levels and platelet count of TMA patients**

PTX3 (A) and CRP (B) levels of acute phase-TMA patients are shown, subdivided based on the platelet count determined at the onset of disease. Data are expressed as mean of technical duplicates, the horizontal line indicates the median of each group. Statistical analysis was performed with the Kruskal-Wallis test corrected for multiple comparisons using the Dunn's post hoc test. Statistical significance is indicated by asterisks (\*\*\*) $p < 0.001$ , (\*\*\*\*) $p < 0.0001$ . (PTX3= pentraxin-3, PLT = platelet, TMA=thrombotic microangiopathy)

### **5.1.3. Association of the systemic pentraxin levels with the acute phase mortality**

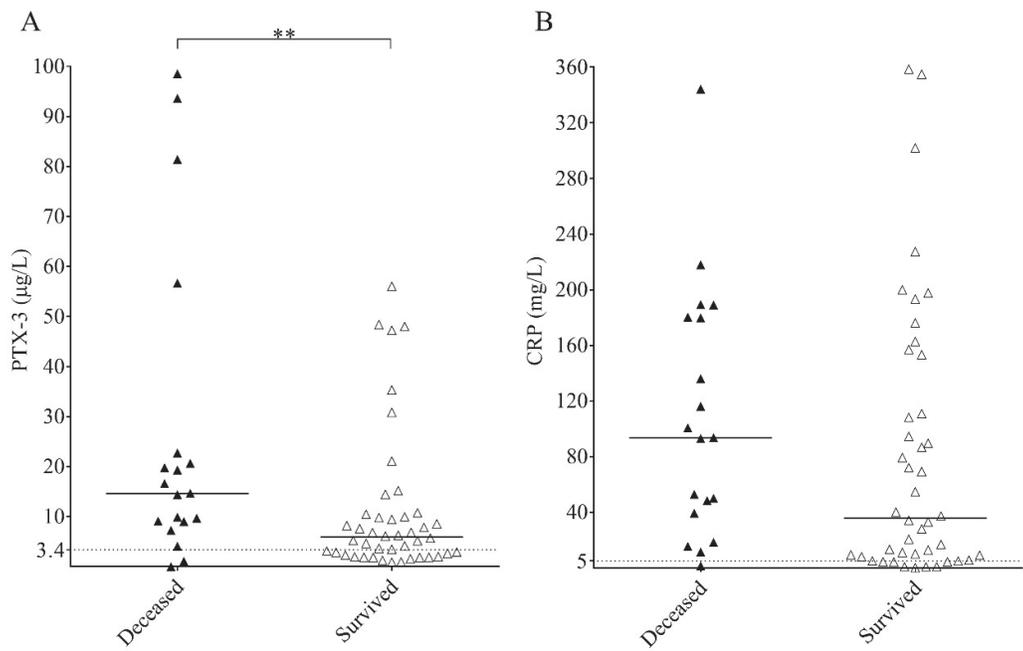
In-hospital mortality was followed-up during the first month of the acute disease flare and the association of circulatory pentraxin levels to disease mortality was analyzed in the secondary TMA subgroup, where mortality exceeded 30% within a 31-day period. Conversely, only one patient died in the TTP group, whereas no deaths occurred among STEC-HUS or aHUS patients. Elevation of the median PTX3 level was associated with a higher acute phase mortality in the secondary TMA group, whereas the median CRP levels did not differ significantly in secondary TMA patients who survived the first month of the TMA episode compared to those who did not (Figure 8 A-B). Since our study augments an earlier publication that focused exclusively on the investigation of secondary TMA patients (Farkas et al. 2017), with considerable overlap of the patient population, we also performed our analysis following the exclusion of patients included in our earlier study. We found that the median PTX3 level remained significantly elevated in deceased patients compared to survivors of the acute episode even in the narrowed patient population (N=33), while elevation of CRP showed no association to disease mortality.

The optimum PTX3 cut-point was 9 µg/mL to differentiate patients who died during follow up, from those who survived (odd's ratio 3.08 (95% CI 1.02-9.33)). One-by-one adjustment for key activity indicators showed that high PTX3 levels are hemoglobin and creatinine independent predictors of mortality, whereas dependent on platelet and LDH levels.

### **5.1.4. Pentraxin levels in disease remission**

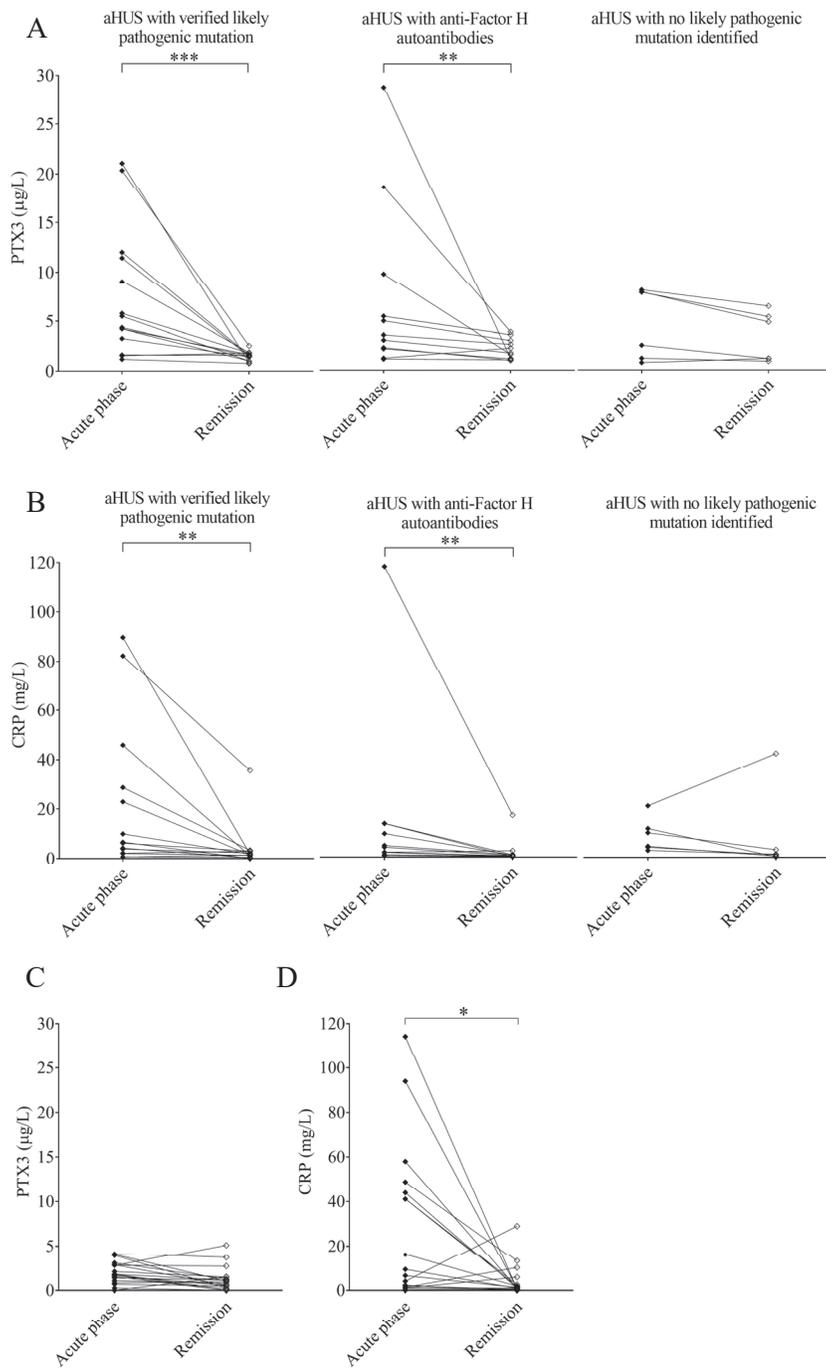
STEC-HUS and secondary TMA are characterized by one acute episode with no disease recurrence upon the eradication of the underlying cause. On the other hand, relapses are common features of aHUS and TTP with a progressive decline of kidney function and neurological deterioration, respectively. To investigate the regression of pentraxin levels in disease remission, we obtained follow-up samples from 31 aHUS patients and 19 of the TTP patients (Figure 9 A-D). Although the PTX3 level remained significantly higher in aHUS remission compared to the healthy control group, the systemic CRP levels in remission were similar to those of the healthy controls. In 80% of aHUS both PTX3 and CRP levels decreased compared to the paired acute phase samples,

nonetheless the extent of decline did not reach statistical significance in patients with no clarified molecular background of the disease. The initially low PTX3 levels of TTP patients showed no remarkable difference in remission, and the CRP levels also normalized in 85% of the cases.



**Figure 8. Association of the systemic pentraxin levels to disease mortality**

Systemic pentraxin levels of the secondary TMA patients are shown at the acute presentation of the disease, grouped based on the 31-day survival of patients (deceased patients indicated with black triangles, those who survived with white triangles). Data are shown as mean of technical duplicates, the horizontal line shows the median of each group, with an intermittent line indicating the calculated cutoff of each pentraxin. Statistical analysis was performed with the Mann-Whitney test. Statistical significance is indicated by asterisks (\*\* $p < 0.01$ ). (CRP= C-reactive protein, PTX3= pentraxin-3, TMA= thrombotic microangiopathy)



**Figure 9. Pentraxin levels in the acute phase and remission of aHUS and TTP**

Measured pentraxin levels in the acute phase (black squares) and in remission (empty squares) are shown in aHUS (A-B) and TTP (C-D) patients. (A-B) The aHUS patients are displayed grouped by the confirmed molecular background of the disease: 22 cases had a confirmed likely pathogenic mutation, 11 patients presented with anti-Factor H

