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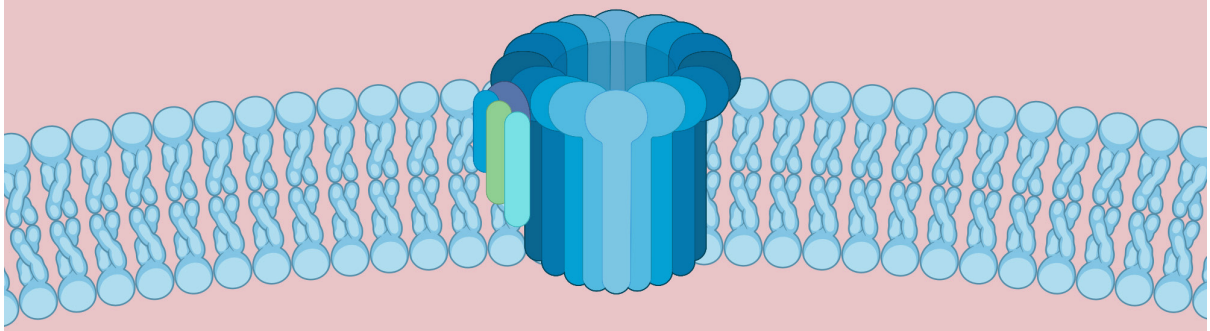

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LUND
UNIVERSITY

DOCTORAL DISSERTATION

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Title

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Abstract

The complement system is an essential effector of both innate and acquired immune responses. Due to its destructive potential, tight regulation is required. The contribution of complement has been associated with the pathogeneses in a wide range of diseases. In this thesis, aspects of complement activation were investigated in disorders characterized by thrombocytopenia.

In **papers I** and **II**, clinical characteristics, complement analyses, and genetic screening for rare variants encoding complement proteins were retrospectively investigated in a cohort suspected to suffer from atypical hemolytic uremic syndrome. In **paper I**, a population (n = 134) was identified whose phenotypes indicated the possibility of a diagnosis of complement-mediated atypical hemolytic uremic syndrome. In conclusion, laboratory complement analyses and clinical data were consistent with a possible underdiagnosis at the time of discharge. In **paper II**, recruited patients (n = 20) were subjected to follow-up investigations. Clinical outcomes and the phenotypical relevance of identified genetic variants were assessed. A diagnostic scheme compliant with the American College of Medical Genetics and Genomics guidelines was presented. In addition to identifying several previously described genetic variants, a novel likely disease-contributing missense variant in the *complement factor H* gene (c.3450A>G, p.I1150M) was identified. In conclusion, the study illustrated the risk of misdiagnosis and the importance of a comprehensive assessment to reach a diagnosis.

In **paper III**, complement and platelet activation biomarkers were prospectively investigated in thrombocytopenic patients (n = 43) receiving prophylactic platelet transfusions during inpatient care in the hematological department. Neither complement nor platelet activation was shown to correlate with the corrected count increment. In conclusion, complement and platelet activation were not demonstrated contributing to a poor post-transfusion platelet response.

In summary, this thesis has contributed to the growing knowledge of diseases potentially affected by complement activation.

Key words: thrombocytopenia; complement activation; classical complement pathway; alternative complement pathway; terminal complement complexes; complement dysregulation; thrombotic microangiopathy; atypical hemolytic uremic syndrome; platelet transfusion refractoriness.

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Cover photo is created with BioRender.com. With permission. It illustrates a membrane attack complex assembled within the lipid bilayer of a cell membrane.

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Till Ottar

Table of Contents

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals:

Paper I

Åkesson A, Blom AM, Klintman J, Zetterberg E. **Indications of underdiagnosis of atypical haemolytic uraemic syndrome in a cohort referred to the Coagulation Unit in Malmo, Sweden, for analysis of ADAMTS13 2007-2012.** *Nephrology (Carlton)*. 2017;22(7):555-561.

Paper II

Åkesson A, Martin M, Blom AM, Rossing M, Gabrielaite M, Zetterberg E, Klintman J. **Clinical characterization and identification of rare genetic variants in atypical hemolytic uremic syndrome: a Swedish retrospective observational study**. *Ther Apher Dial*. 2021. Electronic publication ahead of print.

Paper III

Åkesson A, Ljungkvist M, Martin M, Blom AM, Klintman J, Schött U, Zetterberg E, Kander T. **Biomarkers of complement and platelet activation are not correlated with the one or twenty-four hours corrected count increments in prophylactically platelet transfused hematological patients: a prospective cohort study**. *Platelets*. 2021. Electronic publication ahead of print.

Papers not included in this thesis:

Åkesson A, Zetterberg E, Klintman J. **At the Cross Section of Thrombotic Microangiopathy and Atypical Hemolytic Uremic Syndrome: a Narrative Review of Differential Diagnostics and a Problematization of Nomenclature.** *Ther Apher Dial*. 2017;21(4):304-319.

Contributions to the Papers

Paper I

Eva Zetterberg (E.Z.) and Jenny Klintman (J.K.) were responsible for study conception. E.Z., J.K., Anna M. Blom (A.B.), and I took part in study design. I inventoried referrals and samples received for ADAMTS13 analysis. I performed the medical record requisitions and the clinical screening inclusion process. I performed the laboratory complement screening, including western blots and enzyme-linked immunosorbent assays with the laboratory introduction and appreciated guidance from A.B., Myriam Martin (M.M.), Anna Foltyn-Zadura, and other colleagues in the Medical Protein Chemistry Group at the Department of Translational Medicine, Lund University, Malmö, Sweden. Nephelometry was performed with the appreciated assistance of colleagues at the Department of Clinical Chemistry and Pharmacology, University and Regional Laboratories, Region Skåne, Malmö, Sweden. I compiled the data, performed the statistics, interpreted the results, and wrote the manuscript.

Paper II

E.Z., J.K., A.B., and I took part in study conception and design. I recruited patients for inclusion and organized the reception and logistics for blood sampling. I performed the requisitions of medical records and questionnaires. I performed the enzyme-linked immunosorbent assays and the western blots. M.M. kindly performed the flow cytometry. The nephelometry was performed with biomedical analyst Paola Pascal's appreciated assistance at the Department of Clinical Chemistry and Pharmacology. The whole genome sequencing and variant calling were performed by Maria Rossing and Migle Gabrielaite at the Center for Genomic Medicine, Copenhagen University Hospital, Copenhagen, Denmark. I investigated and classified the detected genetic variants. I compiled the data, performed the statistics, interpreted the results, and wrote the manuscript.

Paper III

Thomas Kander (T.K.) and Ulf Schött (U.S.) in Anesthesia and Intensive Care at the Department of Clinical Sciences, Lund University, Lund, Sweden, were responsible for study conception. T.K., U.S., E.Z., A.B., and I took part in the study design. I was responsible for the reception of samples and the organization of aliquots. Further, I performed the enzyme-linked immunosorbent assays for the complement activation biomarkers. Biomedical analyst Marcus Ljungkvist kindly performed the enzyme-linked immunosorbent assays for the platelet activation biomarkers. I compiled the data, performed the statistics, interpreted the results, and wrote the manuscript.

Abstract

The complement system is an essential effector of both innate and acquired immune responses. Due to its destructive potential, tight regulation is required. The contribution of complement has been associated with the pathogeneses in a wide range of diseases. In this thesis, aspects of complement activation were investigated in disorders characterized by thrombocytopenia.

In **papers I** and **II**, clinical characteristics, complement analyses, and genetic screening for rare variants encoding complement proteins were retrospectively investigated in a cohort suspected to suffer from atypical hemolytic uremic syndrome. In **paper I**, a population (n = 134) was identified whose phenotypes indicated the possibility of a diagnosis of complement-mediated atypical hemolytic uremic syndrome. In conclusion, laboratory complement analyses and clinical data were consistent with a possible underdiagnosis at the time of discharge. In **paper II**, recruited patients (n = 20) were subjected to follow-up investigations. Clinical outcomes and the phenotypical relevance of identified genetic variants were assessed. A diagnostic scheme compliant with the American College of Medical Genetics and Genomics guidelines was presented. In addition to identifying several previously described genetic variants, a novel likely disease-contributing missense variant in the complement factor H gene (c.3450A>G, p.I1150M) was identified. In conclusion, the study illustrated the risk of misdiagnosis and the importance of a comprehensive assessment to reach a diagnosis.

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In summary, this thesis has contributed to the growing knowledge of diseases potentially affected by complement activation.

Selected Abbreviations

ACMG	the American College of Medical Genetics and Genomics	MAC	membrane attack complex (a.k.a. TCC)
ADAMTS13	a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13	MAF	minor allele frequency
aHUS	atypical hemolytic uremic syndrome	MAHA	microangiopathic hemolytic anemia
C	complement component	MCP/CD46	membrane cofactor protein
CCI	corrected count increment	PEX	plasma exchange therapy
CCP	complement control protein domain	PLC	platelet count
CF/F	complement factor	PLG	plasminogen
CFHR	complement factor H related protein	sCD40L	soluble CD40 ligand
CFP	CF properdin	SNV	single-nucleotide variant
C4d	complement degradation product c4d	sP-selectin	soluble P-selectin
DAT	direct antiglobulin test	sTCC	soluble terminal complement complex
DGKE	diacylglycerol kinase-epsilon	THBD	thrombomodulin
EHEC	enterohemorrhagic *Escherichia coli*	TMA	thrombotic microangiopathy
ELISA	enzyme-linked immunosorbent assay	TTP	thrombotic thrombocytopenic purpura
ESRD	end-stage renal disease	VTN	vitronectin
HLA	human leukocyte antigen	VUS	variant of unknown significance
HPA	human platelet antigen	WGS	whole genome sequencing
HSCT	hematopoietic stem-cell transplantation		

Preface

In this thesis, aspects of complement activation were investigated in diseases and conditions associated with thrombocytopenia. The causes of thrombocytopenia varied with the specific disorders. However, a mutual influence between platelet functions and complement activation has previously been established. This association is the fundament upon which several aspects of this thesis rest.

The thesis starts off by introducing the complement system from a historical point of view. It then introduces the main functions and elements of the three separate pathways that constitute the complement cascade. Further, relevant complement regulators are reviewed to subsequently introduce aspects of complement deficiency and dysregulation. Thenceforth, the potential relevance of complement activation is presented for diseases not specifically associated with acquired or inherited complement dysregulation. Current implementations and future aspects of complement therapeutics are thereafter reviewed. Then, functions of platelets and hemostasis are introduced, and central aspects of complement crosstalk are discussed. Thenceforth, brief reviews on general pathogenetic mechanisms and the contribution of complement activation are presented for the two separate disorders that constitute this thesis: atypical hemolytic uremic syndrome and platelet-transfusion refractoriness. Further, the general rationale and aims of the included papers are presented. Finally, methods, major findings, discussions, and future perspectives related to the separate papers are briefly recapitulated. Appended at the end of the thesis are the full reprints of included papers.

The Complement System

The complement system is an evolutionary preserved part of the innate immune system. In higher vertebrates, however, it is involved in important aspects of both innate and acquired immunity [1]. Additionally, it is engaged in crosstalk with other intravascular cascades, which reflects the origins of a common evolutionary ancestor for all plasma cascade systems [2,3]. During the last three decades, several noncanonical functions of complement proteins have been identified [4]. These scientific contributions have indeed humbled clinicians and scientists alike to question the prevailing idea that the complement system purely is an effector of certain archaic functions in our microbial defense. Today, elements of the complement cascade are regarded as a pluripotent battery of proteins supporting a multitude of aspects related to global, local, and cellular homeostasis.

The historical origin of the name traces back to its discovery in the late 19th century, following the synthesis of a series of proposed theses and antitheses. At the time, 'the phagocytic theory of Metchnikoff' prevailed, stating that the function of bacterial defense was synonymous with the capability of immune cells to ingest invading microbes [5]. However, in 1891, Buchner et al. proposed the addition of a 'humoral theory' following the finding of a heat-labile factor capable of neutralizing bacteria in the blood. This factor was named 'alexin', which etymologically corresponds to the Greek meaning of 'fending off' (something) [6,7]. This theory was further supported by Jules Bordet, who demonstrated that immune-mediated lysis of bacteria required the presence of two factors: one heat-labile (similar to alexin) and one heat-stable (in retrospect, an immunoglobulin) [7]. Following the introduction of the side-chain theory of antibody formation by Paul Ehrlich in 1897 [8], it was proposed that these antibodies were multiplied and released as freely circulating receptors upon antigen recognition by immune cells. The antibodies were subsequently fixed to bacterial surfaces as well as to a heat-labile component. The component was at the time renamed to 'complement' on account of it being a complementary contributor to the antibody-mediated killing of pathogens (the heritage of the historical term 'alexin' is, however, presently upheld by the name of a contemporary pharmaceutical company). It was proposed that the antibody and the complement factor formed an enzymatic complex capable of killing bacteria. Bordet argued that the antibody-antigen complex was supported by the nonspecific binding of a single type of complement factor. In contrast, Ehrlich anticipated the potential presence of multiple complementary factors in serum.

In the first half of the 20th century, the discovery of discrete complement components was described by Ferrata and Brand. They proposed two fractions of complement: a midpiece (today corresponding to the complement component 1 complex [C1]) and an endpiece (corresponding to C2) [7]. However, the recognition of three separate complement pathways and the identification of specific cardinal complement components were not fully appreciated until the 1950-60s [9]. During the 1980-90s, the functional characterization of the terminal complement complex (TCC; also termed as the membrane attack complex [MAC]) [10] and various complement receptors and regulators [11] accelerated the understanding of the system [12].

An Outline of Functions and Elements

The complement system and its regulators are now regarded as a family of approximately 50 various proteins [12-16]. Novel complement control proteins are continuously discovered [16] and methodological advances during the last decades have provided unprecedented support to the exploration of molecular structures [17], origins of production [4,18,19], functions [18], and interactions [20].

In recent years, the formerly unanticipated contribution of complement proteins has proven relevant to various physiological processes beyond the microbial defense. One significant finding has been the involvement of complement in the embryonic development of the central nervous system by regulating neurogenesis [21] and neuronal migration [22]. Additionally, complement proteins have been demonstrated to be involved in synaptic plasticity of the adult brain [23]. The latter has led to the discovery of C1q/C3b-tagged synapses and simultaneously decreased density of synapses in various tauopathies, including Alzheimer's disease [24]. This finding has ignited complement research related to cognitive impairment [25], schizophrenia [26], multiple sclerosis [27] and neuromuscular diseases [28]. Additionally, complement activation has been indicated in lipid and glucose metabolism [29], revealing involvement in various overlapping aspects of inflammation, obesity [30], insulin resistance [31-33], and complications to unregulated diabetes mellitus [34,35].

Even though primarily being regarded as acute phase reactants synthesized by hepatocytes in the liver, complement proteins have nevertheless been induced in various tissues. In addition to the central nervous system [18], the secretion of complement proteins has been indicated in peripheral myeloid cells [36] wherein C1q has been synthesized in the absence of serine proteases C1r and C1s [37,38], thereby executing functions independently of these molecules, e.g., related to the clearance of apoptotic cells and cellular debris as well as to the augmentation of adaptive immune responses [38]. Recently, the involvement of complement in tissue regeneration and remodeling [39] has also been indicated following discoveries related to bone homeostasis [40], in

which osteoclast differentiation by anaphylatoxin receptor C5aR [41], local generation of C3 by osteoblasts [42], and the accelerated osteogenic differentiation of mesenchymal stem cells in the presence of anaphylatoxins (C3a/C5a) [43] have been demonstrated. Similarly, the contribution of complement has been recognized in skeletal muscle differentiation and regeneration [44,45] as well as in liver regeneration [46,47]. Further, the intestinal expression of complement proteins has proposed a potential link between complement and inflammatory bowel disease [48]. However, the most distinguished finding during the last years arguably has been the discovery of intracellular complement activity, primarily illustrated by the immune-modulating functions of intracellular C3 in T cells [49,50]. It is proposed that intracellular complement proteins likely are involved in different aspects of cellular metabolism within cells throughout our entire organism, which has given rise to a hypothetical organelle named 'the complosome' [51].

As complement components and regulators constitute an evolutionary preserved system shared by many living organisms, it should come as no surprise that complement proteins are involved in many fundamental aspects of development, and cell differentiation and regeneration [52]. Pathogenetically, it is reflected by the association between complement deficiencies and some malformations [53,54].

Despite new discoveries, the canonical function of complement proteins still is attributed to the amplifying cascade of enzymatic events that follows the initial activation of the upstream complement pathways. Principally, it results in recruitment (chemotaxis) and enhanced phagocytosis (opsonization) by innate immune cells, increased (anaphylatoxin-mediated) inflammation, support to cellular and humoral adaptive immune cells (immunomodulation) [55], and ultimately, it ends in the assembly of membrane attack complexes which subsequently disrupt the targeted pathogen (lysis of membrane) (**Figure 1**). Below is the simplified outline of complement activation by the three initiating pathways. It should be regarded as a presentation of primary elements, enzymatic events, and functions. It does not claim to be exhaustive, neither does it go into biomolecular details. For thorough biomolecular perspectives, several reviews have been published [56-59].

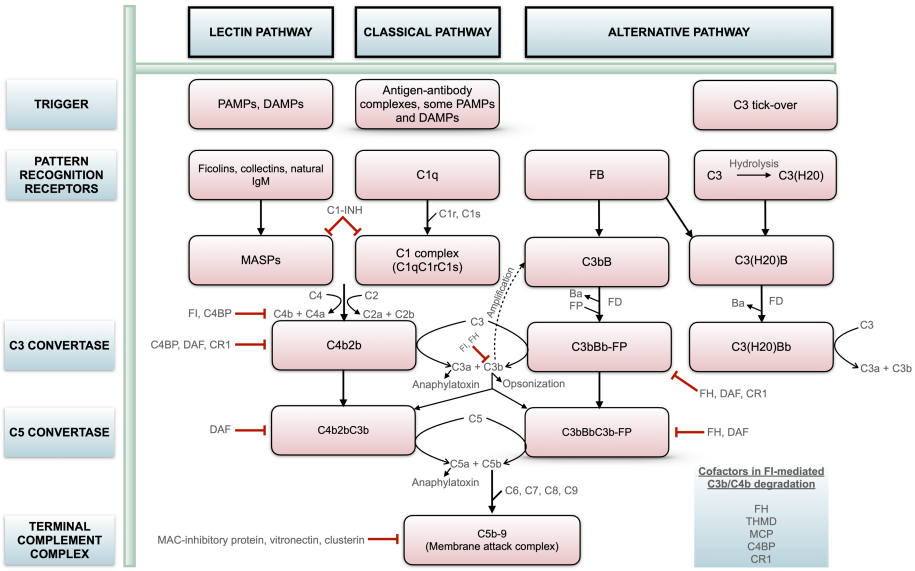

Figure 1. Schematic outline of the complement cascade
The flow chart represents activation of the three separate pathways, which converge into the terminal complement pathway at the level of C5 convertases to ultimately result in the assembly of the terminal complement complex/membrane attack complex. Also presented are complement inhibitors and factor I-related cofactors. Please refer to the text for details. (C: complement component; CR1: complement receptor 1; C4BP: C4b-binding protein; DAF: decay-accelerating factor; DAMP: danger-associated molecular pattern; F: complement factor; Ig: immunoglobulin; MASP: mannose-associated serine protease; MCP: membrane cofactor protein; PAMP: pathogen-associated molecular pattern;THMD: thrombomodulin)

The Classical and Lectin Pathways

Pattern recognition receptors (PRR; e.g., toll-like receptors) are molecules that are abundantly expressed on membranes of cells constituting epithelial barriers but also on migrating cells policing extracellular spaces in various tissues (e.g., macrophages and dendritic cells). These receptors recognize conserved molecular structures of viruses, bacteria, parasites, and fungi denoted pathogen-associated molecular patterns (PAMPs) [60]. From an evolutionary standpoint, these motifs have likely been crucial for pathogen survival. They exhibit molecular or subcellular characteristics that are absent on host cells (e.g., lipopolysaccharides for gram-negative bacteria). Moreover, a class of soluble extracellular receptors called pattern recognition molecules (PRM) that are freely circulating in the bloodstream further facilitates the recognition of PAMPs. These molecules include ficolins and collectins, the latter which includes mannose-binding lectins (MBLs). Moreover, natural immunoglobulin (Ig) M also has the capacity to function as a PRM. Thus, it can activate both the lectin and classical pathways [61]. In addition to PAMPs, PPRs and PRMs also sense host-specific molecules derived from endogenous cells undergoing apoptosis/necrosis or enduring stress with the impending

loss of cellular integrity. These molecules are denoted danger-associated molecular patterns (DAMPs) [60]. The interactions of PPRs and PRMs with PAMPs and DAMPs initiate signaling cascades, leading to the local release of proteins, including chemotactic cytokines, which ultimately results in increased inflammation and recruitment of phagocytes.

Complement activation by the classical (CP) and lectin (LP) pathways is initiated by the activation of complexes formed between pathway-characteristic PRMs and a protease complex. The binding results in activation of the protease complex which cleaves soluble C4, resulting in the deposition of C4b molecules on the targeted surface. In the classical pathway, complement danger sensor C1q is the PRM. It circulates in complex with two types of serine proteases: C1r and C1s. The C1 complex initiates the classical pathway as it recognizes the complex of an antigen and IgG or IgM [62], anionic phospholipids expressed on apoptotic/necrotic cells [63], C-reactive proteins or the lipopolysaccharides of gram-negative bacteria [64]. The interaction between C1q and its binding patterns leads to a conformational change, which activates serine protease C1r to further cleave C1s. Activated C1s subsequently demonstrates the capacity to cleave C4, which results in the minor split product C4a and the major split product C4b. The latter exposes a reactive thioester bond previously hidden within the C4 molecule. Its exposure enables the covalent binding of C4b to amino or hydroxyl groups present on the targeted surface. The conformational change of C4b further promotes the binding of proenzyme C2, which results in the formation of the C4bC2 proconvertase. Within the proconvertase, C2 is cleaved to C2a and C2b by C1s, subsequently resulting in the formation of a C3 convertase [58,62]. In the lectin pathway, freely circulating PRMs (e.g., ficolins and MBLs) recognize and bind to conserved carbohydrate molecules on the targeted surface [65]. These PRMs circulate in complex with MBL-associated serine proteases (MASPs) which are functionally equivalent counterparts to the C1r and C1s of the classical pathway. Upon binding to a targeted surface, this complex effectuates the cleaving of C4 to the C4a and C4b split products, ultimately forming the same C3 convertase by the same mechanisms as the classical pathway. Thus, this C3 convertase is named CP/LP C3 convertase (C4b2b). This convertase cleaves C3 molecules, which are complement components abundantly present in circulation. Similar to C4, the C3 molecule does have an embedded, highly reactive thioester bond. After the cleaving of C3 to the minor C3a and major C3b split products, the thioester bond becomes exposed in the latter molecule, consequently enabling covalent binding to adjacent surfaces in like manner as C4b [58,66].

The Alternative Pathway

Circulating C3 molecules are continuously activated at a slow rate. It is called C3 tick-over and depends on the spontaneous hydrolysis of the C3 molecule. Hydrolyzed C3,

i.e., C3(H20), is a C3b-like molecule capable of binding factor B (FB) [58] to form a proconvertase that makes the FB molecule susceptible to proteolytic cleaving by factor D (FD). This results in the formation of a fluid-phase transient C3 convertase, C3(H20)Bb, that can cleave yet more C3 molecules. It has been proposed that the constitutively active tick-over mechanism guarantees a vigilant defense against microbes, enabling the complement system to enhance its functions rapidly whenever it is required. Recently, however, the relevance of the transient C3 convertase has been questioned, suggesting that *in vivo* formation of C3(H20) is less important for canonical alternative pathway (AP) activation. Instead, it may be important for cell-cell interactions by functioning as a ligand for C3 receptors and as a source of C3 for the intracellular complement reservoir [67], or as a contact-mediated complement activator [68]. Regardless, the primary function of the alternative pathway is that of an amplifier of the classical and lectin pathways. Approximately 80% of the total complement response is estimated to originate from the alternative pathway [69]. Following the proteolytic cleaving of C3 by the CP/LP C3 convertase, molecules of C3b are deposited on a targeted surface and subsequently bind to FB to form the AP proconvertase (C3bB). This proconvertase is activated by the cleaving of FB, which depends on the proteolytic activity of fluid-phase FD. It results in the Ba and Bb split products, of which the latter forms the AP C3 convertase (C3bBb) in association with C3b. The ability of C3b to form an AP C3 convertase amplifies the catalyzation of C3, thus resulting in the generation of additional C3b molecules. This characteristic of the alternative pathway constitutes the amplification loop which augments the initiated activation of the classical and lectin pathways [58]. The AP C3 convertase has a short half-life (approximately 90 s) during normal physiological conditions. However, with the binding of factor Properdin (FP) during increased complement activation, the convertase becomes more stable, increasing its half-time ten-fold [70].

The Terminal Pathway

A high density of deposited C3b molecules on a targeted surface facilitates the binding of newly generated C3b to adjacent C3 convertases. This results in a conformational change within the C3 convertase, shifting its convertase specificity from C3 to C5 molecules [58]. Thus, a C5 convertase has been assembled: C4b2bC3b or C3bBbC3b for convertases originating from the CP/LP and AP pathways, respectively. The C5 convertase cleaves circulating C5 to C5a and C5b split products. The C5 molecule lacks the internal thioester bond present in C3 and C4 molecules. Rather, the resulting C5b split product sequentially forms a complex with circulating C6 and C7 molecules (C5b-7). This leads to a conformational change within the latter molecule, thereby exposing a previously hidden hydrophobic site, which consequently increases the affinity for hydrophobic structures. This enables the stable association of the C5b-7 complex with the targeted membrane. However, penetration of the complex into the

interior of the lipid bilayer requires the binding of a C8 molecule, which gives rise to the anchored C5b-8 complex. This complex enables the insertion of multiple C9 molecules, which increases the size of the formed pore structure. Thus, the MAC/TCC (C5b-9) has been assembled [56,58,71]. The depositions of multiple membrane attack complexes jointly cause a rapid change in the osmotic pressure, thus preventing the cell (or enveloped virus) from maintaining its integrity. Ultimately, this results in the complete lysis of the targeted surface.

Canonical Functions

The terminal goal of complement activation is the assembly of membrane attack complexes. However, the complement cascade provides additional critical functions for microbial defense and against the immunogenicity of autoantigens.

The minor split products C3a and C5a, generated from the proteolytic cleaving of C3 and C5 molecules, respectively, are highly proinflammatory molecules, also referred to as anaphylatoxins. Of the two, C5a has the greatest proinflammatory potential. These molecules function as ligands for G protein-coupled complement receptors (C3aR and subtypes of C5aR) expressed on immune and nonimmune cells. These interactions have cell-specific implications, i.e., immunomodulatory effects [72], including toll-like receptor signaling, interplay with the stimulation and regulation of lymphocytes [73], enhancement of tissue inflammation by increased vasodilatation and cytokine expression, the release of histamines from mast cells and granular enzymes from neutrophils, and increased expression of adhesion molecules on the surfaces of endothelial and epithelial cells. Anaphylatoxins also possess a chemotactic function by the receptor-mediated guiding of immune cells in the direction of increased inflammation [74]. Minor split product C4a, which shares structural similarity with C3a and C5a, does not seem to exhibit any proinflammatory properties [75].

Major split product C3b constitutes an essential component required for the formation of convertases. Additionally, it is a key player in complement-mediated opsonization. C3b is rapidly degraded to inactivated C3b (iC3b; inactivated as in lost capacity to integrate into a convertase), which effectively is the most abundant opsonin. In order of falling relevance, C1q, C4b, and the sequential iC3b degradation products C3dg and C3d exert similar functions. Opsonin-tagged cell surfaces facilitate ingestion primarily by increased phagocytosis of coincidently IgG-coated particles. However, it may also enhance the engulfment of microbes and compromised host-cells independently of immunoglobulins [57]. The complement receptor of the immunoglobulin superfamily (CRIg), mainly presented by Kupffer cell macrophages [76], has a high affinity for C3b and iC3b, whereas iC3b also exhibits affinity for complement receptors 3 and 4 (CR3/4) [77] which primarily are presented on the myeloid subset of leukocytes, and secondarily on NK cells and activated T and B

lymphocytes. Ultimately, complement-mediated opsonization accelerates the elimination of microbes and reduces the risk of autoimmunity [78].

While only to a lesser extent contributing to enhanced phagocyte engulfment of tagged microbes, the generation of the weak opsonins C3dg and C3d promotes a shift in receptor affinity towards CR2 expressed on B cells. Consequently, C3d-tagged particles may further contribute to different aspects of the adaptive immune response, including lowering the activation threshold for B lymphocytes and the induction of immunoglobulins [57,79].

To conclude, the complement system has the potential to directly lyse membranes, initiate inflammation, recruit immune cells, modulate adaptive immune responses and increase the phagocyte potential by the opsonization of intruders and unwanted host cells. This array of functions requires tight regulation to prevent the system from self-inflicted harm.

Complement Regulation

There are some autoinhibitory mechanisms incorporated into the complement system. First, generated anaphylatoxins which have not bound to complement receptors, are rapidly converted to C3a-desArg and C5a-desArg by the activity of carboxypeptidases. This results in negligible and markedly reduced bioactivity for C3a and C5a molecules, respectively [80]. Consequently, this confines their effects to the proximity of the complement activating event. Secondly, the high reactivity of the externalized C3 thioester bond correspondingly dictates C3b to swiftly form a covalent bond to convenient structures on adjacent surfaces, thus significantly restricting the radius of C3b depositions. Thirdly, the half-life of the important AP C3 convertase is limited in the absence of stabilizing FP.

However, specific and fine-tuned regulation is also required. Membrane-bound complement regulators are abundantly expressed to suppress spontaneous complement activation and amplification on uncompromised host cells. Further, several important fluid-phase complement regulators limit complement activation. Apart from FP, there are no known positive regulators of the complement cascade [70]. Given the strong amplification effectuated by the alternative pathway, regulation of C3b and the AP C3 convertase is particularly important.

Several important complement regulators are mapped to the *regulators of complement activation* (*RCA*) gene cluster located on chromosome 1q32. This is a genomic region that likely has expanded by repeated genomic duplications [81]. Thus, proteins encoded by genes in this cluster share significantly similar tertiary configurations. Three proteins are membrane-bound regulators: membrane cofactor protein (MCP), decay-accelerating factor (DAF), and CR1. One is a fluid-phase regulator: C4b-binding

protein (C4BP). Additionally, complement regulator factor H (FH) exhibits essential inhibitory functions. It is mapped adjacent to the *RCA* locus, located in tandem with the mapping of factor H-related proteins 1-5 (FHR1-5) in the *CFH-CFHR* gene cluster (1q31). This cluster stems from incomplete genomic duplications of exons encoding specific *CFH* domains. Genes in this locus lie in a head-to-tail arrangement and are particularly prone to complex genomic rearrangements [82]. Given the conflicting evidence of their regulatory profiles, the exact functions of the small FHR proteins are yet poorly understood [83].

Further, important fluid-phase regulators mapped outside the *RCA* gene cluster are C1 inhibitor (C1-INH; 11q11-13), factor I (FI; 4q25), vitronectin (17q11), and clusterin (8p21). Two additional important membrane-bound regulators are MAC-inhibitory protein (11p13) and thrombomodulin (THMD; 20p11). The latter is primarily involved in anticoagulation [84]; however, it also exhibits complement regulatory properties.

The functional properties of complement regulators principally either consist of decay-accelerating activity, thus expediting the dissociation of assembled C3 and C5 convertases, or cofactor activity primarily supporting FI-mediated degradation of C3b and C4b [78].

Membrane-bound Regulators

a. **MCP** demonstrates cofactor activity for factor I-mediated degradation of C3b and C4b [78,85].

b. **CR1** is multifunctional. Functions unrelated to complement regulation include its expression on circulating erythrocytes which facilitates the removal of C3b/C4b-opsonized pathogens and immune complexes, primarily by transporting them for ingestion in the mononuclear phagocyte system of the liver and spleen [86]. Further, it modulates B cell responses [87]. In terms of complement regulatory functions, it exerts decay-accelerating activity for both types of C3 convertases and cofactor activity for factor I-mediated degradation of C3b and C4b [88]. However, it also provides cofactor activity to CFI in the further sequential degradation of iC3b to C3c and C3dg [78].

c. **DAF** demonstrates decay-accelerating activity for both types of C3 and C5 convertases [78,88].

d. **MAC-inhibitory protein** inhibits the association of C9 molecules with the C5b-8 complex, thus preventing MAC/TCC formation on host cells [78,89].

e. **THMD** enhances cofactor activity of FH, thus increasing the CFI-mediated degradation of C3b [90]. It is also an important cofactor for the thrombin activatable fibrinolysis inhibitor (TAFI)-mediated deargination of anaphylatoxins C3a and C5a [78,84].

Fluid-phase Regulators

a. **C1-INH** irreversibly binds and inactivates C1r, C1s, MASP-1, and MASP-2 [78]. Although participating in complement regulation of the classical and lectin pathways, it is a multifunctional serine protease inhibitor that also exerts central inhibiting functions directed against elements in the kinin-kallikrein system [91].

b. **C4BP** is a major regulator of the classical and lectin pathways. However, it also exerts functions related to coagulation by the binding of protein S [92]. It demonstrates the ability to prevent the assembly of the CP/LP C3 convertase by binding C4b. It also demonstrates decay-accelerating activity for the CP/LP C3 convertase and cofactor activity for factor I-mediated degradation of both C3b and C4b [78].

c. **FH** is a major regulator of the alternative pathway. It demonstrates the ability to prevent the assembly of the AP C3 convertase by competitively binding to C3b against FB. Further, it demonstrates decay-accelerating activity for the AP C3 and C5 convertases and cofactor activity for factor I-mediated degradation of C3b. Importantly, although an important fluid-phase regulator, it may also bind to the membranes of host cells to assist in the inhibition of membrane-bound complement activation [78,93-95].

d. **Vitronectin** demonstrates the ability to bind to the C5b-7 complex and interacts with existent C9 molecules to prevent further polymerization [78,96].

e. **Clusterin** demonstrates the ability to integrate into the C5b-7 complex, which prevents its insertion into the lipid bilayer of the targeted membrane. Further, it may bind to C8 and C9 molecules to inhibit the initiation of C9 polymerization [78,97].

f. **FI** is a major complement regulator. It degrades C3b and C4b with the help of cofactors, as elaborated above [78,98].