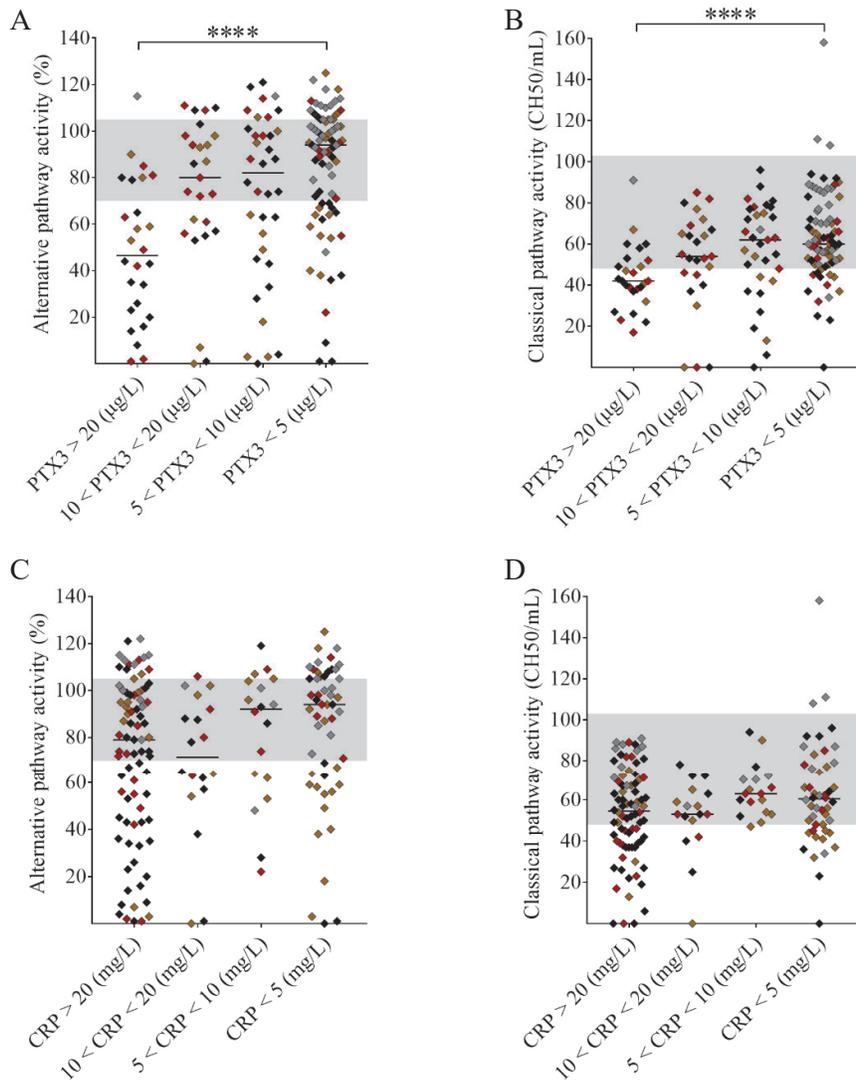
antibodies and the rest (N=11) did not carry any likely pathogenic mutations in the complement genes (*CFH*, *CFHR5*, *CFI*, *CD46*, *C3*, *CFB*), *THBD* or *DGKE*. Data points represent mean of technical duplicates with a continuous line connecting each paired sample, and the group medians indicated by a horizontal line. Statistical analysis was performed with the Wilcoxon matched-pairs signed rank test, statistical significance is indicated by asterisks (\*\*p<0.01; \*\*\*p<0.001). (aHUS=atypical hemolytic uremic syndrome, CRP = C-reactive protein, PTX3=pentraxin-3, TTP = thrombotic thrombocytopenic purpura)

## **5.2. Investigation of the relation of complement overactivation and systemic pentraxin levels in distinct forms of thrombotic microangiopathies**

### **5.2.1. Laboratory signs of complement consumption in acute phase thrombotic microangiopathy and their association to the systemic pentraxin levels**

Complement consumption has been associated to all etiological forms of TMA. Nearly 50% of our patients also presented with decreased C3 levels indicative of complement overactivation, while only 8% of the patients (14/171) showed no signs of complement alteration (with C3, C4, FH, FI, FB levels, CP and AP activities together with the complement activation product levels within the laboratory normal range). Furthermore, we had a notable number of patients with low FH level, originating from all disease subgroups, indicative of complement dysregulation in all investigated forms of TMA.

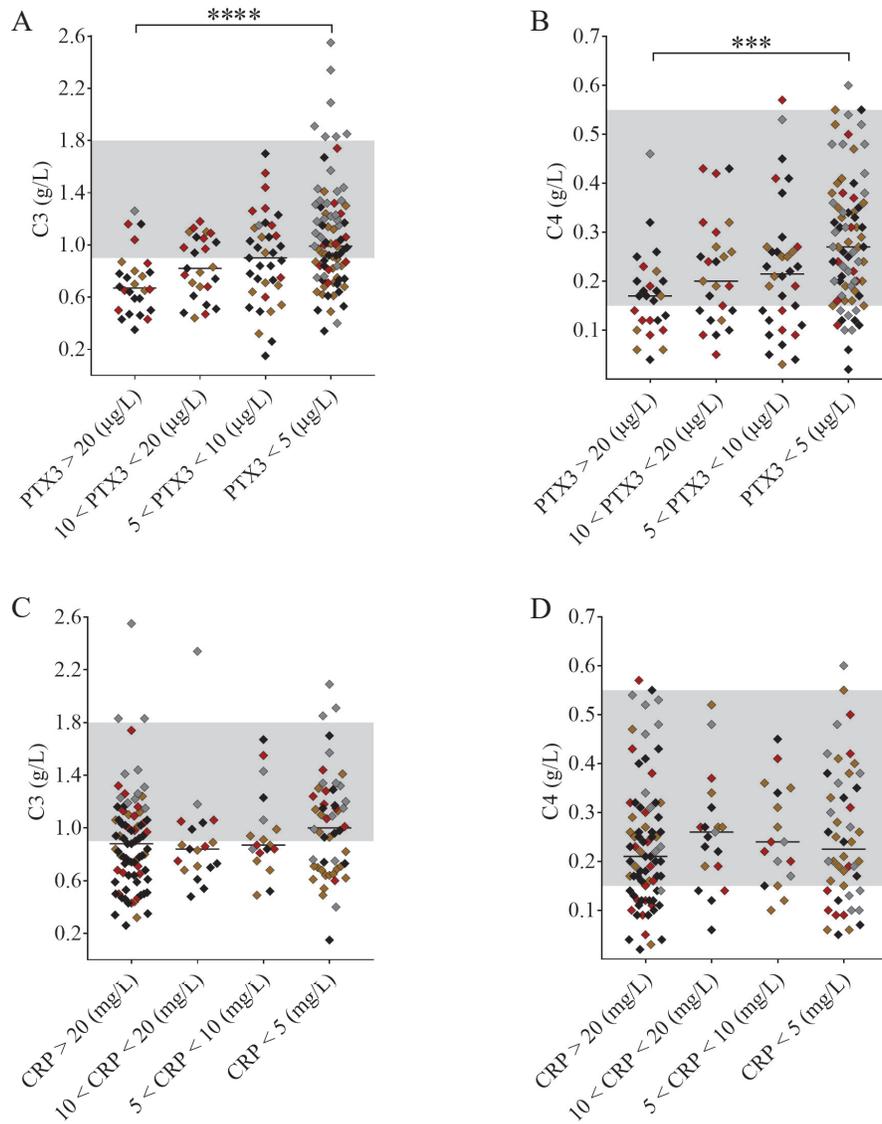
To assess whether elevated pentraxin levels were associated with complement consumption in the acute disease flare, we grouped the patients upon PTX3 and CRP levels and observed a strong linkage between the gradual increase in PTX3 and signs of complement AP and CP consumption (Figure 10 A-D). Complement CP and AP activities were significantly lower in patients with PTX3 above 20 µg/L compared to those with PTX3 below 5 µg/L. Moreover, patients with a PTX3 level exceeding 20 µg/L had a median AP and CP activity below the normal range indicating explicit complement consumption. As a result of complement overactivation and factor consumption, both C3 and C4 levels were significantly lower in patients with PTX3 above 20 µg/L compared to those below 5 µg/L (Figure 11 A-D). However, the gradual increase of PTX3 was not accompanied by a decrease in the FH and FB levels and the level of the complement activation product sC5b-9 was elevated in all groups regardless of the systemic PTX3 level (Figure 12 A-F). By contrast, CRP levels showed no association to any of the measured complement activity parameters (Figures 9-11). Since a considerable number of patients presented with a CRP level exceeding 20mg/L, we analyzed the association between CRP levels and laboratory signs of complement consumption in four additional patient subgroups (20mg/L < CRP < 40mg/L; 40mg/L < CRP < 60mg/L; 60mg/L < CRP < 80mg/L; 80mg/L < CRP), yet we found no association between CRP elevation and laboratory markers of pathological complement activation in TMA.



**Figure 10. Association of the systemic PTX3 and CRP levels to the activity of the alternative and classical pathways**

TMA patients are subdivided based on the measured systemic PTX3 ( $\mu\text{g/L}$ ) or CRP ( $\text{mg/L}$ ) levels indicated on the x axis, with their AP and CP activities shown on the y axis, respectively. The color of each data point indicates the specific form of TMA corresponding to Figure 3 (brown= aHUS, red= STEC-HUS, black= secondary TMA, grey= TTP). Data are expressed as mean of technical duplicates, the horizontal lines indicate the median of each group, whereas the laboratory normal range is shown with grey shading. Statistical analysis was performed with the Kruskal-Wallis test corrected for multiple comparisons using the Dunn's post hoc test. Statistical significance is