To conclude, several different proteins are needed to guarantee tight regulation of the complement cascade. Thus, it is not hard to conceptualize that congenital or acquired complement dysfunctions (including but not limited to genetic alterations) potentially may contribute to clinical disease.

Complement Deficiency and Dysregulation

By analogy with the expanding discoveries of complement proteins involved in various aspects of homeostasis, complement deficiencies and dysregulation correspondingly are indicated in a broad spectrum of clinical diseases.

Complement Deficiencies

Complement deficiencies are considered either primary (i.e., hereditary) or acquired [99]. Acquired deficiencies may be related to increased consumption due to inflammation or the development of complement-specific autoantibodies. It may also be related to decreased production on account of liver failure or cachexia. Generally, acquired deficiencies manifest with fully functional proteins at reduced but not undetectable protein levels. Commonly, acquired deficiencies are presented as a pan-deficiency, with many complement proteins affected coincidently.

For primary complement deficiencies, the mode of inheritance mainly is autosomal recessive [99]. Complement deficiencies either result in loss of production, inhibited secretion, or the presence of a misfolded, nonfunctional protein. Homozygous and compound heterozygous deficiencies typically result in nonfunctional proteins or undetectable protein levels. Heterozygous deficiencies sometimes manifest with reduced but not nonexistent functions or protein levels. Approximately 65% of patients with complement deficiencies suffer from recurring invasive infections, particularly with encapsulated bacteria [100]. Deficiencies associated with the terminal complement pathway have been related to meningococcal disease [101]. Moreover, deficiencies of the classical pathway have been associated with an increased risk of infection with *Streptococcus pneumoniae* [100]. Deficiencies related to the alternative pathway, including the FI and FH complement regulators (secondarily resulting in an increased risk of infection due to low C3 levels caused by persistent consumption), also predispose to infections with *Streptococcus pneumoniae*. However, recurrent risk of infection with other encapsulated bacteria, such as *Hemophilus influenza,* has also been described [101]. Deficiencies of the lectin pathway have been related to recurrent infections with encapsulated bacteria, however, also with fungal, viral, and protozoan microorganisms [102].

Further, complement deficiencies have been implicated in autoimmune disorders. Patients with systemic lupus erythematosus (SLE), dermatomyositis, glomerulonephritis, and rheumatoid arthritis have all been associated with deficiencies of classical pathway components [103,104]. Detectable autoantibodies are often prevalent in these diseases, and for SLE in particular, a fraction of these autoantibodies have shown an affinity for C1q [105,106]. Indeed, in some studies, 90% of patients exhibiting C1q deficiency have demonstrated an SLE or SLE-like phenotype [107].

The corresponding phenotypes have also been correlated with approximately 75% of patients with C4 deficiencies and 60% of patients with C1r/C1s deficiencies [107]. Interestingly, MBL deficiencies have also been identified in patients with SLE and rheumatoid arthritis [108]. It is hypothesized that the development of autoimmune disease is fundamentally conditioned by increased immunogenicity against autoantigens. Various complement deficiencies impair the clearance of compromised host cells, cellular waste, and immune complexes. Failure to clear unwanted material may lead to tissue injuries, resulting in the release of autoantigenic material. Additionally, complement deficiencies have been observed to result in loss of complement-mediated peripheral B cell tolerance [109]. Thus, increased quantities of autoantigens may elicit an abnormal lymphocyte response, resulting in the production of autoantibodies which ultimately may progress and reveal a specific autoimmune phenotype [110]. Of note, however, far from all patients with autoimmune diseases demonstrate complement deficiencies, obviously suggesting multifactorial models of disease development and penetrance.

To conclude, increased risks of infectious and autoimmune diseases have been implied in patients with primary complement deficiencies. Although theoretically appealing, replacement therapy has so far unfortunately been proven unsuccessful, partly due to the rapid metabolism of infused recombinant proteins [111].

Deficiencies in Membrane-bound Complement Regulation

Membrane-bound complement regulators are important in order to inhibit an excessive complement response on host cells.

Deficiencies in the expression or function of MCP result in a loss of cofactor activity for FI. This contributes to dysregulated membrane-bound complement activation and predisposes the development of complement-mediated atypical hemolytic uremic syndrome (aHUS) [85,112]. However, patients with an isolated heterozygous deficiency in the MCP-encoding *CD46* gene exhibit less frequent progress to end-stage renal disease (ESRD) compared to patients with isolated heterozygous mutations in other genes relevant for complement-mediated aHUS [113,114].

Deficiencies in the function of THMD have been described in patients with complement-mediated aHUS [90]. In addition to cofactor activity for FH, it exerts counteracting hemostatic functions. It simultaneously acts as a cofactor for thrombin-mediated activation of protein C and as an indirect inhibitor of fibrinolysis by cleaving TAFI to its active form [115]. Various mutations in the *THMD* gene have anecdotally been associated with bleeding diathesis [116,117] and arterial [118,119], but not venous [120,121], thrombosis.

Recently, a homozygous loss-of-function mutation in the DAF-encoding *CD55* gene has provided the molecular explanation for a rare autosomal recessive disease named CHAPLE syndrome (complement hyperactivation, angiopathic thrombosis, and early-onset protein-losing enteropathy). It is associated with a loss of DAF expression. Patients have demonstrated increased cell surface depositions of complement products as well as elevated plasma levels of the potent anaphylatoxin C5a [122]. Moreover, off-label use with complement inhibiting therapy (eculizumab) has resulted in clinical and laboratory improvements [123].

Deficiency in the expression of the MAC-inhibitory protein due to extremely rare autosomal recessive germline mutations in the *CD59* gene has so far only been described in 13 individuals globally [124,125]. Debuting during early infancy, the resulting loss of membrane-bound complement regulation (on all host cells) leads to chronic hemolytic anemia, thromboembolic events, and the development of chronic inflammatory demyelinating polyneuropathy. It shares erythrocyte phenotype with paroxysmal nocturnal hemoglobinuria (PNH). However, PNH develops due to the clonal expansion of hematopoietic stem cells that have acquired somatic mutations in the *PIGA* gene. These mutations affect the expression of an anchoring protein (glycosylphosphatidylinositol) essential for many membrane-bound proteins, MAC-inhibitory protein and DAF included [126]. Consequently, hematopoietic cells lack entirely the membrane-bound complement regulation effectuated by these proteins. As anucleated cells cannot repair membrane damage, erythrocytes are particularly susceptible to complement-mediated harm. Thus, patients suffering from PNH manifest with the characteristic intravascular hemolysis also observed with primary CD59 deficiency. Of note, PNH was the first disease treatment with eculizumab was approved for [126,127]. Promising clinical improvements have been observed with the off-label use of eculizumab for primary CD59 deficiency [128,129].

To conclude, conditions associated with the loss of essential membrane-bound regulators emphasize the importance of stringently regulated complement activation on host cells. The absence of membrane-bound regulation illustrates the potency of the complement cascade.

Complement Dysregulation

Unsurprisingly, diseases accompanied by an excessive complement response are primarily associated with dysregulation of the alternative pathway. Due to its amplifying capability, the alternative pathway possesses an incredible destructive potential. However, overactivation mainly occurs locally, not systemically, and its specific location is partially explained by tissue-specific characteristics conditioning these cells particularly susceptible to complement dysregulation.

Alternative pathway-mediated complement dysregulation is an engine that drives the pathogenesis of several renal and ocular diseases. The underlying etiologies includes genetic alterations in genes encoding alternative pathway components (gain-of-function) and regulators (loss-of-function). However, it may also be attributed to the development of complement-specific autoantibodies. A combination of rare genetic variants and polymorphisms is commonly observed. The combination of genetic alterations may result in an increased risk of developing specific phenotypes.

The cardinal example of such a disease is complement-mediated aHUS. It is an ultra-rare (incidence approximately one per two million person-years) [130] and life-threatening renal disease, in which unrestricted complement activation takes place at the level of the renal vascular endothelium [130,131]. It is associated with acute flare-ups characterized by acute renal failure, microangiopathic hemolytic anemia, and thrombocytopenia. Histological examination of renal biopsies typically reveals thrombotic microangiopathy. If not promptly treated, it is associated with a high risk of developing ESRD. Additional aspects of complement-mediated aHUS will be presented in a subsequent section.

Moreover, C3 glomerulopathy (C3G) is another group of ultra-rare renal diseases (incidence approximately one per million person-years) which comprises two major subtypes of glomerulopathies: dense deposit disease (DDD) and C3 glomerulonephritis (C3GN) [130]. Dysregulation primarily occurs at the level of the AP C3 convertase. It results in depositions of C3b and degradation products predominantly taking place in glomeruli. Characteristic deposits are identified by electron microscopy, and on account of their appearance, they are classified as either DDD (dense osmiophilic intramembranous deposits) or C3GN (light dense, subendothelial and/or subepithelial deposits, amorphous mesangial and/or paramesangial deposits) [130]. However, immunofluorescence is the required method to make a diagnosis (C3 staining 100x greater than staining for immunocomplexes) [132]. C3 glomerulopathies are often accompanied by the C3 nephritic factor (C3NeF), an autoantibody that stabilizes the AP C3 convertase independently of FP. Other stabilizing and inhibiting autoantibodies directed against C5, C3b, FB, and FH have also been described [133]. Additionally, several rare genetic variants and polymorphisms, encoding alternative pathway proteins, have been demonstrated in patients with C3G disease. While both DDD and C3GN exhibit dysregulated fluid-phase complement activation, C3GN also indicates increased membrane-bound complement activation on the mesangium and the glomerular basement membrane. However, due to overlapping clinical and pathological features, some authors suggest that C3GN and DDD should be regarded as different representations at the same disease continuum [134]. The onset of C3GN and DDD may occur both in children and adults; however, the latter disease is for unknown reasons more frequently diagnosed at young age [135]. Both conditions follow a chronic course, accompanied by continuous alternative pathway-driven disease activity

and a gradually deteriorating renal function. The estimated 10-year renal survival is 50% [133]. Although the molecular background of C3G significantly overlaps with complement-mediated aHUS, the phenotypes vary greatly. This is illustrative of the different implications of fluid-phase and membrane-bound complement dysregulation. While a persistent fluid-phase complement dysregulation, resulting in renal complement depositions, mainly fuels the pathogenesis of C3G, complement-mediated aHUS is prone to a violent overactivation occurring episodically on membranes of endothelial cells in the renal microcirculation, often initiated by complement-triggering events, such as infections.

Regarding ocular diseases, age-related macular degeneration (AMD), the major cause of blindness in the elderly population of developed countries, is occasionally associated with coincident complement mutations [136]. The disease is characterized by a progressive loss of central vision following degeneration of photoreceptor cells in the macula. The degeneration results from the accumulation of pebbles of retinal debris between the retinal epithelial lining and Bruch's membrane. The retinal waste products are referred to as drusen. In addition to aging processes and environmental factors, such as smoking and obesity, genetic factors are also relevant to disease development. Alterations in several complement genes, particularly those related to the alternative and terminal pathways, have been associated with an increased risk of developing AMD [137]. Additionally, results from functional studies have suggested that alternative pathway dysregulation is important. For instance, increased levels of split products C3a and Ba have been shown in the aqueous humor of AMD patients [138]. Of note, several of the rare genetic variants and polymorphisms associated with AMD are also frequent in complement-mediated aHUS. Consequently, for unknown reasons, the same genetic alterations may remarkably result in two very different phenotypes.

To conclude, complement dysregulation encompasses a wide phenotype. Complement-mediated renal disease is often accompanied by high mortality and renal morbidity. Complement-inhibiting therapy has proven effective in diseases wherein treatment previously was limited to plasma exchange therapy, general immunosuppression, and supportive care, often with dismal outcomes as a result.

Complement beyond Dysregulation

The utilization of complement-inhibiting therapeutics has expanded rapidly in recent years. Complement activation has been proven an effective target in diseases that are not characterized by intrinsic complement dysregulation. The etiologies and phenotypes of the diseases vary. However, they are commonly referred to as immune-mediated inflammatory diseases. Therefore, they share the propensity of complement activation. The contribution of complement activation varies greatly within the immune-mediated inflammatory disease panorama, however, in some conditions,

complement activation has proven essential for disease development. Hence, the existence of an extensive series of pending drug trials related to these diseases [139]. For instance, in a subset of patients with generalized myasthenia gravis, detectable acetylcholine receptor (AChR) autoantibodies have been demonstrated. The presence of AChR-specific autoantibodies has been positively correlated with classical pathway-induced TCC depositions in the neuromuscular junctions in both animal models and patients [28]. Experimental myasthenia gravis models have demonstrated that the depositions of TCC are required for disease development. Complement component $3^{-/-}$, $C4^{-/-}$, and $C5^{-/-}$ mice do not develop disease [140]. Drug trials have shown clinically relevant and sustained neurological improvements in patients undergoing complement-inhibiting therapy [141,142].

Additionally, most patients with neuromyelitis optica disorders are accompanied by detectable aquaporin 4 (AQP4)-specific autoantibodies. In animal models, the binding of these antibodies to AQP4 expressed on astrocytes results in complement-mediated astrocyte injury, secondary inflammation, and neuron loss induced by bystander lysis (TCC depositions on adjacent cells) [143]. In a recent drug trial, patients receiving eculizumab exhibited a lower risk of disease relapse [144].

Moreover, the intravascular hemolysis observed with the IgM-mediated cold agglutinin disease (CAD) has been firmly associated with TCC-mediated cell lysis [145]. Anecdotal evidence supports the off-label use of eculizumab, demonstrating remission of hemolytic anemia and the absence of exacerbations during the treatment period [146]. Further, *ex vivo* analyses of samples from CAD patients have demonstrated the potential use of upstream classical pathway inhibition with C1s inhibitors to suppress classical pathway-mediated opsonization. Decreased opsonization may reduce the rate of extravascular hemolysis [147], which is proposed to be the probable cause of anemia in stable CAD disease.

Further, antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) is a category of diseases that has been associated with the destruction of small- and medium-sized blood vessels, primarily engaging the renal circulation, which results in rampant and progressive glomerulonephritis. The diseases are accompanied by autoantibodies targeting specific cytoplasmic proteins in neutrophil granulocytes (proteinase 3 or myeloperoxidase). It consists of three distinct phenotypes: granulomatosis with polyangiitis (GPA; previously known as Wegener's granulomatosis), microscopic polyangiitis (MPA), and eosinophilic granulomatosis with polyangiitis (EGPA; formerly known as Churg-Strauss syndrome) [148]. In an animal model of myeloperoxidase antibody-mediated autoimmunity, the relevance of C5aR1 for the generation of inflammation has been indicated by decreased neutrophil retention and thus attenuated neutrophil-mediated bursts of reactive oxygen species within glomerular capillaries in $C5aR1^{-/-}$ mice [149]. Additionally, a recent randomized controlled trial investigated the use of a C5aR inhibitor (avacopan) in newly diagnosed

or relapsing cases of ANCA-associated vasculitis. It was demonstrated that the use of C5aR inhibition effectively could replace high-dose glucocorticoid tapering without increased risk of relapse in these patients. However, serious adverse events occurred at similar rates in both study arms [150].

Additional examples of potentially significant contributions of complement activation in inflammatory conditions have been indicated in animal models of Alzheimer's disease. These models demonstrate the potential significance of complement activation in diseases that are not elicited by autoantibodies (autoimmunity) but rather driven by autoinflammation. In a mouse model of Alzheimer's disease, the neuron loss and cognitive deficits of $C5aR1^{-/-}$ animals were suppressed relative to C5aR1-expressing animals without observed reduced load of amyloid plaques in the former [151]. Interestingly, ribonucleic acid (RNA) sequencing of isolated microglia cells indicated a decreased induction of inflammatory genes and increased phagosomal-lysosomal gene expression. The authors suggested that the prevention of a C5aR1-induced program of inflammatory gene expression decreased inflammation while maintaining crucial phagocytic functions essential for the clearance of apoptotic cells and extracellular debris. As previously described, the role of upstream classical complement functions has proven important for synaptic pruning as a mechanism of neural plasticity [23]. This highlights the importance of carefully selecting the targets of complement inhibition based on the molecular rationale of disease development.

Finally, the door to cancer therapeutics is just barely opened with the realized significance of complement in cancer immunology. Recent evidence indicates that cancer cells inhibit opsonization and TCC-mediated lysis by overexpressing membrane-bound complement regulators [152-155]. Therefore, cancer cells may escape the regulated necrotic cell death that is part of immune surveillance. It is suggested that the expression of membrane-bound complement regulators may function as biomarkers of malignant transformation in cancer [156]. Therefore, complement-inhibiting therapy may prove relevant for the treatment of numerous cancer types in the future. One emerging area of investigation is the possible use of bifunctional therapeutic antibodies, which, in addition to tumor-specific affinity, also possess the capacity to block membrane-bound complement regulators [157]. In theory, this would increase the chance of further downstream signaling, optimizing both innate and adaptive immune responses, potentially resulting in more effective elimination of malignant cells.

To conclude, complement as a driver or significant contributor of disease is to various extents evident in virtually all immune-mediated inflammation. Thus, complement activation may prove pathogenetically relevant also in the absence of complement dysregulation. The classical pathway has been proven relevant for antibody-mediated effects in some autoimmune diseases. Additionally, the potent anaphylatoxin effects of complement split products C3a and C5a may be relevant for sustaining and

exacerbating autoinflammatory responses, which may be critical for the underlying pathogeneses in several diseases.

Complement Therapeutics

As previously described, the complement system constitutes an interesting target for pharmacological intervention. To date, complement-targeted therapy is approved for clinical use in four different diseases by the European Medical Agency and the Federal Drug Administration: PNH [127], complement-mediated aHUS [158,159], generalized myasthenia gravis [160], and neuromyelitis optica spectrum disorders [144]. It is a fast-moving field, comprising several drugs in the pipeline, which together cover a wide range of indications. The approval of eculizumab (Soliris®, Alexion Pharmaceuticals) for PNH 15 years ago was the steppingstone potentially marking the birth of the complement revolution era. The implications of a dysfunctional complement system fundamentally either result in increased activation or decreased regulatory activity. Regardless of the consequence, the general aim of complement therapeutics is obviously to restore the equilibrium between the two counteracting mechanisms. More specific aims are to reduce the impact of disease-dependent mechanisms induced by a normally functioning complement system, as has been elaborated in the sections above.

Eculizumab is a recombinant humanized monoclonal antibody targeting the terminal pathway by inhibiting C5. Thus, it impairs the production of anaphylatoxin C5a and the downstream assembly of TCC. Previously, concerns were raised regarding the risk of increased infection rates with encapsulated bacteria, particularly *Neisseria meningitidis*, in patients regularly receiving therapy. Thus, meningococcal vaccines are integrated as part of treatment protocols [161]. In 2019, Socié et al. published an extensive ten-year cumulative observational analysis comprising approximately 28,000 patient-years in total, based on Alexion's pharmacovigilance database for eculizumab. Seventy-six cases of meningococcal infection were reported, of which eight cases were fatal (0.03 cases per 100 patient-years). Additionally, the most reported severe nonmeningococcal infection was streptococcus-mediated pneumonia. Considering the previous discussion on complement and cancer, the reporting rates for malignancies were stable throughout the study period [162].

Eculizumab is one of the most expensive ultra-orphan drugs available on the market, and it has been the subject of health economic considerations advising against government coverage. To date, the use of eculizumab and its second-generation derivative ravulizumab (Ultomiris®, Alexion Pharmaceuticals) are generally unadvised for PNH and complement-mediated aHUS by 'Rådet för Nya Terapier' (the New Therapies Council) in Sweden according to their reports dated February 5, 2021

[163,164]. Stated reasons are primarily the inferior cost-effectiveness of therapy (i.e., due to the pricing and the very low prevalence of concerned diseases). Exceptions for use are only granted based on case-specific circumstances following discussions with a national council. Ravulizumab was engineered to have a longer-lasting effect than eculizumab. Otherwise, it is a functionally identical molecule [165].

However, several new therapies in the pipeline will render the position of eculizumab less dominant. For instance, the administration of zilucoplan recently demonstrated positive neurological outcomes in patients with generalized myasthenia gravis [141]. From a molecular perspective, this synthetic peptide belongs to an entirely different drug class. However, by its mechanism of inhibiting C5 cleavage, it is functionally comparable to the monoclonal antibodies mentioned above. The stranglehold of Alexion Pharmaceuticals is further challenged by several pharmaceutical companies, all competing for the trial inclusion of a few eligible patients. Efforts are being made to develop both monoclonal antibodies [147,166,167], recombinant and conjugated proteins [168,169], small molecular peptides and peptidomimetics [170-172], and possibly nanobodies [173] against complement-driven disease. Nanobodies and small peptides/peptidomimetics potentially will enable better tissue access, e.g., as needed for transportation through the blood-brain barrier [174] or for limiting the C3b-mediated extravascular opsonization and hemolysis in PNH [175]. Additionally, by combining complement-inhibiting molecules with peptides or antibodies that specifically bind to tissue-specific molecules, the therapeutic approach can be directed towards the site of interest and avoid complement inhibition in other locations [169,176]. Finally, a vast arsenal of therapeutic options is not only needed due to the current costs of available therapies. It is also needed because of the C5 polymorphism identified in the Japanese population, which renders eculizumab therapy ineffective [177]. Polymorphisms resulting in impaired affinity for specific complement-inhibiting antibodies may be revealed in other populations too. Therefore, a multitude of therapy options may prove relevant for the tailoring of treatment in the future.

To conclude, current research on complement-inhibiting therapy potentially will result in new ingenious approaches to combat several potentially complement-driven diseases in the future. By tailoring the treatment, we may guide the pharmaceutical interventions towards affected tissues and inhibit disease-driving mechanisms without affecting other crucial functions of the complement system.

Hemostasis

Hemostasis represents the intricate homeostatic balance between maintaining blood fluidity and simultaneously protecting the organism from significant blood loss in the event of vascular injury. It is a process orchestrated by several competing and supporting conductors, including platelets, endothelial cells, the coagulation cascade, anticoagulant proteins, and the fibrinolytic system [178].

Platelets

Platelets (thrombocytes) are the smallest and second most prevalent blood component in circulation, corresponding to an estimated concentration of 150-350 x 10^9/L in healthy individuals [179]. Once released into the bloodstream, platelets circulate for approximately seven to ten days before they are spent and removed primarily by splenic sequestration [180]. Platelets are generated from megakaryocyte precursors residing in the bone marrow niche. Megakaryocytes are mature large bone marrow cells with lobated nuclei derived from the myeloid lineage of progenitor cells (megakaryocyte-erythroid progenitors) [181]. However, evidence also suggests that megakaryocytes can arise directly by differentiation from the common multipotent progenitor, thus bypassing the intermediary common myeloid progenitor [182]. Cell differentiation and cellular interactions within the bone marrow niche are complicated and encompass the interplay of several transcription factors, growth factors, and cytokines. However, the stimulation by thrombopoietin via thrombopoietin receptors on megakaryocyte progenitor cells is crucial for the generation of mature megakaryocytes and platelets. The generation of a single mature megakaryocyte can result in the production of several thousand platelets by cytoplasmic fragmentation [183]. Under normal physiological conditions, approximately 200 billion platelets are produced daily in humans [184]. Platelets are anucleated, discoid cell fragments. However, they possess the capacity to synthesize a limited number of proteins by the use of messenger RNA (mRNA) present in their cytoplasm [185]. More importantly, they already contain various molecules formerly produced in the precedent megakaryocyte. These molecules are primarily stored in three different types of granules: dense granules, lysosomes, and α-granules. Dense granules contain small molecules primarily important for initiation and propagation of primary hemostasis, e.g., calcium, adenosine diphosphate, and

serotonin [186]. Lysosomes contain enzymes that degrade glycosaminoglycans, glycolipids, and glycoproteins [187]. Finally, α-granules are the most abundant type of granule. They contain a heterogeneous mix of membrane-bound proteins, growth factors, and cytokines which are released upon platelet activation to assist in a vast array of functions: hemostasis, inflammation, and host defense [188]. Two important constituents are membrane-bound P-selectin and CD40 ligand (CD40L), respectively. Both proteins translocate to the cell surface following fusion of the granule with the cell membrane during platelet activation. Platelet P-selectin provides an important interaction route between hemostasis and inflammation by binding to P-selectin ligand 1 (PSGL-1), primarily expressed on neutrophils, eosinophils, and monocytes. Thus, platelets bound to compromised endothelial cells initiate leukocyte rolling and adhesion by P-selectin/PSGL-1 interactions. It is an important mechanism of leukocyte extravasation, which is reflected by the reduced post-ischemic neutrophil infiltration observed with P-selectin$^{-/-}$ mice in an animal model of renal ischemia [189]. Platelet expression of CD40L provides an important link between innate and adaptive immune responses by influencing antigen-presenting cells and the priming of T-cells [190]. Additionally, CD40L enhances endothelial surface expression of adhesion molecules (e.g., E-selectins and vascular cell adhesion molecules) and endothelial secretion of cytokines, which secondarily promote further migration of leukocytes to the site of vascular insult. Moreover, CD40L has been shown to induce monocyte expression of tissue factor (TF), which augments coagulation. To some extent, both P-selectin and CD40L are also released as soluble proteins, either directly by the secretion of granule-derived proteins [191] or by the proteolytic shedding of membrane-bound proteins following interactions with ligands/receptors [192,193]. Although the membrane-bound expressions of P-selectin and CD40L also are evident on endothelial cells [193] and lymphocytes [194], respectively, platelets are the main source of their soluble equivalents. Thus, levels of soluble P-selectin and soluble CD40L have proven valuable as plasma biomarkers of platelet activation [195,196].

Glycoproteins are abundantly expressed on the surface of platelets, and they are important for various platelet-mediated interactions. Some glycoproteins exert key functions in maintaining hemostasis following vascular injuries. The GPIb-IX-V complex, GPVI, and GPIIa/IIIa (also denoted integrin αIIbβ3) are critically important receptors for mediating platelet adhesion, activation, and aggregation in primary hemostasis [179].

The Platelet Activation Cascade and Primary Hemostasis

Under resting conditions, uncompromised endothelial cells maintain hemostasis with the support of a monolayer of membrane-bound proteins. These include triphosphate

diphosphohydrolases (which hydrolyze extracellular adenine nucleotides) [197], coagulation inhibitors (e.g., thrombomodulin) [120] and heparan sulfate proteoglycans (which, i.a., bind circulating antithrombin) [198]. Moreover, endothelial cells constitutively release prostacyclin and nitric oxide, which prevent the activation of platelets that are generally circulating near the endothelial lining to activate rapidly whenever the endothelial integrity is disrupted.

Upon vascular damage, fibrillar collagen present in the subendothelial, extracellular matrix is exposed to the bloodstream, and the endothelial secretion of platelet-inhibiting molecules is locally decreased. If the vascular injury results in high shear stress, plasma-derived ultra-large von Willebrand factor (vWF) multimers unfold and deposit on the exposed collagen [188]. The unfolding vWF forms a bridge between exposed collagen and platelets by exposing several binding sites for the GPIb-V-IX receptor complex [199]. The activation of endothelial cells and platelets also results in the local release of vWF from Weibel-Palade bodies and α-granules, respectively, which further facilitate the bridging of platelets and collagen [200]. The initial formation of a hemostatic plug leads to lower shear stress and decreased platelet velocity, ultimately enabling the firmer adherence of platelets via the binding of GPIa/IIa to collagen [188]. The lowered shear stress facilitates GPVI-collagen interactions which initiate intracellular platelet activation pathways [180]. The activation of platelets leads to increased cytosolic $Ca^{2+,}$ which results in cytoskeletal changes, transforming the resting discoid shape of platelets to an activated state with numerous pseudopodia, thus increasing the surface area available for adhesion and aggregation [201]. Moreover, it results in the exposure of negatively charged membrane phospholipids named phosphatidylserines, which create binding sites for plasma coagulation factors [202]. Additionally, platelet activation leads to the generation of platelet-activating thromboxane A2, which binds to platelet thromboxane receptors [203], and the translocation of intracellular granules, which results in the release of mediators, such as ADP, serotonin, fibrinogen, and coagulation factors [188]. Mediators released from dense granules activate yet more platelets via G protein-coupled receptors [179]. By the binding of GPVI to collagen, a conformational change also exposes binding sites enabling platelet aggregation. Platelet aggregation is initiated by GPIIb/IIIa receptors binding to plasma-derived vWF multimers, which secondarily bind to GPIb-V-IX receptor complexes of circulating platelets [199]. Consequently, the build-up of the first layer of platelets results in the recruitment, activation, and aggregation of additional platelets. Further, during platelet activation, there is a shift of affinity in the GPIIb/IIIa receptor, favoring the binding of fibrinogen [204]. Ultimately, this leads to the generation of a fibrinogen-rich platelet plug. Molecules of TF exposed on the activated endothelium and expressed by attracted immune cells result in local thrombin generation, which may activate platelets directly via protease-activated receptors 1 (PAR1) and 4 (PAR4) [205]. However, more importantly, thrombin is essential for the formation of a stable clot. This constitutes secondary hemostasis, and it is a

simultaneously initiated process catalyzed by thrombin that ultimately results in the conversion of fibrinogen to a stabilizing mesh of fibrin [206].

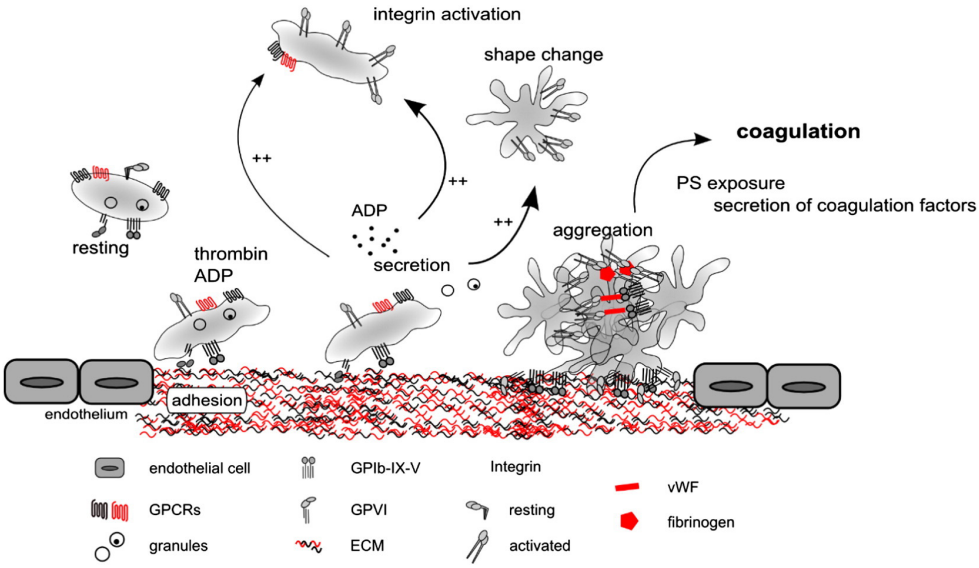

Figure 2. Platelet activation and primary hemostasis
Illustration of an endothelial injury and the subsequent platelet adhesion, activation, and aggregation, ultimately leading to the formation of a hemostatic plug. Please refer to the text for details. The simultaneously initiated coagulation cascade stabilizes the formed thrombus. Reprinted under OA CC-BY 3.0 from [207]. (ADP: adenosine diphosphate; GP: glycoprotein; GPCRs: G protein-coupled receptors; PS: phosphatidylserine; vWF: von Willebrand factor)

Secondary Hemostasis

The conversion of fibrinogen to fibrin is central to the formation of a stable clot. This is a tightly regulated process catalyzed by activated coagulation factor II (FIIa; thrombin). Thrombin is generated from its inactive proenzyme prothrombin (FII). Its enzymatic activity constitutes the final step in a cascading series of events in which proenzymes are cleaved to their enzymatically active equivalents by the sequential activation of upstream coagulation factors. This process is partitioned into three phases: the initiation phase, the amplification phase, and the propagation phase.

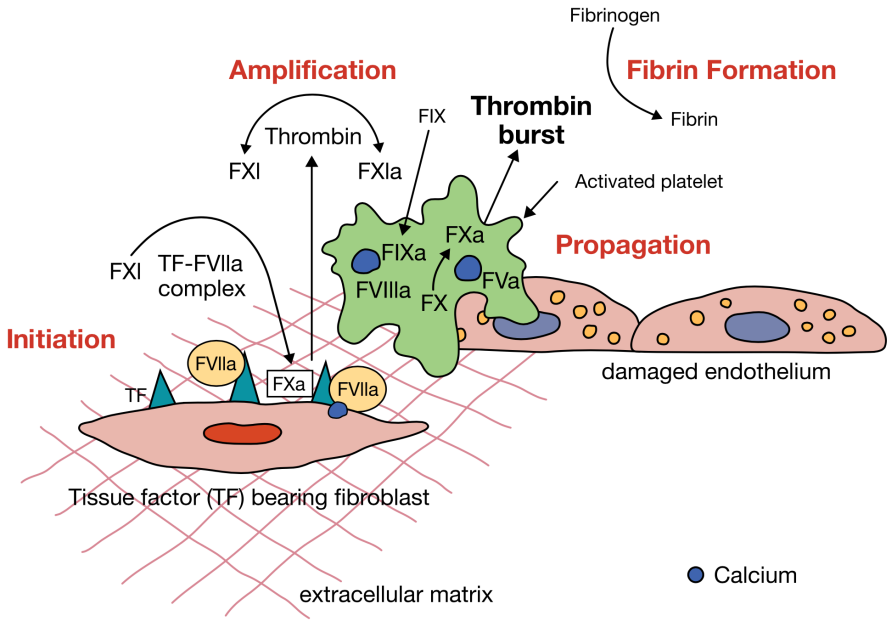

Figure 3. The cell-based model of coagulation
In contrast to the schematic representation of a two-pathway (intrinsic and extrinsic) cascade of serially activated coagulation factors, the cell-based model emphasizes the requisite of engaged cell surfaces for initiation, amplification, and propagation of coagulation *in vivo*. Roman numerals refer to the specific inactive and active (a) coagulation factors. Reprinted with permission from SAGE Publications, Inc. [208].