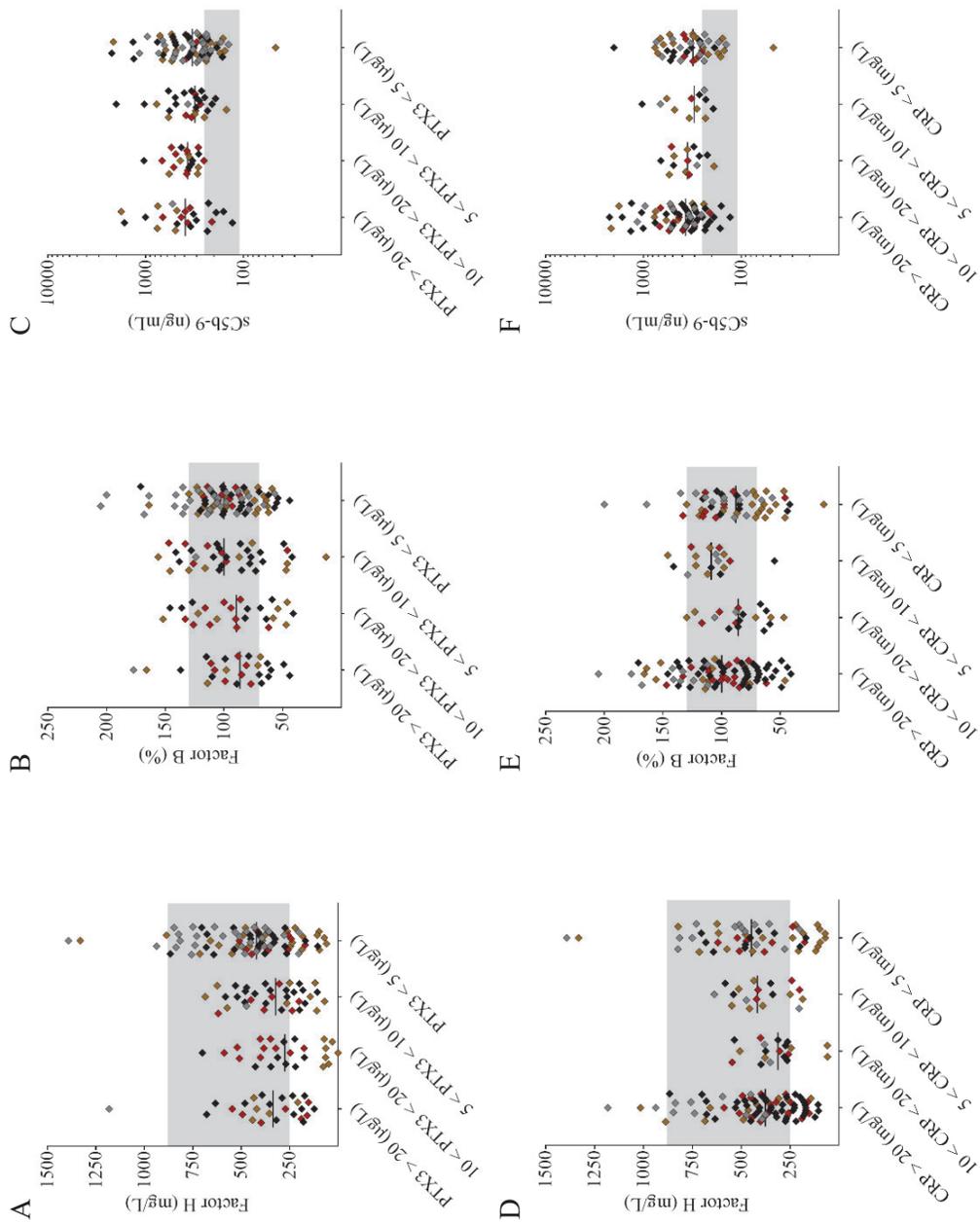
indicated by asterisks (\*\*\*\* $p < 0.0001$ ). (aHUS= atypical hemolytic uremic syndrome, CRP= C-reactive protein, PTX3= pentraxin-3, STEC-HUS= Shiga-like toxin associated HUS, TMA= thrombotic microangiopathy, TTP= thrombotic thrombocytopenic purpura)



**Figure 11. Association of the systemic PTX3 and CRP levels to the complement factor levels C3 and C4.**

TMA patients are subdivided based on the measured systemic PTX3 ( $\mu\text{g/L}$ ) or CRP ( $\text{mg/L}$ ) levels indicated on the x axis, with their C3 and C4 levels shown on the y axis, respectively. The color of each data point indicates the specific form of TMA corresponding to Figure 3 (brown= aHUS, red= STEC-HUS, black= secondary TMA, grey= TTP). Data are expressed as mean of technical duplicates, the horizontal lines indicate the median of each group, whereas the laboratory normal range is shown with grey shading. Statistical analysis was performed with the Kruskal-Wallis test corrected for multiple comparisons using the Dunn's post hoc test. Statistical significance is

indicated by asterisks (\*\*\*\* $p < 0.0001$ ). (aHUS= atypical hemolytic uremic syndrome, CRP= C-reactive protein, PTX3= pentraxin-3, STEC-HUS= Shiga-like toxin associated HUS, TMA= thrombotic microangiopathy, TTP= thrombotic thrombocytopenic purpura)



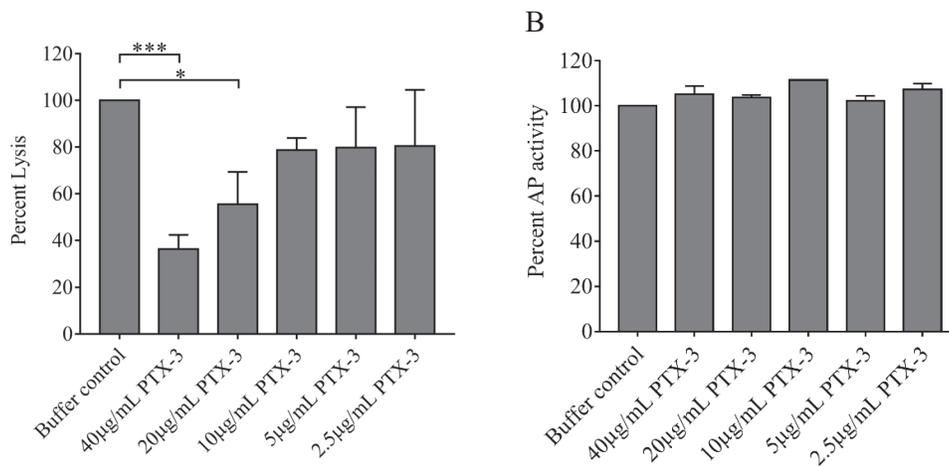
**Figure 12. Complement regulator Factor H, Factor B and the complement activation products according to the pentraxin levels in TMA**

Association of the systemic PTX3 and CRP levels to the complement regulators Factor H, Factor B and the complement activation product sC5b-9 levels. TMA patients are subdivided based on the measured systemic PTX3 ( $\mu\text{g/L}$ ) or CRP (mg/L) levels indicated on the x axis, respectively. The color of each data point indicates the specific

form of TMA corresponding to Figure 3 (brown= aHUS, red= STEC-HUS, black= secondary TMA, grey= TTP). Data are expressed as mean of technical duplicates, the horizontal lines indicate the median of each group and the laboratory normal range is shown with grey shading. Statistical analysis was performed with the Kruskal-Wallis test corrected for multiple comparisons using the Dunn's post hoc test. (aHUS = atypical hemolytic uremic syndrome, CRP= C-reactive protein, PTX3= pentraxin-3, STEC-HUS= Shiga-like toxin associated HUS, TMA= thrombotic microangiopathy, TTP= thrombotic thrombocytopenic purpura)

### **5.2.2. Impact of excess pentraxin-3 on alternative pathway activity *in vitro***

Since *in vivo* complement consumption was accompanied by a gradual increase in the systemic PTX3 level in our acute phase-TMA patients, to explore the functional relevance of this phenomenon, we tested whether PTX3 attenuates or stimulates the AP activity on the cellular surface *in vitro*. In a modified hemolytic assay allowing for activation of solely the AP, we built up the AP C3-convertase on sheep erythrocytes and determined the hemolytic activity of NHS with the addition of recombinant human PTX3 or buffer control, respectively (Figure 13A). We found that addition of PTX3 dose-dependently decreased the AP hemolytic activity on sheep red blood cells. Conversely, addition of PTX3 to NHS did not influence the AP activity on the surface of ELISA plates and thus resulted in no remarkable change in C9 deposition through LPS-induced activation of the AP (Figure 13B).



**Figure 13. Effect of PTX3 on alternative pathway activity *in vitro***

The effect of recombinant human PTX3 on AP hemolytic activity (A) and lipopolysaccharide induced complement C9 deposition (B) is shown in the respective figure panels. Data are expressed in percent, compared to buffer control added to pooled serum of healthy individuals (100%). Data represent mean of three times repeated experiments with technical duplicates, error bars indicate the standard error of mean. Statistical analysis was performed with the Kruskal-Wallis test corrected for multiple comparisons using the Dunn's post hoc test. Statistical significance is indicated by asterisks (\*p<0.05; \*\*\*p<0.001). (AP=complement alternative pathway, PTX3=pentraxin-3)

### **5.3. Epitope analysis of anti-Factor H antibodies in atypical hemolytic uremic syndrome**

#### **5.3.1. Clinical and laboratory characteristics of patients with autoimmune atypical hemolytic uremic syndrome**

Sera of children with the diagnosis of autoimmune aHUS were obtained for the epitope analysis of FH antibodies. For accurate confinement of the antibody binding epitopes on FH, only sera of treatment-naïve patients were applied in the epitope mapping experiments in the acute phase of aHUS. The enrolled patients' anti-FH antibody titers in the acute disease flare and remission, as well as the results of MLPA with characteristic *CFHR1* deficiency are shown in Table 5. The median age of the children at the time of diagnosis was 9 years, therefore sera of age-matched control individuals (N=10) were applied in the epitope analysis experiments. Serum samples of the eight aHUS patients were taken before the initiation of plasmapheresis during the first acute episode of the disease, however two of the study subjects received FFP prior to sampling. We obtained remission phase sera samples from seven of the patients, who were followed-up on a monthly basis after the termination of aHUS-specific therapy. This included plasmapheresis, immunosuppression with corticosteroids or cyclophosphamide, and rituximab, respectively. The remission phase samples were collected after a minimum follow-up period of 6 months in clinical remission, during which the patients received no immunomodulation or any forms of plasma therapy (FFP or PEX). By definition, the diagnosis of autoimmune aHUS was established based on the clinical signs of acute kidney failure and laboratory markers of hemolysis and thrombocytopenia with an anti-FH antibody level exceeding the cutoff titer of 110 AU/mL. Even though the anti-FH antibody level of all patients was higher than the internationally established upper-normal limit, there was a notable difference in the initial titers of the patients ranging from 209-10067 AU/mL. Furthermore, albeit the follow-up periods elapsed devoid of relapse in all of the aHUS patients, the level of free anti-FH IgG remained low-titer positive in three out of seven cases (Table 5).

**Table 5. Patients enrolled in the epitope analysis of anti-Factor H antibodies**

Patients with the diagnosis of aHUS enrolled in this study who had a positive ELISA result for anti-FH antibodies (>110 AU/ml) at the time of acute phase presentation. Major clinical characteristics and results of the MLPA are displayed by each enrolled subject. (aHUS = atypical hemolytic uremic syndrome, anti-FH antibodies = anti-Factor H antibodies, ELISA = enzyme-linked immunosorbent assay, MLPA = multiplex ligation-dependent probe amplification)

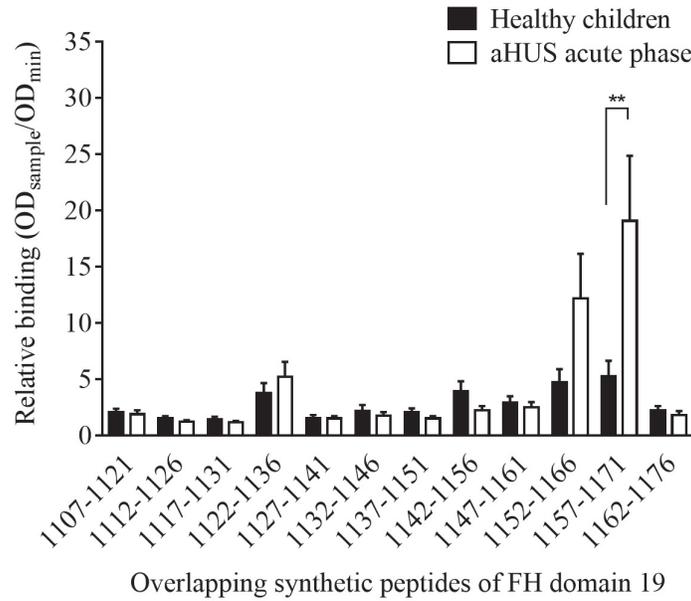
Patient code	Age at disease onset (years)	Gender	Anti-FH antibody level in the acute phase of aHUS (AU/ml)	Anti-FH antibody level in remission of aHUS (AU/ml)	MLPA results for <i>CFHR1</i> and <i>CFHR3</i>
P1	6.5	male	10067	136	Homozygous deletion of <i>CFHR1</i> and <i>CFHR3</i>
P2	8.5	male	2190	125	Homozygous deletion of <i>CFHR1</i> and <i>CFHR3</i>
P3	10.5	female	1306	93	Homozygous deletion of <i>CFHR1</i> and <i>CFHR3</i>
P4	8	female	2221	99	Homozygous deletion of <i>CFHR1</i> and heterozygous deletion of <i>CFHR3</i>
P5	8	male	2725	213	Heterozygous deletion of <i>CFHR1</i> and <i>CFHR3</i>
P6	8	male	209	55	Homozygous deletion of <i>CFHR1</i> and <i>CFHR3</i>
P7	11	male	329	89	Homozygous deletion of <i>CFHR1</i> and <i>CFHR3</i>
P8	11	female	9152	no sample available	Homozygous deletion of <i>CFHR1</i> and <i>CFHR3</i>

### **5.3.2. Localization of the linear autoantibody epitopes on Factor H in the acute phase and remission of atypical hemolytic uremic syndrome**

Autoantibody binding to the synthetic peptides was investigated by ELISA. Reactivity of serum anti-FH antibodies to the synthetic peptides of ten control children and eight aHUS patients is shown in Figure 14 A-B. Peptides with a significantly increased relative autoantibody binding in the acute disease flare (patients versus control, Mann-Whitney test) included amino acids 1157-1171 on FH domain 19 (Figure 14A), and amino acids 1177-1191 and 1207-1226 on FH domain 20 (Figure 14B). The highest average binding was observed to peptides covering the amino acid residues 1212-1226. Albeit average autoantibody binding to the linear peptides is presented in the graphs, it is noteworthy that all three of the linear epitopes displayed increased autoantibody binding in the acute phase sera of every child. In disease remission, the epitope recognition pattern of the autoantibodies remained similar compared to what we had observed at the acute disease onset, although with weaker signals (Figure 15 A-B). The decline of antibody binding reached statistical significance by one of the epitopes (peptide 1177-1191) although in case of all epitopes a decrease of at least 25% was measured at the level of group mean.

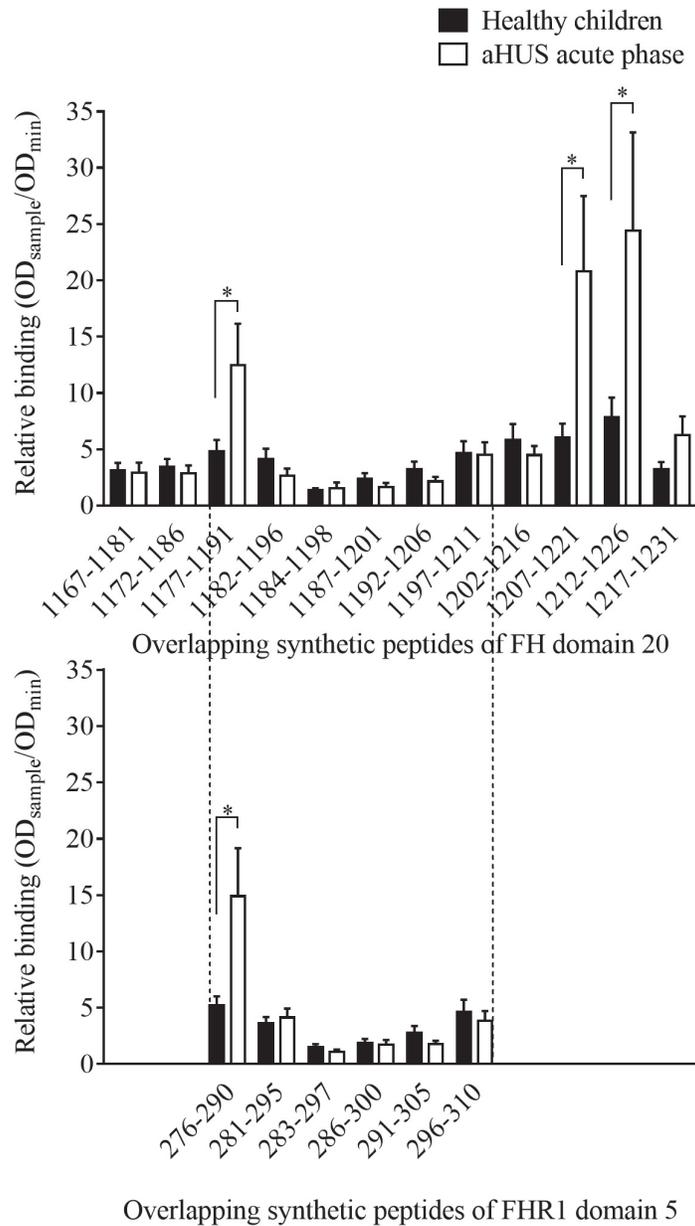
### **5.3.3. Comparison of Factor H autoantibody binding to linear epitopes on Factor H and Factor H-related protein 1**

The C-terminal domains of FH and FHR1 share a 99% homology and differ only in two amino acids (Table 2). Since seven of our patients carried a homozygous, and one a heterozygous deletion of *CFHR1* (Table 5), we also synthesized overlapping peptides of the region containing the two residues which are different in FH and FHR1 (Table 2). We identified significantly increased autoantibody binding to peptide 276-290 of FHR1 compared to controls in the acute phase of aHUS (Figure 14B), which covered the exact same location as the previously identified autoantibody epitope on FH (peptide 1177-1191). Even though in remission antibody binding decreased significantly to the homologous epitopes of FH and FHR1 (Figure 15B), the decline only reached statistical significance by the FH peptide. The extent of autoantibody binding however, was comparable to each of the homologous peptides, suggesting that the serine-leucine exchange did not influence the autoantibody binding to the complement proteins.



**Figure 14A. Detection of linear antibody binding epitopes in the acute phase of disease**

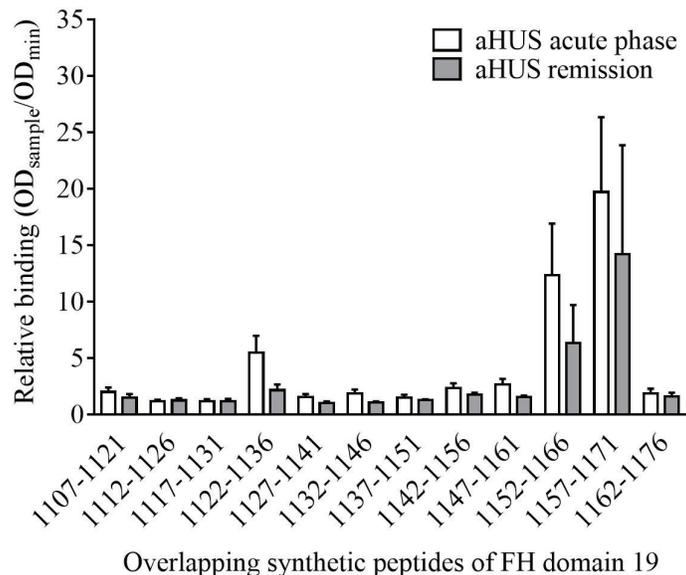
Relative binding of serum anti-FH antibodies to the synthetic FH peptides of SCR domain 19 in control patients and in the acute phase of aHUS. The sera of ten control children (black bars) and eight children in the acute phase of HUS (white bars) were analyzed, data are displayed as mean relative autoantibody binding per group with the standard error of the mean. Relative autoantibody binding is expressed as the ratio of  $OD_{\text{sample}}/OD_{\text{min}}$ , where  $OD_{\text{sample}}$  is the mean of duplicate OD values of the test samples and  $OD_{\text{min}}$  is the mean binding to the negative control HSP60 peptide. Numbering on the x axis shows the initial and final amino acid of each tested peptide. Difference in autoantibody binding to the peptides was determined with Mann-Whitney test. Statistical significance is indicated by asterisks (\*\* $p < 0.01$ ). (aHUS = atypical hemolytic uremic syndrome, FH = Factor H, anti-FH antibodies = anti-Factor H antibodies, HSP60 = heat shock protein 60, OD = optical density)