During vascular injury, TFs expressed by subendothelial cells are exposed to the bloodstream. This marks the start of the initiation phase, during which TF binds to FVII and enables its transformation to FVIIa [209]. Membrane-bound complexes of TF-FVIIa can proteolytically activate FIX, FX, and yet more FVII. The latter results in the increased formation of TF-FVIIa complexes. Factor Xa, bound to TF-bearing cells, cleaves FV, subsequently forming a FVa-FXa complex, referred to as the prothrombinase complex. This complex cleaves FII at a low rate, thus producing small quantities of FIIa, which diffuse away from the TF-bearing cells to adjacent membranes [210]. Factor IIa activates adjacent platelets, induces the release and activation of FVIII from vWFs, and activates molecules of FV and FXI that are primarily bound to the surfaces of platelets [211]. The effects that follow the initial generation of FIIa constitute the amplification phase. Platelet activation then marks the start of the propagation phase. Factor XIa bound to the surface of activated platelets subsequently activates additional FIX molecules. Further, activated platelets expose phospholipid phosphatidylserine molecules on their surfaces, which subsequently facilitate the assembly of FIXa-FVIIIa and FVa-FXa complexes [206,211]. This results in a rapid

increase in the generation of FXa and FIIa, respectively. In addition to converting fibrinogen to fibrin, FIIa also activates FXIII. This results in FXIIIa-mediated cross-linking of the generated fibrin strands, ultimately creating a network of fibrin molecules which increases the strength and elasticity of the hemostatic plug [212].

Anticoagulation and Fibrinolysis

To avoid excessive coagulation, the cascading process of secondary hemostasis is tightly regulated. First, endothelial membrane-bound regulators exert several inhibitory functions. Tissue factor pathway inhibitor impairs the activity of FXa and TF-FVIIa complexes [213]. Additionally, thrombomodulin acts as a cofactor for thrombin-mediated activation of protein C, thus subsequently inhibiting thrombin generation, which is further augmented in the presence of protein C's cofactor protein S [115]. Secondly, endothelial heparan sulfate proteoglycans bind circulating antithrombin molecules, secondarily resulting in the inhibition of several activated coagulation factors and complexes: FIIa, FXa, FIXa, FXIa, and TF-FVIIa [198]. Thirdly, activation of secondary hemostasis simultaneously leads to increased fibrinolysis. The fibrinolytic system prevents excessive coagulation following the acute loss of endothelial integrity. Additionally, it is responsible for the controlled degradation of the fibrin-rich clot as the endothelial integrity gradually is restored during healing. Fibrinolysis is induced by endothelial-derived tissue plasminogen activators (tPA) and monocyte/macrophage-derived urokinase plasminogen activators (uPA) [214]. These enzymes cleave the inactive proenzyme plasminogen to an active protease denoted plasmin. Plasmin cleaves and degrades the formed fibrin polymers. The process of fibrin degradation is also tightly regulated by several inhibitors, such as TAFI, which reduces the generation of plasmin, and plasminogen activator inhibitor-1, which inhibits the enzymatic activity of tPA and uPA [215]. Additionally, autoregulatory mechanisms of the fibrinolytic system are at work. For instance, compared to fluid phase tPA, the enzymatic activity of fibrin-bound tPA demonstrates a 500-fold increase in catalytic efficiency [214].

Complement and Secondary Hemostasis: Crosstalk

The complement system and the systems of secondary hemostasis (coagulation and fibrinolysis) share phylogenetic origins. Genes responsible for these systems are derived from genes encoding an archaic serine protease cascade that predates vertebrate evolution. Phylogenetic studies have indicated that the central cleaving processes of C3 and fibrinogen constitute the most ancient elements [216]. Therefore, it is not surprising that the systems share structural and functional similarities [217]. According to several (primarily *in vitro*) studies, there is significant crosstalk between the systems [216-220]. For instance, several coagulation factors demonstrate complement

convertase activity [216]. Thus, it is suggested that the two systems have remained functionally intertwined during vertebrate evolution. The relevance of crosstalk under physiological conditions, however, remains to be elucidated. The contribution of complement activation, and the effect of complement-inhibiting therapy, in thrombosis-related diseases, such as PNH [126], complement-mediated aHUS [130], and antiphospholipid syndrome [221], suggest the existence of a clinically relevant influence.

Platelets and Complement: Crosstalk

Lately, the contributions of platelets in tissue remodeling [222], inflammation [223], and the modulation of immune responses [188] have been recognized. However, the importance of platelets in maintaining hemostasis has been appreciated ever since the initial discovery by Giulio Bizzozero in 1882 [224]. In this respect, the interplay between platelets and the coagulation system is of critical importance. Therefore, reports on crosstalk between platelets and the ancestrally related complement system are unsurprising. In fact, that platelets express complement receptors and that complement proteins may induce platelet activation have been known for years [225,226]. Below is a short exposé of investigations supporting the existence of relevant platelet-complement crosstalk.

Influence of Complement on Platelets

Both *in vitro* and *in vivo* studies have reported on the existence of TCC-mediated stimulation of platelets. Sublytic formation of TCC on the membranes of platelets results in transient membrane depolarisation [227], translocation of granules [228], increased expression of phosphatidylserines, and enhanced platelet-derived thrombin generation [227]. Thus, TCC mediates various aspects of platelet activation, including the release of mediators supporting platelet activation, such as serotonin, and secondary hemostasis, such as FV [229]. Lytic quantities of TCC assembled on the surface of platelets mediate the release of highly procoagulant platelet-derived microvesicles and the exposure of binding sites for FVa, as is seen in *in vitro* models of PNH [230]. Accordingly, the administration of eculizumab has been shown to reduce the production of procoagulant microvesicles in PNH [231].

Deposits of C1q on the surface of platelets may induce platelet rolling by interacting with vWF [232]. Moreover, C1q binding to C1q receptors on the surface of platelets initiates platelet activation and aggregation. Platelet expression of these receptors is also upregulated during increased shear stress, a common effect of vascular injury [233].

In vivo studies have reported the capacity of C3 to activate platelets directly in a TCC-independent manner. In a study by Subramaniam et al., $C3^{-/-}$ mice demonstrated prolonged bleeding times and reduced platelet activation while $C5^{-/-}$ mice exhibited no apparent defects in platelet activation or primary hemostasis [234]. By contrast, in a model of extracellular histone-induced arterial thrombosis in mice, $C5^{-/-}$ animals did not suffer from lethal thrombosis and demonstrated less thrombocytopenia compared to $C5^{+/+}$ animals. The extent of platelet activation was not directly investigated in the latter study [235].

Factor properdin has demonstrated relevance for the formation of platelet-leukocyte aggregates in models of thromboinflammation [236].

The binding of complement degradation products to CR2 [237] and CR3 [238] expressed on platelets has been reported to induce platelet activation. Additionally, anaphylatoxin C3a and its derivative C3a-desArg have demonstrated the capacity to initiate platelet activation and aggregation *in vitro* [239,240]. Additionally, C3a and C4d deposited on platelets in trauma patients have been correlated to enhanced platelet aggregation [241].

Finally, MASP-1 shares structural similarities with thrombin [242] and has been shown to induce platelet activation [243]. On a similar note, complement split product C4a has demonstrated the ability to bind to PAR1 and PAR4 [244]. For platelets, the binding of C4a to PAR1 or PAR4 may potentially induce platelet-activating pathways in a similar manner as thrombin [205].

Influence of Platelets on Complement

Activated platelets secrete chondroitin sulfate, which triggers complement activation and secondarily may activate yet more platelets [245].

Platelet α-granules have been reported to contain C3 split products [246] and FD [247], which may result in increased local inflammation and complement activation upon secretion. However, α-granules have also been reported to contain FH, which impairs alternative pathway activation [248]. Therefore, the exact role and implications of secreted complement proteins following platelet activation remain to be elucidated.

Recently, activated platelets have been shown to bind to ficolins, leading to the activation of MASP-1 and MASP-2. Secondarily, this may result in increased complement activation mediated by the lectin pathway. However, MASP-1 and MASP-2 have also been demonstrated to potentially contribute to thromboinflammation [249].

A casein kinase released from activated platelets has been shown to alter C3 by means of phosphorylation. This is suggested to enhance C3b-mediated opsonization of immune complexes [250].

Platelet-derived vWF has been demonstrated to impair complement activation, thereby revealing a novel mechanism protecting endothelial cells from excessive complement depositions [251].

Finally, the increased expression of P-selectin on the surface of activated platelets has demonstrated the potential to activate complement, primarily by fixing C3b molecules generated in fluid-phase cleaving [252].

To conclude, there is a mutual influence between complement and platelet activation. However, the current understanding is fragmented, and it heavily relies on *in vitro* investigations. The intersection between complement activation, inflammation, platelets, and coagulation is an intricate field of study. In the future, it will likely prove important for the understanding of thromboinflammation in trauma, sepsis, and diseases associated with systemic thromboembolism. Additionally, the potential significance of complement activation in conditions associated with platelet activation, consumption, and isolated thrombocytopenia remains to be clarified.

Brief Reviews

Below are summaries of the diseases and conditions upon which the investigations of this thesis are based. They share the common denominator of thrombocytopenia; however, as will be shown, the pathogeneses are diverse.

Atypical Hemolytic Uremic Syndrome

Patients presenting with direct antiglobulin test (DAT)-negative hemolytic anemia, schistocytosis (fragmented erythrocytes on peripheral blood smears), thrombocytopenia, and acute renal failure are generally suspected to suffer from diseases that may reveal thrombotic microangiopathy (TMA) in the histological examination of renal biopsies. However, since renal biopsies are not performed in the acute setting of diseases manifesting with low platelets, the surrogate use of TMA as a tentative clinical diagnosis has been widely applicated in cases associated with the laboratory tetrad described above. Historically, the phenotypical presentation has been commonly referred to as hemolytic uremic syndrome (HUS), and it was first described by von Gasser et al. in 1955 [253].

Nomenclature

During the last 20 years, major developments in etiologic and pathogenetic understanding have been achieved for diseases complicated by TMA. Previously, nomenclature mainly relied on symptomatologic definitions. As the research field has evolved, associated nomenclature has had a hard time keeping up. Thus, an outdated nomenclature has generated much confusion concerning the labeling of newly identified pathogenetic mechanisms within the conceptual framework of TMA and HUS [254]. Further, inconsistent use of terminology has hampered the validity of systematic reviews for these rare diseases.

Lately, the nomenclature has caught up, and a shift from symptomatic to etiologic definitions has been established. Thus, diseases that manifest with the laboratory tetrad (and which may reveal TMA histologically) are currently categorized as outlined:

a. **Typical HUS**. Caused by shiga/shiga-like toxin-producing bacteria, predominantly observed with shiga-like toxin-producing *Escherichia coli* (STEC-HUS) [255]; however, also reported secondary to shiga toxin-producing *Shigella dysenteriae* [256]. Renal circulation is primarily affected.

b. **Thrombotic Thrombocytopenic Purpura (TTP)**. Rarely caused by a congenital, more often by an acquired (autoantibody-mediated), deficiency of a vWF-cleaving protease identified as a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13 (ADAMTS13). Significantly reduced functional activity of ADAMTS13 (<10%) is the hallmark of TTP. Enzyme deficiency results in the aggregation of large complexes of vWF multimers and platelets, thereby causing thrombus formation in primarily small vessels, clinically often engaging the renal and cerebral circulations [257].

c. **Complement-mediated atypical HUS (aHUS)**. Caused by alterations in genes encoding complement proteins, resulting in complement dysregulation and excessive complement activation by the alternative pathway. The disease is episodically occurring secondary to complement triggering events, such as infections, hypertension, or pregnancy. It predominantly involves the microvasculature of the renal circulation, thereby resulting in complement-mediated loss of endothelial integrity and the subsequent formation of microthrombi [131].

d. **Noncomplement-mediated aHUS** (i.e., aHUS of other causes, also denoted secondary aHUS). Caused by comparably more prevalent diseases and conditions which comprise a broad spectrum of pathogenetic mechanisms. They share the common denominator of interfering with vascular function and hemostasis. Thus, if the renal circulation is engaged, they manifest with the full laboratory tetrad and may indicate TMA in renal biopsies. Differential diagnostics are often complicated as many reported diseases and conditions also may trigger flare-ups of complement-mediated aHUS [131,254,258].

Microangiopathic Hemolytic Anemia and Thrombotic Microangiopathy

Loss of unidirectional laminar blood flow results in turbulence and increased shear stress. In microangiopathic hemolytic anemia (MAHA), the loss of laminar blood flow results from vessel stenoses formed in the microvasculature. These stenoses may develop secondary to the formation of fibrin-rich thrombi as observed with, e.g., disseminated intravascular coagulopathy, heparin-induced thrombocytopenia, or catastrophic antiphospholipid syndrome. Histologically, an intact endothelium (or secondary

endothelial disruption) is often demonstrated [259]. Moreover, microvascular stenoses may develop secondary to the formation of vWF/platelet thrombi, as seen in TTP, which histologically mainly reveal an intact endothelium (or secondary endothelial disruption). Further, it may result from small vessel vasculitis, histologically indicated by immune cell infiltration, fibrinoid necrosis and fibrous proliferation, disruption of the internal elastic lamina, and endothelial swelling and detachment. Secondarily, the comprised endothelium may trigger the formation of fibrin-rich thrombi [259]. Additionally, microvascular stenoses may emerge from the intravascular clustering of cancer cells (with or without secondary fibrin-rich thrombus formation). Finally, it may result from primary TMA, histologically associated with subendothelial expansion and cell proliferation, interstitial edema, an intact internal elastic membrane, and endothelial swelling and detachment. Generally, the latter results in fibrin-rich thrombus formation [260]. Diseases associated with a primary endothelial impact, i.e., primary thrombotic microangiopathy, are enumerated in **Figure 4**.

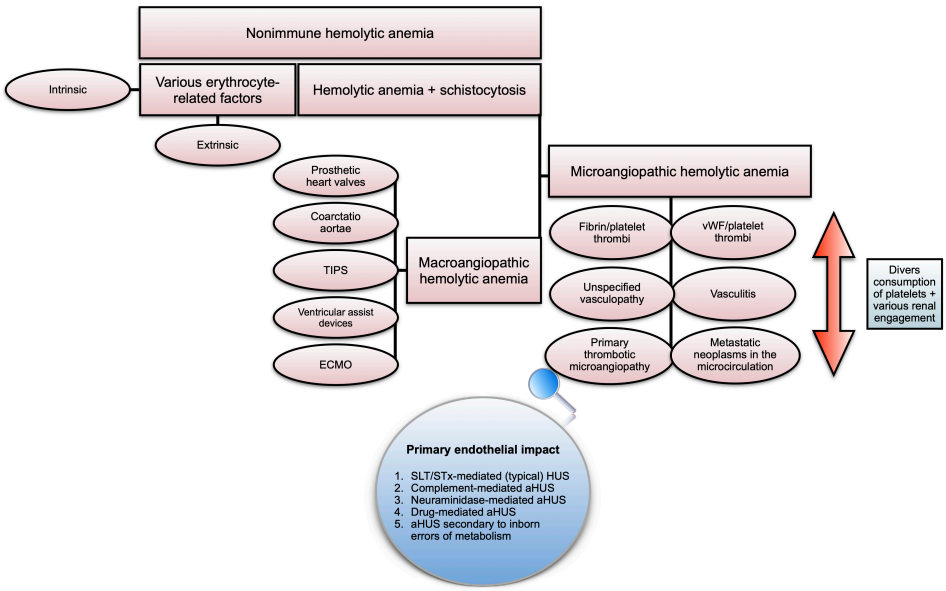

Figure 4. An outline of diseases associated with nonimmune hemolytic anemia
Erythrocyte-related factors of nonimmune-mediated hemolysis are not presented. Diseases and conditions resulting in thrombotic microangiopathies due to a primary endothelial impact are specified in the magnifier. Other causes of microangiopathic hemolytic anemia presented may potentially result in thrombotic microangiopathy due to a secondary endothelial impact related to tissue ischemia or inflammation. Adapted from Åkesson et al. [254] with permission from John Wiley & Sons, Inc. (aHUS: atypical hemolytic uremic syndrome; ECMO: extracorporeal membrane oxygenation; MAHA: microangiopathic hemolytic anemia; SLX: shiga-like toxin; STx: shiga toxin; TIPS: transjugular intrahepatic portosystemic shunt; TMA: thrombotic microangiopathy; TTP: thrombotic thrombocytopenic purpura; vWF: von Willebrand factor)

Evidently, microvascular stenoses and occlusions impair perfusion and cause tissue ischemia with the impending risk of necrosis and loss of organ function. Practically, there are significant histological overlaps between the histological presentations of primary thrombotic microangiopathy and other causes of MAHA. The extent of overlap primarily depends on the magnitude of secondary endothelial disruption in the latter category. Additionally, different histopathological characteristics are observed within the primary thrombotic microangiopathy group. For instance, in complement-mediated aHUS, the histopathological pattern is particularly observed in preglomerular arterioles, whereas it predominantly occurs in glomerular capillaries in typical HUS [261]. In conclusion, however, it is currently not possible to safely deduce specific etiologies from the histopathological findings of renal biopsies using conventional optical microscopy [130,260].

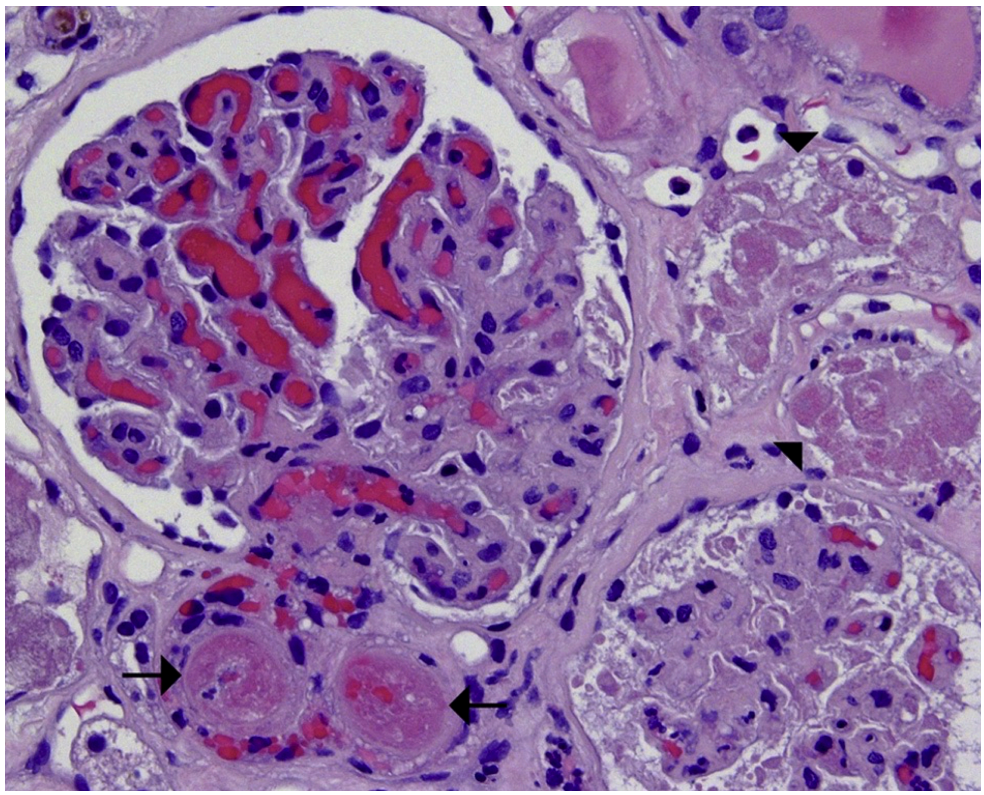

Figure 5. Thrombotic microangiopathy
Histopathological assessment of a renal biopsy using optical microscopy and conventional hematoxylin-eosin staining. Arrows indicate the endoluminal occlusions of thrombi in arterioles. Arrowheads indicate the necrosis-related detachment of cells from the basement membranes into the tubular lumina. Reprinted with permission from Elsevier, Ltd. [262].

The massive formation of microthrombi evidently requires platelets, thereby resulting in consumptive thrombocytopenia. Increased shear stress due to endothelial swelling and endoluminal fibrin meshes of formed microthrombi mechanically fragment erythrocytes, causing nonimmune hemolytic anemia. It is generally characterized by the finding of significant numbers of schistocytes (>1%) in peripheral blood smears [263] (**Figure 6**).

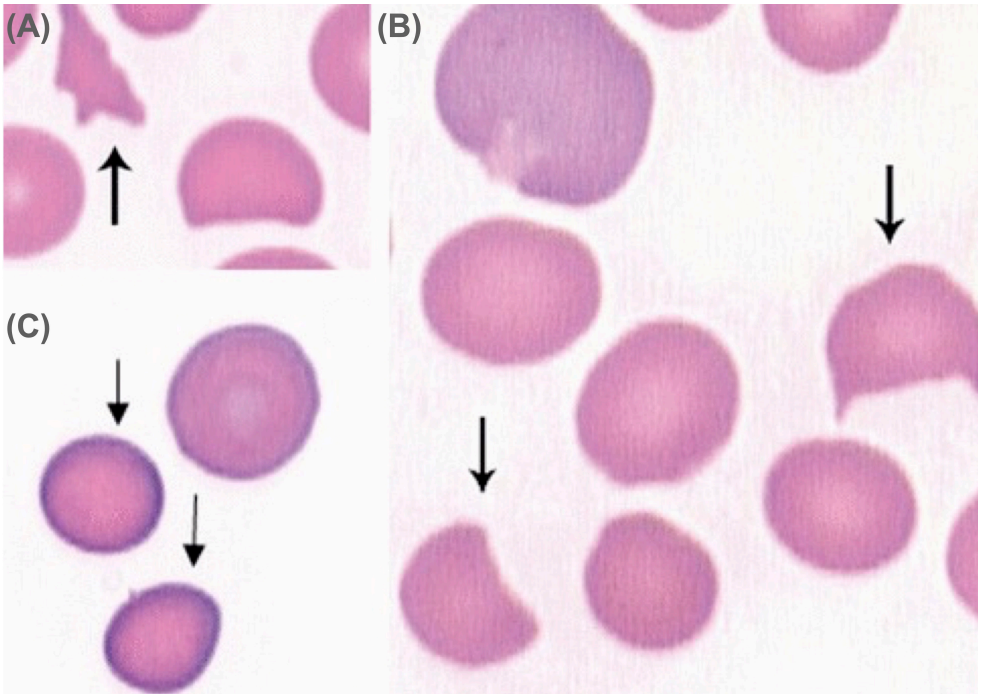

Figure 6. Shapes of schistocytes
According to the 2021 update [263] of the 2012 International Council for Standardization in Hematology Recommendations for identification, diagnostic value, and quantitation of schistocytes [264], the number of schistocytes should be assessed using an optical microscope at medium (x400) or high (x1000) magnification. The prevalence of schistocytes should be expressed as a percentage of the total number after counting at least 1000 erythrocytes. (A) shows a triangle schistocyte (arrow) and a helmet cell (lower right quadrant). (B) shows a helmet cell (left arrow) and a keratocyte (right arrow). (C) shows two microspherocytes (arrows), i.e., small-sized hyperdense erythrocytes derived from schistocytes and regarded as secondary manifestations of fragmentation. Morphologically, there is a partial overlap with the larger spherocytes identified in hereditary spherocytosis or immune hemolysis. Thus, microspherocytes should only be included in the schistocyte count in the presence of other forms of schistocytes. Reprinted with permission from John Wiley & Sons, Inc. [264].

The combination of nonimmune (DAT-negative) anemia and significant schistocytosis is often referred to as MAHA [265]. Generally, anemia developed secondary to erythrocyte fragmentation owing to, e.g., prosthetic heart valves or extracorporeal membrane oxygenation, is also categorized as microangiopathic hemolytic anemia, although it more accurately should be referred to as large vessel erythrocyte fragmentation syndrome or macroangiopathic hemolytic anemia [254]. To conclude, thrombocytopenia and MAHA are regarded as epiphenomena of microthrombus

formation and the subsequent trapping and fragmentation of passing erythrocytes. The combined manifestations of thrombocytopenia, MAHA, and acute renal failure raise a strong suspicion of widespread thrombus formation in the renal microcirculation.

Etiology and Pathogenesis

Table 1. Causes and triggers of atypical hemolytic uremic syndrome
The conditions presented may be causes of noncomplement-mediated aHUS, triggers of complement-mediated aHUS, or both.

INFECTIONS		DRUGS		MALIGNANCY	AUTOIMMUNE DISEASE
Neuraminidase-mediated aHUS Influenza A virus *Streptococcus pneumoniae* Viruses HHV6, VZV, CMV EBV HCV, HAV HIV Coxsackie B virus Parvovirus B19 Dengue virus Norovirus	Bacteria *Haemophilus influenzae* *Bordetella pertussis* *Clostridium difficile* *Campylobacter upsaliensis* *Fusobacterium necrophorum* Parasites *Plasmodium falciparum* Potentially all infections resulting in sepsis with MOF and DIC	Calcineurin inhibitors Cyclosporine Tacrolimus VEGF inhibitors Bevacizumab Sunitimib mTOR inhibitors Sirolimus Everolimus CD52 inhibitors Alemtuzumab General cytotoxics Mitomycin C Cisplatin Vincristine Gemcitabine	Antibiotics and antiparasitics Ciprofloxacin Quinine Platelet inhibitors Clopidogrel Interferon alpha/beta Contraceptives Illicit drugs Heroin Ecstasy Cocaine	Metastatic microvascular neoplasms Lymphoma HUS phenotypes have also been reported in the following cancers: Prostatic Gastric Hepatic Pancreatic Breast Ovarian Lung Colon	Systemic lupus erythematosus Antiphospholipid syndrome Renal scleroderma crisis Dermatomyositis
SYNDROMES OF HSCT	PREGNANCY-MEDIATED	VASCULITIS	RENAL CORTICAL NECROSIS	UNSPECIFIED GLOMERULAR DISEASE	MALIGNANT HYPER-TENSION
Radiation Graft-versus-host disease CMV infections Associated drugs	Pregnancy Postpartum period Preeclampsia HELLP syndrome	Polyarteritis nodosa Infectious vasculitis (e.g. *Rickettsia rickettsii*)	Regardless cause	Regardless cause	Regardless cause
HEPARIN-INDUCED THROMBO-CYTOPENIA AND THROMBOSIS	PAROXYSMAL NOCTURNAL HEMOGLOBINURIA	PANCREATITIS	INBORN ERRORS OF METABOLISM	DISSEMINATED INTRAVASCULAR COAGULOPATHY	HEPATITIS B VACCINATION

Adapted from Åkesson et al. [254] with permission from John Wiley & Sons, Inc. (CMV: cytomegalovirus; DIC: disseminated intravascular coagulation; EBV: Epstein-Barr virus; HAV: hepatitis A virus; HCV: hepatitis C virus; HELLP: hemolysis, elevated liver enzymes, low platelets syndrome; HHV6: human herpesvirus 6; HIV: human immunodeficiency virus; HSCT: hematopoietic stem cell transplantation; MOF: multiple organ failure; mTOR: mammalian target of rapamycin; VEGF: vascular endothelial growth factor; VZV: varicella-zoster virus)

Typical HUS is generally confirmed by the combination of symptoms, patient history, and microbiological testing. Correspondingly, confirmation of TTP is strictly dependent on an analysis of ADAMTS13. Currently, no swift and easy gold standard exists for diagnosing complement-mediated aHUS [266]. Noncomplement-mediated aHUS comprises a heterogeneous collection of diseases and conditions, superficially

united by their shared phenotype, although diverse in pathogeneses and treatments. Therefore, the relevance of 'second hit' physiological complement activation in these diseases is difficult to assess as the pathogenetic heterogeneity limits comprehensive analyses. However, for some subtypes, e.g., in hematopoietic stem cell transplantation (HSCT)-mediated aHUS, the contribution of complement activation for the development of TMA has been implicated [267]. Additionally, the physiological (but potentially disease-contributing) effect of complement activation in cases without genetic or acquired complement dysregulation may explain the ambiguous outcomes observed in studies investigating eculizumab treatment for noncomplement-mediated aHUS [268,269]. Of note, these small study series could not be adjusted for the confounding effects of coincident disease-specific therapies. Thus, to date, no evidence supports the administration of eculizumab in noncomplement-mediated aHUS. However, in like manner as some autoimmune and autoinflammatory diseases, complement-inhibiting therapy may prove beneficial in a subset of patients with noncomplement-mediated aHUS in the future.

The phenotypical resemblance between noncomplement-mediated and complement-mediated aHUS constitutes a complicating factor with respect to differential diagnostics. Especially so as anecdotal observations indicate that several noncomplement-mediated conditions may elicit an excessive complement response in patients that are genetically prone to complement dysregulation (**Table 1**). For instance, phenotypical HUS (and the histopathological finding of TMA) has been observed with cases of noncomplement-mediated malignant hypertension. However, malignant hypertension may also constitute both a trigger and a consequence of complement-mediated aHUS [270-272]. It is beyond the scope of this thesis to review further examples. However, several reviews have recapitulated pathogeneses, treatments, and complications of noncomplement-mediated aHUS [130,131,254,255,268].

Complement-mediated Atypical Hemolytic Uremic Syndrome

Complement-mediated aHUS is an ultra-rare, life-threatening disease that predominantly manifests in a relapsing manner. The association between complement dysregulation and disease development was first described in 1998 with the finding of alterations in the *CFH* gene in families suffering from hereditary HUS [273]. Before the introduction of complement-inhibiting therapy with eculizumab in 2011 [159], treatment relied on plasma exchange therapy and approximately 70% of patients died or developed ESRD within three years from the first flare-up [114,274,275]. Complement-inhibiting therapy was a game-changer, generally showing superior outcomes compared to plasma exchange therapy [158]. Moreover, early initiated complement-inhibiting treatment has been associated with better renal outcomes [276].

Disease onset occurs at any age, ranging from neonates to adults. During childhood, the incidence is equally frequent in boys and girls [274]; however, in adults, there is a 2:1 female to male ratio [277]. It can be sporadic or familial (20%) [94,277], and in the latter case, genetic variants are mainly transmitted as autosomal-dominant traits with incomplete penetrance [274]. Half of the family members harboring an inherited genetic variant do generally not present with the disease by age 45 [278]. Thus, genetic alterations constitute susceptibility factors for clinical breakthrough rather than causative drivers [94] (**Table 2**).

Table 2. Genetic findings and related features in complement-mediated aHUS
Data are based on several multicenter cohorts and reflect the prevalence of homozygous, compound heterozygous and heterozygous rare variants. Aggregated prevalence of unspecified CNVs and NAHR events and the prevalence of anti-FH antibodies are also presented. References: [94,114,279-286].

IMPACT	PROTEIN	GENE (locus)	PREVALENCE (%)	LOW C3 (%)	ESRD (%)	RELAPSE (%)	
						Total	Renal Tx
Gain-of-function comlement activation	C3	*C3* (19p13)	2-10	70-80	60-80	50	40-50
	FB	*CFB* (6p21)	1-4	100	50-70	>80	u
Loss-of-function complement regulation	FH	*CFH* (1q31)	20-30	30-50	50-70	50	80–90
	FHR1-5	*CFHR1-5* (1q31)	1-2	u	u	u	u
	FI	*CFI* (4q25)	4-10	20-30	50-60	10-30	70-80
	MCP	*CD46* (1q32)	5-15	25	<20	70-90	15-20
	VTN	*VTN* (17q11)	<1	u	u	u	u
	FP	*CFP* (Xp11/21)	u	u	u	u	u
	CNV/NAHR	Primarily 1q31	5-10	n/a	n/a	n/a	n/a
	Anti-FH antibodies	u/1q31 (DEAP-HUS)	5-20	40-60	30-40	10-60	u
Additive or combined	Combined complement alterations	n/a	2-12	n/a	n/a	n/a	n/a
Coagulation -related (increased risk of thrombosis)	THBD	*THBD* (20p11)	3-5	50	50-60	30	u
	PLG	*PLG* (6q26)	u	u	u	u	u
	DKGE	*DGKE* (17q22)	2	<2	u	u	u

(C: complement component; CF/F: complement factor; MCP: membrane cofactor protein; CNV: copy number variation; DEAP-HUS: deficiency of FHR plasma proteins and autoantibody-positive form of hemolytic uremic syndrome; DKGE: diacylglycerol kinase-epsilon; ESRD: end-stage renal disease; FHR: factor H-related protein; n/a: not applicable; NAHR: nonallelic homologous recombination; PLG: plasminogen; RCA: regulators of complement activation gene cluster; Tx: transplantation; THBD: thrombomodulin; u: unknown; VTN: vitronectin)

Regardless of familial or sporadic presentation, the risk of developing disease or suffering from relapse rests on a complex synergy of contributing factors:

a Rare (**gain-of-function**) genetic variants in genes encoding **complement components** [284].

b. Rare (**loss-of-function**) genetic variants in genes encoding **complement inhibitors** [284].

c. **Copy number variations** and **complex genomic rearrangements** in genes encoding complement inhibitors, primarily in the **1q31 locus** [114].

d. **Complement risk haplotypes** and **polymorphisms** [130,266].

e. The development of **complement-specific antibodies** [287].

f. Rare (**loss-of-function**) genetic variants in genes encoding **coagulation-related proteins** [284].

g. Flare-ups of **coincident diseases and conditions** that **trigger complement activation** or which may **jeopardize endothelial integrity** (e.g., infections, autoimmunity, pregnancy) [254].

To date, more than 500 rare (<0.1% minor allele frequency) genetic variants have been identified in genes encoding complement proteins in patients with aHUS [284]. However, genetic data from global genome databases, e.g., the Genome Aggregation Database (gnomAD), which currently comprises +15,000 whole genomes and +125,000 exomes [288], indicate the presence of any rare complement variant in 3-4 % of healthy individuals. Hence, it is the enrichment of rare genetic variants in cohorts of aHUS, as compared to the general population, that suggests a relevant contribution of identified genetic findings in the absence of functional studies.

Genetic variants in the *CFH* gene are frequently identified in patients with complement-mediated aHUS. It reflects the key role of FH in the regulation of the alternative complement pathway. However, it also provides clues regarding the renal predisposition of complement dysregulation, which indeed is incompletely explored. Factor H is important for membrane-bound complement regulation by its binding to endothelial glycosaminoglycans, sialic acids, and heparan sulfate molecules [289]. Essentially, it is the only complement regulator engaged in alternative pathway complement inhibition on the surface of host structures lacking membrane-bound regulators, such as the glomerular basement membrane [290]. These characteristics may provide some explanation for the renal susceptibility. However, other possible causes include the fenestrated characteristics of the glomerular endothelial cells, which may expose the basement membrane to increased concentrations of complement proteins as plasma water is filtered [291]. Moreover, the kidneys are one of the largest extrahepatic

complement production sites [292,293]. The tubulointerstitium in particular has adapted to synthesize its own complement proteins. By contrast to glomeruli, which are constantly exposed to liver-derived complement proteins, tubulointerstitial cells have limited access to circulating proteins [294]. Thus, they synthesize their own complement proteins, possibly as part of the defense against microorganisms ascending through the renal calyces. The local production of complement proteins and the relative deficiency of complement regulators on surfaces of renal tubule cells [295] may compose a vicious cocktail in patients genetically susceptible to complement dysregulation. Moreover, healthy kidneys accept 25% of cardiac output per unit of time. More specifically, renal plasma flow is approximately 600-720 ml per minute [296]. Thus, the kidneys are heavily exposed to the potential depositions of circulating immune complexes and inflammatory mediators, which may predispose complement activation.