**Figure 14B. Detection of linear antibody binding epitopes in the acute phase of disease**

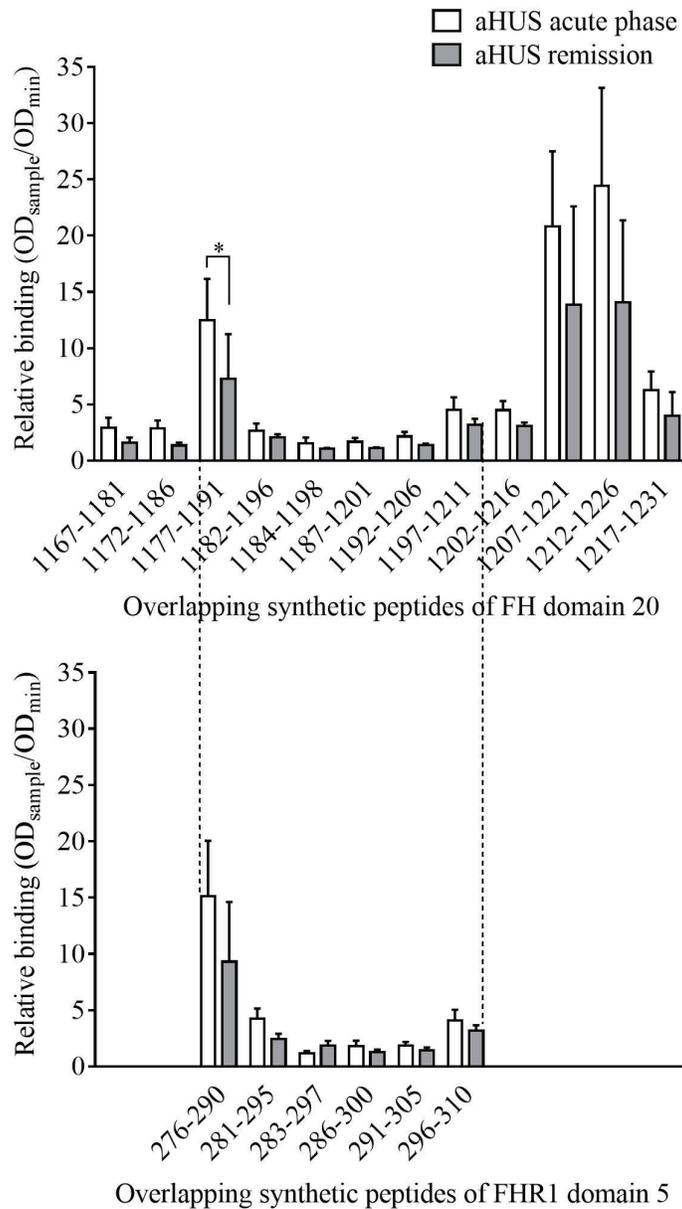
Relative binding of serum anti-FH antibodies to the synthetic peptides of FH SCR domain 20 (upper panel) and the fragment of FHR1 SCR domain 5 (lower panel) homolog to that of FH amino acids 1177-1211 in control patients and in the acute phase of aHUS. The sera of ten control children (black bars) and eight children in the acute phase of HUS (white bars) were analyzed, data are displayed as mean relative

autoantibody binding per group with the standard error of the mean. Relative autoantibody binding is expressed as the ratio of  $OD_{\text{sample}}/OD_{\text{min}}$ , where  $OD_{\text{sample}}$  is the mean of duplicate OD values of the test samples and  $OD_{\text{min}}$  is the mean binding to the negative control HSP60 peptide. Numbering on the x axis represents the initial and final amino acid of each tested peptide. Difference in autoantibody binding to the indicated peptides was determined with Mann-Whitney test. Statistical significance is indicated by asterisks (\* $p < 0.05$ ). (aHUS = atypical hemolytic uremic syndrome, FH = Factor H, anti-FH antibodies = anti-Factor H antibodies, HSP60 = heat shock protein 60, OD = optical density)



**Figure 15A. Detection of linear antibody binding epitopes in remission**

Epitope specific relative anti-FH antibody binding to the linear epitopes of FH SCR domain 19 in remission (grey bars) versus the acute phase (white bars) of atypical hemolytic uremic syndrome (aHUS). Analysis of seven patients is displayed as mean relative autoantibody binding per group with the standard error of the mean. Relative autoantibody binding is expressed as the ratio of  $OD_{\text{sample}}/OD_{\text{min}}$ , where  $OD_{\text{sample}}$  is the mean of duplicate OD values of the test samples and  $OD_{\text{min}}$  is the mean binding to the negative control HSP60 peptide. Numbering on the x axis shows the initial and final amino acid of each tested peptide. Statistical analysis for the difference in autoantibody binding to the peptides was performed using the Wilcoxon matched-pairs signed rank test (aHUS = atypical hemolytic uremic syndrome, FH = Factor H, anti-FH antibodies = anti-Factor H antibodies, HSP60 = heat shock protein 60, OD = optical density).



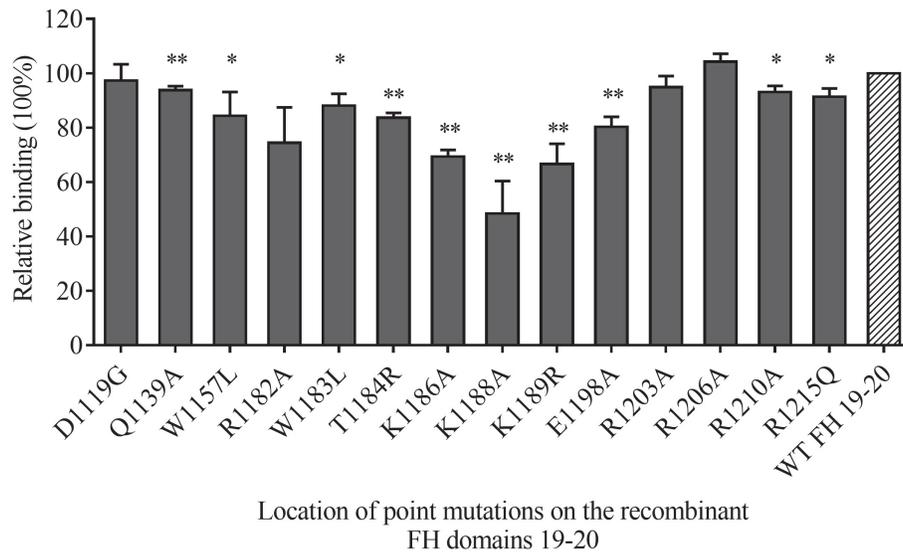
**Figure 15B. Detection of linear antibody binding epitopes in remission**

Epitope specific relative anti-FH autoantibody binding to the linear epitopes of FH SCR domain 20 (upper panel) and the fragment of FHR1 SCR domain 5 (lower panel) homolog to that of FH amino acids 1177-1211 in remission (grey bars) versus the acute phase (white bars) of atypical hemolytic uremic syndrome (aHUS). Analysis of seven patients is shown as mean relative autoantibody binding per group with the standard error of the mean. Relative autoantibody binding is expressed as the ratio of  $OD_{\text{sample}}/OD_{\text{min}}$ , where  $OD_{\text{sample}}$  is the mean of duplicate OD values of the test samples

and  $OD_{min}$  is the mean binding to the negative control HSP60 peptide. Numbering on the x axis shows the initial and final amino acid of each tested peptide. Statistical analysis for the difference in autoantibody binding to the peptides was performed using the Wilcoxon matched-pairs signed rank test. Statistical significance is indicated by asterisks (\* $p < 0.05$ ). (aHUS = atypical hemolytic uremic syndrome, FH = Factor H, anti-FH antibodies = anti-Factor H antibodies, HSP60 = heat shock protein 60, OD = optical density)

#### **5.3.4. Fine epitope mapping of recombinant Factor H domains 19-20 expressing atypical hemolytic uremic syndrome-associated point mutations by anti-Factor H autoantibodies of atypical hemolytic uremic syndrome patients**

Pathogenic mutations associated with aHUS are most frequently located on the C-terminal SCR domains of FH and their presence results in the loss of host recognition and C3b binding by the complement regulator. Since antibody binding to FH also arrests these functions of the protein, we applied various recombinant FH19-20 constructs displaying single amino acid changes associated with aHUS to locate those that are involved in antibody binding, and to validate the results of the linear epitope mapping on the folded FH domains. For this purpose 14 different constructs were tested in ELISA (Figure 16) using serum samples of three patients. On the folded FH domains a distinct antibody binding epitope appeared between amino acids 1183-1198, with a symmetric gradual decline in antibody binding towards amino acid 1188. We observed the highest decrease in anti-FH binding to the FH 19-20 construct with a point mutation at amino acid position 1188. Additional positions, where an amino-acid switch significantly decreased the anti-FH antibody binding were detected on domain 19 (amino acids 1139 and 1157) and the C-terminal end of domain 20 (amino acids 1210 and 1215) concordant to the location of the identified linear epitopes.

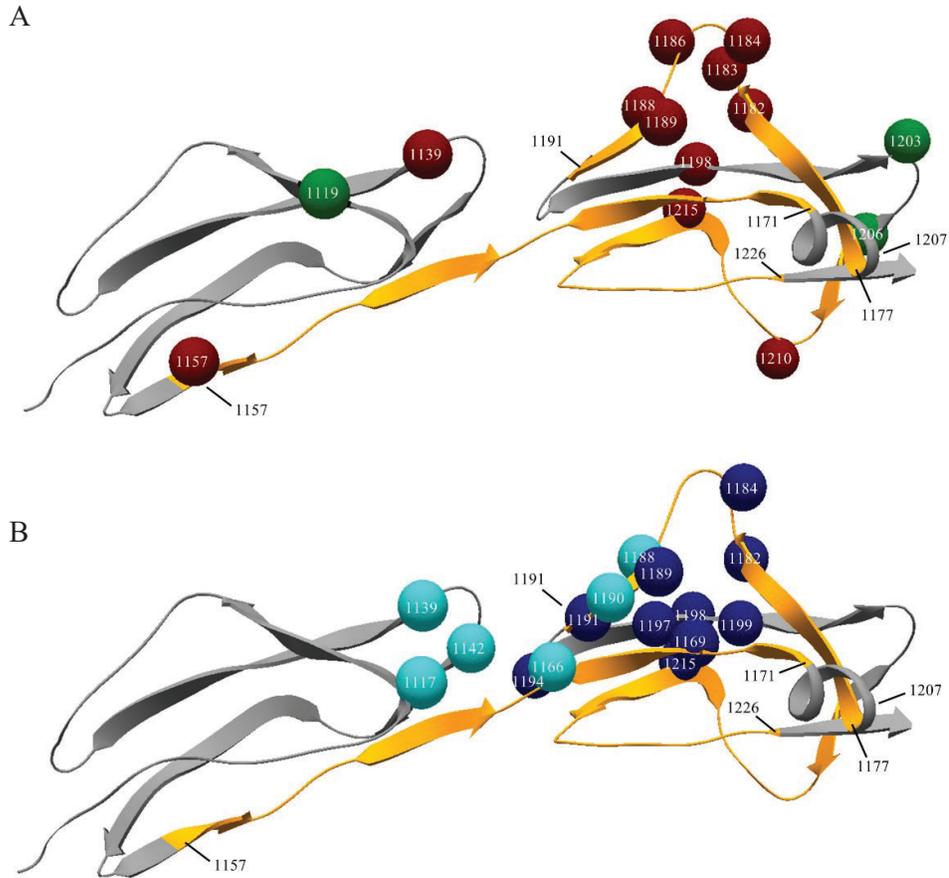


**Figure 16. Epitope mapping of single amino acid-substituted FH constructs by anti-FH autoantibodies of aHUS patients**

Anti-FH antibody binding to recombinant human FH domains 19-20, substituted with single amino acid-mutations (grey bars) is shown compared to the wild type protein domains (striped bar). The single letter amino acid codes of the substitutions are indicated on the x axis with capital letters and numbers marking their location. Binding is expressed on the y axis in percent relative to that of the wild type (100%). We tested the sera of three patients in the acute phase of aHUS, mean and standard error of the mean from three independent experiments is displayed on the graphs. Statistical analysis was performed with the one-sample Wilcoxon Signed Rank test, statistical significance is indicated by asterisks (\* $p < 0.05$ ; \*\* $p < 0.01$ );. (aHUS = atypical hemolytic uremic syndrome, anti-FH antibody = anti-Factor H antibody, WT FH= wild type Factor H)

### **5.3.5. Position of the linear Factor H epitopes and atypical hemolytic uremic syndrome-associated point mutations on the crystal structure of Factor H**

To verify the observations of the distinct approaches of epitope mapping we visualized the location of the linear epitopes and single amino acid-substitutions in the tertiary structure of FH obtained from the Protein Data Bank (ID: pdb2g7i) (Figure 17A). We also positioned the linear epitopes on the amino acids participating in C3b and sialic acid binding (Figure 17B), to explore the potential functional relevance of FH blockade by autoantibodies. In the steric conformation, epitope 1157-1171 appears as a linear segment in the hinge region between FH domains 19 and 20, while epitopes 1177-1191 and especially 1207-1226 are in close sterical proximity with the C-terminal end of peptide 1157-1171. The data obtained from the linear and fine epitope mapping experiments is summarized in Table 6, linked to the role of each amino acid in C3b as well as sialic acid binding, respectively.



**Figure 17. Visualization of the linear epitopes and point mutations on Factor H**

Ribbon model of the folded structure of Factor H (FH) short consensus repeat (SCR) domains 19-20 obtained from the Protein Data Bank (ID: pdb2g7i). Linear epitopes of the anti-Factor H autoantibodies are highlighted with orange (amino acids 1157-1171, 1177-1191, 1207-1221), arrowheads point towards the C-terminal end of the protein and the initial and final amino acid (aa) of each segment is indicated with black numbers. **(A)** The location of the generated amino acid switches is displayed as colorful spheres on the backbone of the protein, with white numbers pinpointing their location and colors representing their effect on autoantibody binding by FH (red: significantly decreased binding when the aa substitutions is present, green: no significant effect on binding). **(B)** Colorful spheres refer to amino acids forming the C3b (Morgan et al. 2011, Strobel et al. 2011) (light blue) and sialic acid (Blaum et al. 2015) (dark blue) binding sites of the molecule with numbers within the spheres showing the aa location on FH.

**Table 6. Overlap of the antibody binding epitopes with sites of C3b binding and sialic acid recognition on Factor H**

Association of the identified linear epitopes and the atypical hemolytic uremic syndrome-associated point mutations on Factor H domains 19-20 to C3b and sialic acid binding sites of the protein. The position of the investigated amino acids is shown in the first column with the single letter amino acid code of the indicated location. Single amino-acid switches that overlapped the identified epitopes are highlighted in orange, corresponding to Figure 17, whereas their involvement in C3b or sialic acid binding is shown with a plus sign (+).

Location of amino acids	C3b binding	Sialic acid binding	Significant decrease in anti-FH Ab binding when point mutation is present	Linear epitope
N1117	+		no	
D1119			not tested	
Q1139	+		yes	
Y1142	+		not tested	
W1157			yes	1157-1171
P1166	+		not tested	
I1169		+	not tested	
R1182		+	yes	1177-1191
W1183			yes	
T1184		+	yes	
K1186			yes	
K1188	+		yes	
L1189		+	yes	
Y1190	+		not tested	
S1191		+	not tested	
G1194		+	not tested	
V1197		+	not tested	
E1198		+	yes	
F1199		+	not tested	
R1203			no	
R1206			no	
R1210			yes	
R1215		+	not tested	1207-1226

## 6. DISCUSSION

### 6.1. Elevation of the systemic pentraxin levels in the acute phase of thrombotic microangiopathies and their association to complement overactivation and consumption

TMA is a life-threatening condition with pathological activation of both the coagulation and complement cascades that evoke a systemic inflammatory response in the acute phase of disease (Noris et al. 2012). Our understanding of the pathophysiology and characteristic course of distinct TMA forms has substantially improved in recent years with novel genes, pathways and mechanisms described as a result of intensive research of this field. The distinct etiological forms of TMA have recently been associated with neutrophil cell activation and the release of NETs (Walters et al. 1989, Fitzpatrick et al. 1992, Ishikawa et al. 2000, Fuchs et al. 2012, Mikes et al. 2014), as a component of which PTX3 may be secreted at the site of tissue damage. During the acute phase of the disease, activation of innate immunity also stimulates the production of CRP. We chose to investigate the relation of these two pentraxins with laboratory markers of disease activity, to provide observational data on the systemic levels of pentraxins in the acute phase of TMA. We hypothesized that there is a potential association between the complement activation status and the systemic level of pentraxins *in vivo*, based on the reported interaction of pentraxins with members of the complement system, and the complement mediated tissue injury present in TMA.

We report on the significant elevation of the fluid phase PRMs CRP and PTX3 in the acute phase of TMA and their association with laboratory markers of disease severity, signs of complement consumption and laboratory markers of the ongoing inflammatory response. We detected elevated CRP levels in all subgroups of TMA, regardless of the underlying etiology. PTX3 levels on the other hand were only elevated in STEC-HUS, aHUS and secondary TMA, however not in TTP (Figure 5C).

Elevation of the CRP levels could be detected in over half of the acute phase-TTP patients, which remained so in 20% during disease convalescence. The sole elevation of CRP without a concomitant increase in the PTX3 levels may be attributed to a considerable IL-6 response in the acute phase of TTP (Westwood et al. 2014) that drives CRP production in the liver (Bottazzi et al. 2006) whereas local generation of PTX3 is IL-6-independent (Alles et al. 1994, Basile et al. 1997, Luchetti et al. 2000), which

could in part explain the lack of PTX3 elevation in TTP. However, neutrophil cell activation together with complement dysregulation have been noted in TTP (Reti et al. 2012, Mikes et al. 2014) and the white blood cell as well as the absolute neutrophil counts were similar in all etiological subgroups of TMA, suggesting a similar extent of PTX3 production by neutrophils in all subgroups, including TTP. On the other hand, absence of elevation in the systemic PTX3 levels in TTP may also be associated with an enhanced consumption of the released protein. PTX3 was reported to inhibit thrombocyte aggregate formation through the binding of activated platelets under pathological conditions (Maugeri et al. 2011). This phenomenon could also be present in TTP, where thrombocytopenia is more severe than in the rest of TMA (Maugeri et al. 2011). Accelerated platelet consumption therefore may result in the depletion of free PTX3, thus causing low or undetectable systemic levels of the protein in the sera of TTP patients. However, it cannot be excluded that additional factors arising from the distinct pathogenesis of TTP (e.g. lack of kidney damage) have also contributed to the observed difference, the detailed exploration of which were beyond the scope of our investigation.

In approximately 60% of aHUS genetic alterations in the complement genes or antibodies directed against FH are accountable for the evoked dysregulation of the AP (Kavanagh et al. 2013). In our aHUS cohort the representation of autoimmune aHUS was somewhat higher than the previously reported prevalence (25% versus 10%) together with a relatively small proportion of unexplained cases (25% versus 30-40%) (Kavanagh et al. 2013). When dividing the patients based on the confirmed molecular background of the disease, we found that elevated pentraxin levels were present in all aHUS subgroups independent of the molecular etiology. PTX3 and CRP elevation was also prominent in STEC-HUS and secondary TMA, regardless of the heterogenic etiological background of the patients.

Acute phase disease mortality was associated with a higher median PTX3 level in our secondary TMA patients. This relationship was independent of the subjects' hemoglobin and creatinine levels, but was related to platelet and LDH. We recorded the highest acute phase mortality (30.2%) in this TMA subgroup, an observation similar to those reported in literature (Farkas et al. 2017, Sun et al. 2018), whereas no deaths occurred in the aHUS and STEC-HUS patient groups and only one patient was lost

among TTP. In contrast with PTX3, no considerable difference could be detected between the median CRP levels of the deceased patients compared to those who survived the first month of the acute episode. Albeit CRP is used as an established marker of inflammation in the clinical practice, PTX3 has also been reported to predict both long and short-term disease outcome in an increasing number of conditions (Lin et al. 2013, Sjoberg et al. 2014, Zhou et al. 2016, Krzanowski et al. 2017, Hu et al. 2018, Lee et al. 2018), whereas some studies even place PTX3 superior to CRP as a predictor of mortality (Lin et al. 2013).

We discovered a strong association between the systemic PTX3 levels and laboratory signs of complement consumption in the acute phase of TMA, but no connection in this regard could be detected with CRP. According to previously published observations, under definite experimental conditions the net result of the PTX3-complement interaction is restraint of terminal pathway activity on non-activator surfaces, which aids the homeostatic mechanism of phagocytosis and clearance of cellular debris (Inforzato et al. 2013, Daigo et al. 2016). This finding is corroborated by human studies, where PTX3 has been shown to correlate with established markers of disease severity in cardiovascular and renal diseases (Maekawa et al. 2011, Lech et al. 2013, Speeckaert et al. 2013) and molecular characterization of this association suggests that PTX3 is involved in the fine tuning of inflammation with an overall host-protective effect (Maugeri et al. 2011, Bonacina et al. 2013). However reports on animal models of tissue damage are controversial in regard to the contribution of PTX3 to the evoked cellular damage and inflammatory response, depending on the applied disease model (Souza et al. 2002, Salio et al. 2008, Souza et al. 2009, Lech et al. 2013). Neutrophil cell activation and NET formation has been reported in all forms of TMA (Walters et al. 1989, Fitzpatrick et al. 1992, Ishikawa et al. 2000, Fuchs et al. 2012, Mikes et al. 2014). As a component of NETs, excess PTX3 may be released on demand from the assembled leukocyte infiltrates (Jaillon et al. 2007, Kunes et al. 2012), albeit its production by injured endothelial cells and innate immune cells at the site of tissue injury also significantly contributes to the locally increased PTX3 levels (Kunes et al. 2012). As a NET component, PTX3 may either aggravate or restrain the overactivation of complement. A potential regulatory role of PTX3 on AP activity is suggested by its recruitment of complement regulators to the cell surface or the ECM (Deban et al. 2008,

Kopp et al. 2012) that may limit the expansion of complement-mediated tissue damage. Activated complement proteins on the other hand may stimulate the formation of NETs that in turn provide a platform for further activation of both coagulation and complement (de Bont et al. 2018).