Diagnostic Approach and Management

Since the approval of complement-inhibiting therapy for complement-mediated aHUS, the treatment approach no longer relies on empirical plasma exchange therapy. This treatment has demonstrated inferior renal outcome and increased overall mortality compared with complement therapeutics. Further, it is associated with significant complications, such as anaphylaxis, serum sickness, and catheter-related infections and hemorrhages [297]. Early initiated complement-inhibiting therapy is critical for the restoration of kidney function [276]. Therefore, it is essential to assess the probability of complement-mediated aHUS rapidly. The measurement of plasma complement components is far from indicative of diagnosis. For instance, low C3 levels are only present in 30% of the patients [114]. Additionally, potential differences in routine laboratory aberrations do not reliable differentiate complement-mediated aHUS from TTP [298] nor likely complement-mediated from noncomplement-mediated disease. Assays have been developed to assess the capacity of patient plasma to increase complement depositions either on endothelial cells *in vitro* [299,300] or by using a modified *in vitro* Ham test, thus taking advantage of the increased susceptibility to complement depositions associated with PNH-like cells [301]. However, assays based on patient plasma do not consider complement dysregulation caused by impaired membrane-bound regulation. Currently, no complement deposition-based assay has been clinically implemented.

Owing to the absence of a swift diagnostic gold standard, the diagnostic approach requires a tiered weight of evidence strategy. First, non-TTP-specific ADAMTS13 activity and negative microbiological testing tentatively rule out TTP and typical HUS, respectively. Secondly, the potential presence of noncomplement-mediated aHUS must be assessed according to patient history, age, and clinical suspicion. Thirdly,

complement testing, including hemolytic assays to determine the extent of involvement for the separate complement pathways, and assays to determine plasma concentrations of complement components, complement-specific antibodies, complement degradation products, and terminal complement complexes, are warranted. Additionally, flow cytometric assessment of MCP expression on blood cells should be conducted to evaluate the presence of germline mutations affecting the endothelial expression of MCP. Based on analyses of the compiled results, the probability of complement-mediated aHUS should be assessed, and the initiation of complement-inhibiting therapy should be considered. Ultimately, however, results from genetic analyses are required to make a robust diagnosis (**Figure** 7).

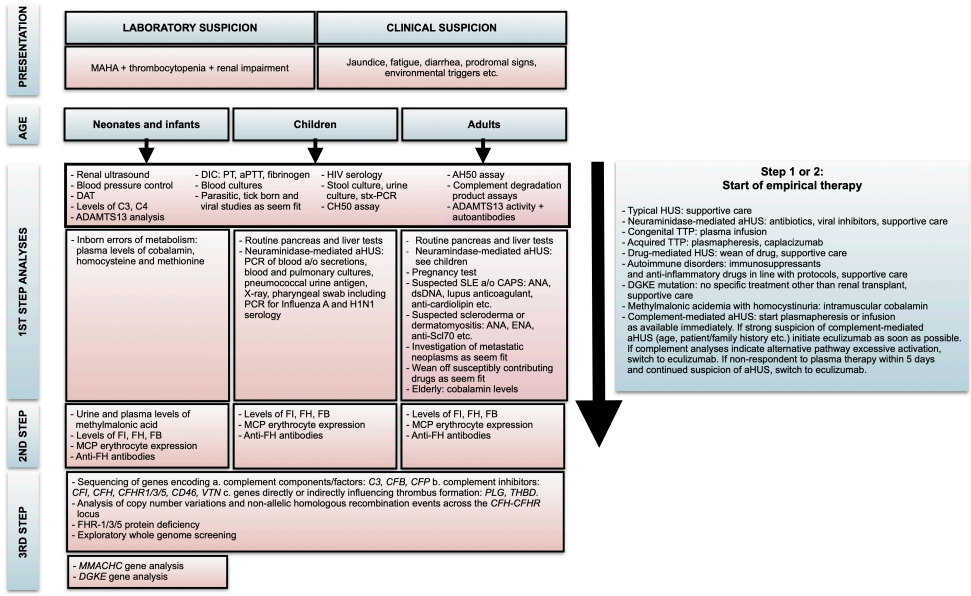

Figure 7. Differential diagnostics and laboratory workup for suspected aHUS
Empirical therapy should be initiated as soon as possible. Step 3 is a diagnostic step; however, due to the time-consuming analyses required, results from step 3 are not considered a decision basis for the start of therapy. Adapted from Åkesson et al. [254] with permission from John Wiley & Sons, Inc. (ADAMTS13: a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13; ANA: antinuclear antibody; aPTT: activated partial thromboplastin time; CAPS: catastrophic antiphospholipid syndrome; CFHR; complement factor H-related protein; CH50/AH50 assays: classical and alternative pathway complement activation assays; DAT: direct antiglobulin test; DGKE: diacylglycerol kinase-epsilon; DIC: disseminated intravascular coagulopathy; dsDNA: double-stranded DNA; ENA: extractable nuclear antigen; F: complement factor; MAHA: microangiopathic hemolytic anemia; MCP; membrane cofactor protein; MMACHC: methylmalonic aciduria and homocystinuria type C protein; PCR: polymerase chain reaction; PT: prothrombin test; SLE: systemic lupus erythematosus; stx: shiga toxin)

To conclude, the lack of a gold standard for the resolute identification of cases with complement-mediated aHUS poses a differential diagnostic challenge on account of the phenotypical overlap with noncomplement-mediated aHUS. A correct diagnosis is important as the renal outcome is time-dependent, effective complement-inhibiting

therapy expensive, and treatment associated with an increased risk of infectious adverse effects.

Platelet Transfusion Refractoriness

Since the initial discovery of platelets in the 19th century, it took more than thirty years before the first therapeutic platelet transfusion in a man with severe thrombocytopenia and mucocutaneous bleedings was reported by W.W. Duke in 1910 [302]. However, it was first during the 1960s that platelet concentrates became an integrated part of the clinical routine, effectively cutting the hemorrhage-mediated mortality rate among leukemia patients by almost half [303]. Since then, several improvements have been achieved to preserve the hemostatic function of platelet concentrates during storage [302], including the finding of 22 °C optimal storage temperature [304]. Today, platelet concentrates can be stored up to seven days before use [302]. Approximately 50% of available platelet concentrates worldwide are transfused to patients with hematological malignancies and/or patients undergoing HSCT [305]. Platelet transfusions can be categorized as therapeutic or prophylactic. While guidelines for therapeutic transfusions, i.e., during active bleeds, are unanimous and generally suggest a transfusion threshold for platelet counts < 50 x 10^9/L [306,307], guidelines for prophylactic transfusions appear heterogeneous. Empirical transfusion thresholds for similar-risk surgeries and invasive procedures vary across different countries and disciplines [308,309]. By contrast, however, effects and complications of platelet transfusions have been extensively studied in patients with hematological malignancies undergoing chemotherapy and allogeneic HSCT [310-313]. Therefore, these guidelines are evidence-based and practically identical worldwide, generally recommending a platelet transfusion threshold for platelet counts < 10 x 10^9/L [306,314-316]. Prophylactic platelet transfusions in these patients significantly reduce WHO grade 2 bleedings and worse [313]. Of note, in patients undergoing autologous HSCT, prophylactic transfusions have not been proven superior to therapeutic strategy [310,313].

Platelet concentrates can be obtained by two principally different techniques: random-donor platelets (RDP) obtained from the pooled concentrate of four to six whole blood donors or single donor apheresis platelets (SDAP) obtained by apheresis technique. Benefits vary with the two techniques. Historically, a better post-transfusion platelet response and fewer complications have been achieved with SDAP. Today, however, given advancements in bacterial growth surveillance [317], universal leukoreduction [318] and special platelet additive solutions [319], there is no definite evidence favoring one type of platelet product over the other in terms of hemostatic efficacy and adverse outcomes [316]. Slightly better post-transfusion increments have been shown with

SDAP [311]. However, previous studies on hematologic malignancies and HSCT have demonstrated insignificant differences in the incidence of bleeding between patients receiving high, medium, and low dose prophylactically administered platelet concentrates [320]. Thus, it is not certain that the slightly better post-transfusion increments observed with SDAP are clinically relevant. In the USA, over 80% of transfusions are still administered as SDAP [308], whereas RDP is considered standard of care in Europe [306]. Random-donor platelet products can be manufactured by two techniques: the buffy coat method and the platelet-rich plasma method. Comparative studies have shown similar hemostatic qualities in platelet products obtained by the two techniques and stored up to seven days [321].

Leukoreduction by filtration of platelet concentrates is associated with a 25% loss of viable platelets. However, it also reduces the risk of alloimmunization [322], the transmission of cytomegalovirus [323], and febrile transfusion reactions [324]. Today, universal leukoreduction is adapted as part of the protocol in most blood centers around the world. Importantly, it has led to negligible differences between SDAP and RDP regarding the incidence of febrile transfusion reactions [325] and alloimmunization [322,326]. Consequently, the lower costs and more effective use of resources associated with RDP have warranted its extensive use. In many European countries, the use of SDAP is reserved for the pediatric patient population and selected patients who have developed immune platelet transfusion refractoriness [316]. Of note, leukoreduction does not result in the complete elimination of viable leukocytes. For specific patient populations, e.g., patients undergoing allogeneic HSCT or other severely immunocompromised patients, irradiation of platelet products is performed before use to reduce the risk of developing post-transfusion graft-versus-host disease [327].

Definitions

Repeated platelet transfusions increase the risk of platelet transfusion refractoriness (PTR), i.e., a lower-than-expected platelet increment following completed transfusion. It is associated with increased morbidity and mortality [328], and increased care-related costs [329]. Isolated suboptimal responses to platelet transfusions are common. Therefore, two serial platelet transfusions are needed to assess the presence of PTR. On average, one unit of platelets should increase the platelet count by approximately 25 $x10^9$/L as measured within 10 minutes to one hour after transfusion [330]. A post-transfusion platelet increment < 10 x 10^9/L is indicative of PTR. However, utilizing formulas such as percent platelet recovery (PPR) and corrected count increment (CCI) [331] are better options to more precisely assess the post-transfusion response. These calculations adjust for the number of platelets transfused and the individual estimate of blood volume and body surface area, respectively [330]. Most guidelines define PTR as a CCI < 5000 or PPR < 20% at one hour post-transfusion on two separate transfusion

occasions using ABO-identical platelet units less than 72 hours old [316,322,332]. However, higher cutoffs (< 7500 and < 30%, respectively) have also been applied in some studies [333]. Importantly, a good correlation between the CCI calculated at one and 20 hours post-transfusion, respectively, has been demonstrated, with values of the latter averaging two-thirds of the former. The correlation is useful as it enables an ideal timing of transfusion before planned interventions. However, it also enables the initial assessment of PTR utilizing blood samples obtained during routine morning sampling the day after transfusion (i.e., generally the time during which the initial suspicion of PTR is raised) [334].

Pathogenesis

Platelet transfusion refractoriness is often underdiagnosed. It has been estimated to occur in at least half of all platelet transfusions administered to patients with hematologic malignancies [335,336]. The development of PTR is often multifactorial. However, causes of PTR are generally classified as either nonimmune or immune, with the former category responsible for up to 80% of all cases. **Table 3** summarizes common nonimmune and immune-related factors. Nonimmune-related factors are attributed to increased platelet consumption by antibody-independent platelet activation, aggregation, and sequestration [332,337].

Table 3. Nonimmune and immune-related factors in platelet transfusion refractoriness
ABO mismatch is presented within brackets as it is subject to ongoing debate and conflicting evidence [338-340].

NONIMMUNE-RELATED FACTORS	IMMUNE-RELATED FACTORS
Infection, sepsis, fever	HLA class I alloantibodies
Bleeds	HPA alloantibodies
Splenomegaly	Antibodies against platelet glycoprotein-drug complexes
Poor platelet product quality or extended storage time	(ABO mismatch)
Thromboinflammation (DIC, MAHA, VOD)	
Graft-versus-host disease	
Total body irradiation	
Drugs (vancomycin, amphotericin B, interferons, ATG)	

(ATG: anti-thymocyte globulin; DIC: disseminated intravascular coagulation; HLA: human leukocyte antigen; HPA: human platelet antigen; MAHA: microangiopathic hemolytic anemia; VOD: veno-occlusive disease)

Immune-related factors are attributed to antibody-mediated clearance of platelets. It may increase i. antibody-mediated opsonization [341], ii. antibody-dependent cell-mediated cytotoxicity by NK cells [342] and iii. antibody-dependent complement fixation, which further may enhance opsonization or directly induce cell lysis [343]. Immune PTR is mainly caused by acquired alloantibodies against class I human leukocyte antigens (HLA), corresponding to approximately 75% of the immune-related

factors [344] and 10-25% of all PTR cases [345]. Solid-organ transplantations [346], pregnancies [347], and multiple blood and/or platelet transfusions [348] are risk factors. Interestingly, in the Trial to Reduce Alloimmunization to Platelets (TRAP), not all patients with anti-HLA alloantibodies suffered from PTR [322]. This may indicate varying immunogenic properties of alloantibodies or the need for coincident mechanisms of platelet clearance in immune PTR.

In 2-8% of repeatedly platelet-transfused thrombocytopenic patients, alloantibodies against human platelet antigens (HPA) have been reported [348]. Human platelet antigens demonstrate less antigenic variability compared to HLA, which might explain its lower prevalence [349]. Of note, however, PTR is unfrequently seen in cases of isolated HPA alloimmunization [350,351]. Rather, the coincident presence of HLA alloimmunization is often proven relevant [352].

Finally, drug-induced antibodies formed due to specific drug interactions have been demonstrated to result in PTR. These antibodies emerge as a consequence of drugs altering and forming complexes with platelet membrane glycoproteins. These scenarios are often characterized by rapid onset of thrombocytopenia which resolves within a couple of days after drug cessation [353].

Management

In patients diagnosed with PTR, apparent nonimmune-related factors must be excluded or treated. Treatment for nonimmune PTR primarily focuses on the underlying cause as general recommendations to transfuse fresh and ABO compatible platelet products [307] often are ineffective. If suspicion of immune PTR remains, a workup for immune-related causes should be initiated. Generally, a rapid screening test to confirm the presence of anti-HLA alloantibodies is performed. If positive, other tests are utilized to assess the antibody specificity. Solid-phase testing assays, also termed single-antigen bead assays (SAB), are commonly used. In these assays, beads coated with different HLA antigens are exposed to patient serum. Subsequently, the degree of antibody binding is primarily assessed by flow cytometry using fluorescent-labeled anti-human immunoglobulins. The assay reports a list of antibody specificities and related mean fluorescence intensities (MFI) reflecting the strength of each antibody bond [354].

Various methods can be used to identify compatible platelet products in HLA alloimmunized patients. Platelet cross-matching is frequently used. It is the fastest method to identify eligible transfusion products without the need to assess the specific anti-HLA antibody specificity or the HLA genotype of the patient. With this method, a panel of donor platelets is mixed with patient serum and visualized with indicator erythrocytes coated with anti-immunoglobulin G [337]. Thus, it is a compound test

assessing the simultaneous presence of both anti-HLA and anti-HPA alloantibodies. For the same reason, it may be futile to use the test in extensively alloimmunized patients as results would render it difficult to identify compatible platelet products. Moreover, the administration of platelet products chosen by cross-matching is associated with the risk of further alloimmunization due to potentially mismatched HLA antigens [337]. Therefore, HLA-matched platelet products might be preferred to reduce the risk of future alloimmunization. HLA matching requires HLA genotyping and aims to identify SDAPs with a perfect match (4/4) for the patient's HLA class IA and IB alleles. A perfect match is associated with a significantly lower risk of future alloimmunization; however, it also limits the available donor pool. In the event of unavailable perfect matches, the antibody specificity profile assessed by solid-phase testing may be utilized to identify SDAPs that do not express the corresponding antigen. This method is referred to as the antibody specificity prediction method, and it aims to identify HLA-compatible platelet products [332]. Thus, the method expands the available donor pool. However, it also increases the risk of further alloimmunization [355]. It is a practical compromise when cross-matched platelet products result in PTR, and available HLA-matched products are scarce. Of note, HLA-matched platelet products do not guarantee acceptable post-transfusion increments. Previous studies have demonstrated that 75% of cross-matched and 70% of HLA-matched platelet products fail to reach acceptable post-transfusion increments in alloimmunized patients suffering from PTR [356]. Thus, the finding supports a multifactorial model of PTR development in which coincident nonimmune-related factors, and perhaps the failure of available assays to detect other important antibodies, may be relevant. Furthermore, a recent study demonstrated that a subset of HLA alloantibodies is capable of crosslinking HLA and FcγRIIa molecules on the surface of platelets, thereby inducing platelet activation which facilitates phagocytosis [357]. This finding is consistent with the observation that not all HLA alloimmunized patients suffer from PTR. On another note, patients may, for some reason, lose their antibody positivity over time although regularly transfused. Consequently, occasional reassessments of the antibody status are warranted as some patients may be able to shift from HLA-matched/compatible platelet products to cross-matched transfusions [333], further emphasizing the multifactorial dynamics of PTR.