We aimed to explore the functional relevance of the described association through the measurement of alternative pathway activity in the presence of excess PTX3. We explored the *in vitro* effect of PTX3 on the overall activity of the AP using two modified assays routinely used in the determination of the whole complement AP activity, where we replaced the patients' sera with NHS with or without external PTX3. First, we built up the C3 convertase on sheep erythrocytes under conditions allowing for AP activation only to assess the AP hemolytic activity. Second, we assessed C9 generation on the surface of ELISA plates via LPS-induced AP activation. The major difference between the two methods is the composition of the platform provided for the assembly of the consecutive steps in the activation of the AP. In the hemolytic assay the surface of red blood cells provides the activation scaffold, whereas in the AP ELISA C9 deposition occurs on the surface of a plastic well. Based on the gradual decline of the hemolytic activity parallel to the increment of PTX3 concentration in pooled human serum, we conclude that local release of PTX3 may indeed play an important role in the limitation of AP activity. However, this restraint is most probably due to an indirect effect (e.g. via recruitment of complement regulators to the cellular surface) rather than direct inhibition of the activation pathway, as indicated by the lack of AP decline, when the activation was induced on the surface of an ELISA well. Nonetheless, local regulation of the AP via excess PTX3 could possibly aid the attenuation of the endothelial damage in TMA.

Although we could not detect an association between the patients' CRP levels and complement consumption, *in vitro* evidence suggests that through the binding of complement regulators and the restraint of excess terminal pathway activity, CRP as well as PTX3 are able to regulate the AP and CP of complement (Sjoberg et al. 2006, Biro et al. 2007, Inforzato et al. 2013, Thiele et al. 2014, O'Flynn et al. 2016). However, since CRP production is induced in the liver in frames of a systemic inflammatory response, it may not closely reflect the degree of local endothelial damage and subsequent overactivation of complement.

Our observations may indicate a potential practical use of PTX3 determination as a biomarker and determinant of complement consumption in the acute phase of TMA. However, since this is the first investigation that provides *in vivo* data on the association of elevated systemic PTX3 and laboratory signs of complement consumption, further independent investigations are required to confirm the potential use of the PTX3 measurement as a biomarker of this disease. Nonetheless, the strength of our data is its additional value to so far *in vitro* investigations that described the PTX3-complement interaction, and thus our results provide proof for the *in vivo* relevance of this connection. Furthermore, the observed increment in the production of PTX3 in TMA may indicate a role for this molecule in the disease pathogenesis. However, apparent limitations of our study are the retrospective enrollment of patients and the rare nature of this disease that together may have caused some of our analyses to be underpowered. The limited number of study subjects and subsequently low case and event numbers in this study precluded multivariate analysis in different etiology based subgroups of TMAs, therefore some of our observation may represent overestimation of true effects due to the lack of adjustment for important clinical and/or laboratory covariates. These factors further substantiate the need for the independent confirmation of our observations before firm conclusions can be reached on the contribution of PTX3 to the pathogenesis of TMA. Ultimately, our results add to a better understanding of TMA disease pathogenesis, and the reported association of elevated PTX3 levels and complement consumption hopefully may fuel further *in vitro* investigations that characterize the molecular role of PTX3 in TMA.

## **6.2. Epitope analysis of anti-Factor H antibodies**

In frames of the investigation of the FH-autoantibody interaction in aHUS our specific aim was to identify the antibody binding epitopes on the C-terminal domains of the complement regulator. Here, we tested the binding capacity of serum antibodies to synthetic linear peptides and recombinant folded domains of FH and to synthetic peptides of FHR1. We identified for the first time three linear, extended autoantibody binding epitopes on FH, one of which was also recognized on its homologous region on FHR1. The described epitopes were recognized by serum autoantibodies both in the acute phase and remission of aHUS, albeit we observed a considerable decline in

autoantibody binding in convalescence. We propose that the autoantibody binding site is formed by the linear epitopes in the tertiary structure, and overlaps with multiple ligand binding epitopes of the complement regulator, thus inducing the dysfunction of FH similar to that observed in the presence of aHUS-associated FH mutations (Figure 17). Besides, detection of a linear epitope on FHR1 confirms its previously observed cross-reactivity with FH autoantibodies (Moore et al. 2010, Strobel et al. 2011), which further substantiates the role of FHR1 in aHUS pathogenesis.

We chose two complementary approaches for the localization of the autoantibody binding epitopes on FH. Our primary goal was to identify the anti-FH antibody epitopes on the amino acid level, however for the modelling of the tertiary protein structure, application of recombinant, folded FH domains was inevitable. The obtained results of the mutant domain and linear epitope mapping were concurrent on both domains 19 and 20 of FH. The biggest reduction in target recognition could be observed when mutations to amino acids 1183-1198 of FH domain 20 were present (Figure 16). The hence emerging binding motif overlapped the location of the linear epitope 1177-1191, while on the C-terminal end of domain 20 the identified linear epitope 1207-1226 was validated by the reduced domain recognition of R1210A and R1215Q (Figure 17). These data are in line with previously published observations (Bhattacharjee et al. 2015, Nozal et al. 2016) that tested antibody binding to FH fragments both from aHUS patient sera and isolated IgG. Nonetheless, our results supplement literature data with the identification of the antibody binding sites on the amino acid level.

The location of the identified epitopes corresponds to the described impairment of the cell-associated regulator functions of FH in aHUS. The mutation Q1139A on SCR domain 19 and two of the linear epitopes (1157-1171; 1177-1191) involve amino acids reported to participate in the binding of C3b (Kajander et al. 2011, Morgan et al. 2011), hence explaining the reduced C3b recognition of FH in the presence of autoantibodies (Jozsi et al. 2007). Furthermore, the linear epitope on the C-terminal end of domain 20 (amino acids 1207-1226) covers the reported heparin and sialic acid binding sites of FH (Kajander et al. 2011, Blaum et al. 2015), which suggests a hindered attachment to host surface sialic acids and heparin for autoantibody-bound FH. In addition to direct binding of surface elements, FH may be recruited to the host membrane by surface-bound pentraxins, too (Deban et al. 2008). The interaction with fluid phase PRMs

involves the middle SCRs 6-7 and the most C-terminal domains of FH (Sanchez-Corral et al. 2018). Namely, the reported PTX3 binding site on SCR 20 of FH involves amino acids 1180-1186 and 1198-1204 (Kopp et al. 2012), which partially overlaps with the identified autoantibody specific epitope 1177-1191. Since complement regulation through FH is fulfilled via discrimination between specific surface elements of intact host tissues and foreign moieties or damaged self-structures, inhibition of target recognition could lead to a severe dysfunction of AP regulation by FH. In the presence of autoantibodies, their simultaneous interference with both C3b binding and host-discrimination provides molecular basis for AP dysregulation on host surfaces, most probably via the inhibition of the C3b-sialic acid-FH complex formation (Hyvarinen et al. 2016). Moreover, due to impaired binding of surface-attached PRMs, antibodies may also interfere with the clearance of apoptotic and damaged cells (Kopp et al. 2012), further aggravating the overactivation of complement.

Similar to the patients' antibody titers at the presentation of aHUS (Table 5), the extent of autoantibody binding to the linear epitopes also showed a matching, however considerable difference in the individual samples. It is therefore important to mention that all three of the identified linear epitopes showed increased autoantibody binding compared to the background reactivity in every patient's serum. Besides, although the level of epitope specific antibodies decreased in disease remission, their binding pattern remained the same with autoantibody positivity (>110 AU/ml) in three of our patients, but no relapse recorded during the follow-up period. The documented presence of anti-FH antibodies in remission together with the reported possibility of anti-FH positivity in healthy individuals (Sinha et al. 2014) further corroborate the concept that in addition to the known predisposing factors of aHUS, an environmental trigger event is necessary to evoke the clinical manifestation of disease, not only by the first acute episode but also before relapses.

One of these hypotheses outlines the importance of the homozygous deficiency of *CFHR1*, which is by itself not a prerequisite for antibody production, yet it is a very common genetic alteration in aHUS patients with anti-FH positivity (Zipfel et al. 2007, Jozsi et al. 2008, Dragon-Durey et al. 2010, Geerdink et al. 2012, Hofer et al. 2013). Lack of FHR1 together with an environmental trigger (e.g. infection) was proposed to induce the formation of a neoepitope on FH domain 20, similar in structure to its

homologous region on FHR1 domain 5, and hence prompt the production of autoantibodies targeting this region (Bhattacharjee et al. 2015). Our results are in line with this hypothesis in regard to the observed similar pattern and extent of antibody binding to the linear epitopes of both FH (1177-1191) and FHR1 (276-290), despite 7 of our patients being deficient for *CFHRI*. Albeit the C-terminal domains of FHR1 have a lower avidity towards the anti-FH antibodies than their counterparts on FH (Bhattacharjee et al. 2015), the two proteins share a high sequence homology and FHR1 was reported to cross-react with the anti-FH antibodies as well as to neutralize their effect *in vitro* (Moore et al. 2010, Strobel et al. 2011). These observations further conform the observed similarities in the antibody binding epitopes of FH and FHR1 in our experiments. However, an important limitation of our study was the application of linear synthetic peptides with an equal accessibility, whereas under physiological circumstances some of the identified epitopes may be hidden in the cryptic conformation of FH. Furthermore, a considerable proportion of autoimmune aHUS patients do not carry a genetic alteration in the *CFHR* genes (Dragon-Durey et al. 2009, Moore et al. 2010, Strobel et al. 2011, Geerdink et al. 2012), while anti-FH antibodies may also bind additional domains of FH (Moore et al. 2010, Blanc et al. 2012), which together suggest the imperfection of our knowledge on the molecular background of disease pathogenesis.

To visualize the spatial composition of the linear antibody binding epitopes, we positioned them on the folded domains of FH together with the generated single-amino acid switches and known binding sites of C3b and sialic acid (Figure 17). Based on the tertiary structure of FH downloaded from the Protein Data Bank, we conclude that the identified epitopes are located close to each other on the folded domains of the complement regulator. The middle of fragment 1177-1191 protrudes from the surface of the protein with high accessibility of the amino acid residues 1185-1187, which could explain the significantly decreased antibody binding when point mutations are present in this region. The C-terminal epitope 1207-1226 forms a loop, centering in a turn structure of 1219-1222, and the residues of the turn are close to the C-terminal end of peptide 1157-1171 (1163-1167), just like the C-terminal residues of the linear epitope 1177-1191. All in all, the C-terminal residues of 1177-1191 and the turn region of 1207-1226 sandwich the middle of the 1157-1171 sequence and this configuration of the

linear epitopes overlaps the reported ternary complex of FH, sialic acids and the C3b thioester-containing domain (Figure 17). Therefore, binding of the autoantibodies to any of the linear regions either alters the conformation of the combined binding site or interferes with C3b and sialic acid recognition by FH through steric hindrance, a hypothesis further substantiated by the clustering of aHUS-associated FH mutations at this region (Bhattacharjee et al. 2015).

Taken together we have shown that the aHUS specific autoantibodies recognize three distinct linear epitopes on FH and one on FHR1, which show increased autoantibody binding not only in the acute phase of aHUS but also in disease remission. The presence of an autoantibody specific epitope on FHR1 underlines its role in aHUS pathogenesis, although its exact function in disease pathogenesis remains to be defined in future studies. We validated the presence of the autoantibody binding epitopes using recombinant FH domains 19-20 that carried aHUS-associated point mutations on the C-terminal domains of the protein. We found that the linear binding sites on FH are located close to each other in the tertiary structure, which may suggest the formation of a conformational antibody binding site during *in vivo* folding. The novelty of our work lies in the direct mapping of the autoantibody binding site on FH and FHR1 using synthetic peptides of the complement proteins, which in the long term may help us to design specific inhibitors of anti-FH antibodies and also adds to the exploration of the in detail mechanisms involved in aHUS pathogenesis.

## 7. CONCLUSIONS

In frames of our investigations we explored the role of pentraxins in TMA and the molecular background of complement dysregulation in autoimmune aHUS. We studied the role of PTX3 and CRP in association with complement consumption in the acute phase of TMA. In frames of this we provide a detailed description of acute phase-TMA patients' complement profile linked to changes in the systemic pentraxin levels. We report that PTX3 elevation is present in the acute phase of STEC-HUS, aHUS and secondary TMA but is exceptional in TTP, whereas an elevation in the systemic CRP level was detected regardless of disease etiology during the acute disease flare. We observed the highest acute phase mortality in secondary TMA patients, which was associated with high PTX3 but not CRP levels. Furthermore, laboratory signs of complement activation and consumption were detected in the majority of our patients, regardless of the etiological background of TMA. We show for the first time that AP and CP consumption are associated with elevated PTX3 in the acute phase of the disease, and we confirmed *in vitro* that PTX3 limits AP activity on the surface of red blood cells, with no effect on terminal pathway assembly during LPS-induced AP activation on ELISA plates. This is the first study where the association of PTX3 and CRP elevation has been investigated in a complement mediated disease *in vivo*, and thus our results provide a missing link between the numerous *in vitro* observations that described the interaction of PTX3 with the complement system under defined experimental conditions. Through the epitope analysis of anti-FH antibodies we identified three linear, extended autoantibody binding epitopes on FH and one on FHR1. These epitopes were recognized by both acute phase and remission phase serum autoantibodies of aHUS patients and their close sterical proximity on the folded FH domains suggests that they form of joint antibody binding site in the tertiary structure. The identified epitopes overlap with the reported clustering of aHUS-associated FH mutations, as well as with the FH fractions necessary for ligand binding, which provides molecular basis for the reported dysfunction of autoantibody-bound FH, ultimately leading to the dysregulation of the complement AP. In conclusion, we aimed for the investigation of specific molecular aspects of complement dysregulation in aHUS and TMA and our results hopefully add to the better understanding of the complex molecular mechanisms that contribute to the pathogenesis of these conditions.

## 8. SUMMARY

The complement system composes a main branch of the humoral immune response that plays an important role in the elimination of pathogens and altered self-structures. However, dysfunction or inefficiency of its regulatory mechanisms may lead to overactivation of the cascade with substantial host-tissue injury, such as endothelial damage in TMA. Since complement overactivation may contribute to the vascular damage in all forms of TMA, the detailed characterization of the molecular factors interfering with complement regulation is of particular importance in the research of this disease. In frames of our investigations we chose to explore the association of CRP and PTX3 elevation with complement consumption and disease activity in the acute phase of TMAs, and furthermore to analyze the interaction between FH and anti-FH autoantibodies to reveal the structural bases of FH inhibition in aHUS. We determined the systemic levels of CRP and PTX3 to reveal the potential association of the circulatory pentraxin levels with complement consumption and disease activity in the acute phase of TMA. In this regard we conclude that the acute phase of all etiological forms of TMA might be accompanied by the elevation of CRP, whereas PTX3 elevation is present in aHUS, STEC-HUS and secondary TMA but is exceptional in TTP. Both PTX3 and CRP correlated to laboratory markers of disease severity, whereas PTX3 elevation was even associated with the acute phase disease mortality in secondary TMA. PTX3 levels showed an association to laboratory signs of complement consumption, and inhibited the whole complement AP activity *in vitro*, which suggests a protective role of the locally released PTX3 at the site of endothelial damage in TMA. During the investigation of the FH anti-FH interaction we identified three linear epitopes on the C-terminal domains of the regulator and one on its related protein, FHR1. Based on the location of the identified epitopes on the folded domains of FH, we propose that the linear epitopes might jointly form a conformational antibody binding site in the tertiary structure that overlaps with reported ligand binding sites of FH. Antibody binding to the identified epitopes was detected both in the acute phase and remission of aHUS, and we recorded a similar extent of antibody binding to the homologous regions on FH and FHR1, confirming the previously reported cross-reactivity of FHR1 with the autoantibodies.

## 9. ÖSSZEFOGLALÁS

A komplement rendszer fontos szerepet játszik a patogén kórokozók és megváltozott saját struktúrák szervezetünkbeli való eltávolításában. A kaszkád szabályozó mechanizmusainak károsodása azonban túlaktivációhoz és saját szöveteink (például endotél sejtek) jelentős károsodásához és ezen keresztül komplement-mediálta betegségek, többek között trombotikus mikroangiopátiák (TMA) kialakulásához vezethet. A közelmúlt intenzív kutatási eredményei jelentősen hozzájárultak a TMA-k patogenezisééről szerzett ismereteink bővüléséhez és a betegség kialakulásában szerepet játszó molekuláris tényezők megismeréséhez. Mivel a komplement rendszer túlaktivációja minden TMA forma kialakulásában szerepet játszhat, a kaszkád regulációját károsító molekuláris mechanizmusok azonosítása különös fontossággal bír ezen betegségcsoport vizsgálatában. Munkánk során célul tűztük ki a szisztémás C-reaktív protein (CRP) és pentraxin-3 (PTX3) szint emelkedés összefüggésének vizsgálatát a betegségaktivitással és a komplement rendszer túlműködésének laboratóriumi jeleivel különböző etiológiájú TMA formákban. Ezt követően a H faktor (HF) és az ellene termelt autoantitestek közötti kapcsolatot karakterizáltuk, hogy feltérképezzük a HF gátlás molekuláris mechanizmusát atípusos hemolitikus urémiás szindrómában (aHUS). A pentraxin szintek TMA akut szakaszában és remissziójában való meghatározása során a következőket állapíthatjuk meg. Az etiológiai háttértől függetlenül emelkedett CRP szint jelen lehet minden TMA formában, PTX3 szint emelkedés azonban csak atípusos és típusos HUS-ban valamint szekunder TMA-ban volt kimutatható, trombotikus trombocitopéniás purpurában hiányzott. Mindkét pentraxin összefüggést mutatott a betegség súlyosságának laboratóriumi jeleivel. Emellett a PTX3 szint emelkedése korrelált a komplement konzumpció mértékével *in vivo* és csökkentette az alternatív út hemolitikus aktivitását *in vitro*, ami a lokálisan emelkedett PTX3 szint endoteliális károsodással szembeni védő szerepére utal TMA akut szakaszában. A HF és anti-HF antitestek kötődésének vizsgálata során azonosítottunk három lineáris antitest kötő epitópot a regulátor C terminális szakaszán és egyet a HF-ral homológ H faktor rokon-1 fehérjén. Az epitópok térbeli elhelyezkedése arra utal, hogy a lineáris struktúrák együttesen alkotják az antitest kötőhelyet a fehérje harmadlagos szerkezetén, és ezek átfedése a HF egyéb ligandjainak kötőhelyeivel alátámasztja az autoantitestek által okozott funkciókiesést.

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## 11. LIST OF PUBLICATIONS

### 11.1. Publications related to the Ph.D. dissertation of the candidate

- I. **Trojnár E**, Józsi M, Szabó Zs, Réti M, Farkas P, Kelen K, Reusz GS, Szabó AJ, Garam N, Mikes B, Sinkovits G, Mező B, Csuka D, Prohászka Z. Elevated systemic pentraxin-3 is associated with complement consumption in the acute phase of thrombotic microangiopathies. *Front Immunol.* 2019 Feb 25;10:240.
- II. **Trojnár E**, Szilágyi Á, Mikes B, Csuka D, Sinkovits Gy, Prohászka Z. Role of complement in the pathogenesis of thrombotic microangiopathies. *Magazine of European Medical Oncology* 2018 September, 11;3:227–234.
- III. **Trojnár E**, Józsi M, Uray K, Csuka D, Szilágyi Á, Milosevic D, Stojanović VD, Spasojević B, Rusai K, Müller T, Arbeiter K, Kelen K, Szabó AJ, Reusz GS, Hyvärinen S, Jokiranta TS, Prohászka Z. Analysis of Linear Antibody Epitopes on Factor H and CFHR1 Using Sera of Patients with Autoimmune Atypical Hemolytic Uremic Syndrome. *Front Immunol.* 2017 Mar 30;8:302.

### 11.2. Publications independent of the Ph.D. dissertation of the candidate

- IV. Petro CD, **Trojnár E**, Sinclair J, Liu ZM, Smith M, O'Brien AD, Melton-Celsa A. Shiga toxin (Stx) type 1a reduces the toxicity of the more potent Stx2a *in vivo* and *in vitro*. *Infect Immun.* 2019 Mar 25;87(4). pii: e00787-18.
- V. Bhattacharjee A, Reuter S, **Trojnár E**, Kolodziejczyk R, Seeberger H, Hyvärinen S, Uzonyi B, Szilágyi Á, Prohászka Z, Goldman A, Józsi M, Jokiranta TS. The major autoantibody epitope on Factor H in atypical Hemolytic Uremic Syndrome is structurally different from its homologous site in Factor H related protein 1 supporting a novel model for induction of autoimmunity in this disease. *J Biol Chem.* 2015 Apr 10;290(15):9500-10.

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