Complement Activation

The complex and incompletely explored dynamics between platelets and complement activation have been discussed in a previous section. Data on PTR in general and HLA alloimmunization in particular, suggest that complement activation is intimately entangled in the pathogenesis of PTR in a subset of patients [343,344,358]. The potential contribution of complement activation in the development of PTR raises several serially related questions. Is *in vitro* complement activation during platelet

product storage inversely correlated with the post-transfusion response? Is increased complement activation *in vivo* in patients suffering from, e.g., systemic inflammatory responses inversely correlated with the post-transfusion response, as a nonimmune-related risk factor of PTR? Do platelet transfusions in themselves result in increased complement activation *in vivo*, potentially affecting the post-transfusion response negatively? Is complement activation relevant for immune PTR, and if so, how?

First, regarding storage, previous studies have demonstrated a positive correlation between storage time and levels of complement activation biomarkers in platelet products [359]. However, to the best of my knowledge, no studies have investigated *in vivo* effects of complement activation on the post-transfusion response in this setting. Secondly, no studies have prospectively measured complement biomarkers in patients characterized by nonimmune-related risk profiles for PTR. Thirdly, no studies have investigated the potential correlation between the post-transfusion response and transfusion-mediated complement activation. Fourthly, there is, however, convincing evidence suggesting a relevant role for complement activation in a subset of patients with immune PTR.

Alloantibody-mediated complement activation is a recognized complication contributing to graft rejection in solid-organ transplants [360-362]. The development of the IgG-based SAB assay overcame the previous lymphocytotoxicity assay-related problem of not being able to identify the HLA specificities of potentially coincident alloantibodies. However, the implementation of the new assay also introduced a new problem, i.e., how to determine clinically relevant thresholds for positive anti-HLA antibodies. Therefore, a C1q-based SAB assay was introduced in 2011, appreciating the complement-dependent methods utilized in the precedent cytotoxicity assays. The new assay revealed a good correlation between the C1q-binding capacity of anti-HLA antibodies and the risk of antibody-mediated rejection in solid-organ transplants. Notably, the correlation was independent of antibody levels assessed with the IgG-based SAB assay [363]. Subsequently, the C1q assay was retrospectively evaluated in a population of alloimmunized patients receiving platelet transfusions [344]. The results indicated that the C1q-based compared to the IgG-based SAB assay was better at predicting the chance of achieving an acceptable post-transfusion response. Thus, the C1q-based SAB assay more effectively guided in the selection of compatible platelet products. Consequently, the complement-fixing capacity of anti-HLA antibodies, which likely facilitates complement-mediated opsonization or direct lysis of transfused platelets, presumably is of clinical importance. Of note, the C1q-based SAB assay was also implemented in a study using samples from the TRAP trial. In this study, a good agreement was unsurprisingly shown between patients demonstrating positive results in the (complement-dependent) lymphocytotoxicity assay and patients with high levels of C1q-binding alloantibodies. Importantly, the study did not demonstrate any significant difference in levels of C1q-binding alloantibodies between clinically

refractory and nonrefractory cases within the lymphocytotoxicity assay-positive group [364]. The study was, however, limited by the small sample size and further studies are evidently needed to better reveal the clinical relevance of the C1q-based SAB assay.

On another note, Rijkers et al. performed a study *in vitro* to confirm the route of complement activation associated with anti-HLA antibody-tagged platelets [343]. The aim was to investigate whether complement activation was initiated by antibody-mediated complement activation (i.e., mediated by the classical complement pathway) or by the complement-binding capacity of P-selectin. In other words, was complement activation a cause or consequence of platelet activation? By using a blocking antibody targeting C1q, the authors demonstrated a radical reduction in the quantities of C3b and C4b depositions on platelet surfaces, suggesting that anti-HLA antibody-mediated complement activation was strictly dependent on the classical complement pathway. Further, it was shown that complement activation led to the assembly of TCC and subsequent Ca^{2+} influx, thereby facilitating platelet activation and the enhanced membrane expression of P-selectin. Ultimately, complement activation may induce direct lysis and/or enhanced opsonization. In the presence of eculizumab, TCC levels and expression of P-selectin were shown to decrease. These results suggest that anti-HLA antibody-mediated complement activation may be an important pathogenetic mechanism in immune PTR. The contribution of direct lysis is further supported by an *in vitro* study by Meinke et al. in which extracellular levels of the cytosolic dye calcein red-orange (indicating a damaged membrane) were elevated in platelets demonstrating increased staining for C1q and C3c [365]. Interestingly, acid treatment was used to strip off the HLA class I molecules from platelet membranes. These platelets demonstrated significantly reduced levels of complement depositions and calcein in the presence of anti-HLA antibodies compared to platelets with normal HLA class I expression. Thus, the authors suggested that transfusions with acid-treated platelet products may prove valuable in the treatment of immune PTR in the future. Of note, the viability of platelet glycoproteins was not investigated after the acid treatment, which could be of interest since desialylated glycoproteins increase the hepatic uptake of platelets via Ashwell-Morell receptors [366]. Another possible treatment alternative would be the use of complement-inhibiting therapy. One small study has demonstrated *in vivo* that four out of ten alloimmunized subjects overcame PTR following treatment with eculizumab [367]. Eculizumab-treated patients exhibited a reduction in the requirement for further platelet transfusions by approximately 50% and demonstrated an increase in the 1 hour CCI despite the use of mismatched platelet products. Importantly, however, six patients did not respond to therapy, and the sample size was obviously limited.

To conclude, disease-contributing complement activation is indicated in a subset of patients suffering from immune PTR. The pathogenesis of PTR is multifactorial, and it likely constitutes a mix of immune and nonimmune-related factors in most

alloimmunized patients. A potential challenge of future research is to determine eligibility criteria for complement-inhibiting therapy. Moreover, the potential role of complement activation in nonimmune PTR has not been fully explored. The current pathophysiologic understanding of PTR primarily relies on *in vitro* studies and limited investigations *in vivo*. Clearly, large multicenter trials are needed to study the effects of complement activation and the potential benefits of complement-inhibiting therapy.

General Rationales

Complement activation is intertwined with different aspects of platelet function and viability, including platelet activation and elimination. The preceding sections have reviewed various pathogenetic mechanisms contributing to thrombocytopenia that are potentially dependent on, or influenced by, coincident complement activation and/or dysregulation.

In complement-mediated aHUS, nonimmune consumptive thrombocytopenia results from the massive formation of renal microthrombi induced by endothelial injuries which are caused by dysregulated complement activation. The making of a diagnosis is challenging due to the lack of a diagnostic gold standard, phenotypical overlaps with noncomplement-mediated disease, and the time-consuming and complicated genetic workup required to make a robust assessment. The potential underdiagnosis of complement-mediated aHUS has not been assessed in a Swedish setting before.

In platelet transfusion refractoriness, complement activation potentially contributes to the activation, opsonization, and direct destruction of transfused platelets in the presence of anti-HLA antibodies. However, potential *in vivo* effects of complement activation during platelet product storage and in nonimmune PTR have remained unexplored.

Aims of the Thesis

Paper I/II – Prevalence of Atypical Hemolytic Uremic Syndrome

The aim of *paper I* was to retrospectively investigate the prevalence of clinically suspected complement-mediated and noncomplement-mediated aHUS in a cohort referred for ADAMTS13 analysis during the years 2007 - 2012.

The primary aim of *paper II* was to apply a diagnostic scheme compliant with the American College of Medical Genetics and Genomics (ACMG) guidelines to assess the prevalence of complement-mediated and noncomplement-mediated aHUS among subjects formerly included in *paper I*, utilizing clinical data and whole genome sequencing. Secondary aims were to compare acute episode features and renal outcomes between subjects with complement-mediated and noncomplement-mediated disease.

Paper III – Platelet Transfusions in Hematological Conditions

The primary aim was to investigate correlations between biomarkers of complement (soluble TCC) and platelet (soluble P-selectin and soluble CD40L) activation, respectively, and corrected count increments (CCI) assessed at one hour and 24 hours after transfusion. Biomarkers were assessed *in vitro* (in the platelet concentrates) and *in vivo* before and at one and eight hours after prophylactic platelet transfusions. Secondary aims were to compare differences in CCI, biomarker levels, and clinical characteristics between different categories of patients.

Materials and Methods

This section briefly describes central aspects of materials and methods relevant for the included papers. For full reviews on study designs, patient populations, and laboratory methods, refer to the reprinted papers appended at the end of the thesis.

Study design

Study Inclusion and Study Design Specifics

Paper I/II

The investigations were designed as retrospective cohort studies. Subjects referred nationwide to the Coagulation Unit, Skane University Hospital, Malmö, Sweden, for ADAMTS13 analysis during the years 2007 - 2012 were eligible for inclusion. Subjects who at the time of referral exhibited ADAMTS13 activity > 0.05 AU/L and coincident routine laboratory findings of i. hemolytic anemia (decreased hemoglobin accompanied by increased lactate dehydrogenase and hypohaptoglobinemia and/or reticulocytosis and/or unconjugated hyperbilirubinemia), ii. thrombocytopenia, and iii. renal failure (increased creatinine and/or cystatin C) were included in a cohort of clinically suspected aHUS. Assessments of peripheral blood smears and results from DAT were not required for study inclusion. Exclusion criterium was a positive microbiological testing for enterohemorrhagic *Escherichia coli*. In *paper I*, included subjects were categorized into two clinical groups based on information extracted by medical record reviewing: suspected aHUS with and without clinically suspected triggers or causes, respectively. In *paper II*, included subjects from the precedent investigation were asked for participation in a follow-up study, including complement analyses, whole genome sequencing, medical record reviews, and self-reported inquiry forms regarding current diseases and medications.

In *paper II*, included subjects were categorized into four groups:

a. Definite complement-mediated aHUS: subjects harboring ≥ 1 disease-contributing genetic variant (pathogenic or likely pathogenic) and/or subjects featuring significant titers of FH-specific antibodies.

b. Highly suspected complement-mediated aHUS: subjects harboring ≥1 likely disease-contributing genetic variant (a variant of uncertain significance located in a mutational hotspot and/or a critical functional domain) and/or subjects featuring distinct alternative pathway complement activation during a flare-up and confirmed TMA in a subsequent renal biopsy.

c. Noncomplement-mediated aHUS: subjects harboring no disease-contributing/likely disease-contributing genetic variants, who were coincidently afflicted by conditions acknowledged to cause phenotypical HUS by means of other mechanisms.

d. HUS-like phenotype: subjects harboring no disease-contributing/likely disease-contributing genetic variants and in whom post-inclusion assessments of clinical presentations and/or laboratory results deemed aHUS unlikely.

Detected genetic variants were classified according to the ACMG guidelines [368] with some disease-specific modifications concerning the assessment of minor allele frequency (MAF) and the significance of *CFB*, *CFP*, and *C3* gene products. First, regarding the MAF-related criterion for benign variant classification, i. 0.1% ≤ MAF < 1% provided supporting evidence of benign impact, ii. 1% ≤ MAF < 5% provided strong evidence of benign impact, and iii. MAF ≥ 5% provided stand-alone evidence of benign impact. Secondly, regarding the MAF-related criterion for pathogenic variant classification, i. 0.01% ≤ MAF < 0.1% provided supporting evidence of pathogenic impact for single-nucleotide variants in *CFH*, *CFI*, *CD46*, *C3, DGKE*, and *VTN* [280], and ii. MAF < 0.01% provided moderate evidence of pathogenic impact for single-nucleotide variants and insertion/deletion variants in *CFH*, *CFI*, *CD46*, *C3*, and *DGKE* [284]. Thirdly, regarding exceptions to *the Rules for Combining Criteria*, if a MAF-related criterion was the only present evidence in favor of pathogenic impact, variants with conflicting evidence were classified as likely benign. Fourthly, regarding the reclassification of *CFB*, *CFP*, and *C3* variants of uncertain significance (VUS), loss-of-function variants were reclassified as likely benign if i. in-silico predictions alone resulted in conflicting evidence or ii. available evidence was insufficient to determine a classification according to *the Rules for Combining Criteria to Classify Sequence Variants*.

Paper III

The investigation was designed as a prospective cohort study. Patients hospitalized in the hematological department at Skane University Hospital, Lund, Sweden, between February 12th and September 28th, 2016, were eligible for inclusion. A need for prophylactic platelet transfusion during office hours, patient age of 18 years or older, and an established central venous access catheter (except for subjects recruited to study group 4) were inclusion criteria. Exclusion criteria were ongoing bleeds and

coincidental conditions and therapies associated with increased platelet consumption, including a diagnosis of acute promyelocytic leukemia and the treatment with anti-thymocyte globulin or amphotericin B less than three days before transfusion. Cases with confirmed HLA- or HPA-mediated alloimmunization were also excluded.

According to protocol, a morning platelet count (PLC) < 10 x 10^9/L (applicable for groups 1 - 3) and a PLC < 50 x 10^9/L prior to a scheduled minor intervention (applicable for group 4) were triggers to consider a patient for prophylactic platelet transfusion. Included subjects were categorized into four transfused study groups and one nontransfused control group:

a. Group 1 (Chemo): hypo-proliferative thrombocytopenia due to induction or consolidation chemotherapy.

b. Group 2 (Auto-HSCT): hypo-proliferative thrombocytopenia due to autologous HSCT.

c. Group 3 (Allo-HSCT): hypo-proliferative thrombocytopenia due to allogeneic HSCT.

d. Group 4 (Intervention): scheduled insertion of a central venous catheter and a platelet count (PLC) < 50 x 10^9/L.

e. Controls: nontransfused hematological control subjects exhibiting a morning PLC of 10 - 20 x 10^9/L, who were sampled according to study protocol to account for nontransfusion-related temporal variations in the levels of measured biomarkers.

The corrected count increment (CCI) was calculated using the following formula [305,369]:

$$CCI = \frac{\Delta PLC(10^9 \cdot L^{-1}) \times BSA(m^2) \times 10^5}{platelets\ transfused(10^9)}$$

where ΔPLC was assessed for the one and 24 hours post-transfusion time point, respectively (the PLC at one or 24 hours post-transfusion – the pretransfusion PLC). Applying the formula above, CCI should be presented with a unit of [(count x L^{-1}) x m^2] per platelet. However, CCI units are commonly left out when presenting data. In *paper III*, platelet transfusion refractoriness was defined as a single CCI measurement < 5000 at one hour after transfusion.

The body surface area (BSA) was calculated using the Mosteller formula [370]:

$$BSA(m^2) = \sqrt{\frac{height(cm) \times weight(kg)}{3600}}$$

To estimate the calculated post-transfusion concentration per biomarker *in vivo* caused by the passive infusion of plasma residuals, the following formulas were applied.

Estimated total blood volume (TBV) was calculated using Nadler's equation [371]:

$$TBV(L)_{male} = 0.3669 \times [height(m)]^3 + 0.03219 \times weight(kg) + 0.6041$$

$$TBV(L)_{female} = 0.3561 \times [height(m)]^3 + 0.03308 \times weight(kg) + 0.1833$$

Estimated plasma volume (PV) was calculated using the following equation:

$$PV(mL) = \frac{TBV(mL) \times [100 - hematocrit(\%)]}{100}$$

The approximate fraction of residual plasma (RP) in apheresis and pooled concentrates was 32% (80/250 mL) and 40% (120/300 mL), respectively. Therefore, the residual plasma volume per concentrate was estimated using the following formula:

$$RP(mL)_{concentrate} = \frac{volume(mL)_{concentrate} \times plasma\ fraction(\%)_{concentrate}}{100}$$

Finally, to calculate the estimated *in vivo* concentration per biomarker after transfusion, the following formula was applied:

$$Calculated\ concentration(unit \cdot mL^{-1})_{in\ vivo\ post\text{-}transfusion} = \frac{concentration(unit \cdot mL^{-1})_{in\ vivo\ pretransfusion} \times PV(mL) + RP(mL)_{concentrate} \times concentration(unit \cdot mL^{-1})_{concentrate}}{PV(mL) + RP(mL)_{concentrate}}$$

Ethics Approval

All studies were conducted in compliance with the Declaration of Helsinki [372].

Paper I/II

The Regional Ethics Review Board at Lund University approved the study (diary number: 2013/514) and the use of healthy control samples for the laboratory assays (diary number: 2017/582). In *paper II*, written informed consent was obtained from all participating individuals (or by proxy legal guardians) prior to study inclusion.

Paper III

The Regional Ethics Review Board at Lund University approved the study (diary number: 2015/628) and it was a priori registered as a clinical trial at ClinicalTrials.gov (identifier: NCT02601131). Written informed consent was obtained from all participating individuals prior to study inclusion.

Laboratory Methods

Major developments regarding the role of complement in health and disease during the last 30 years have called for the standardization of sample preparation, international harmonization of complement methods, and the global establishment of reference intervals and uniform units' nomenclature. Previously, complement research was flawed by inter-laboratory variances, evidently hampering the comparability of study results [373]. Consequently, the International Complement Society and the International Union of Immunological Societies formed the International Complement Standardization (ICS) Committee to battle these challenges. Today, many relevant complement assays have been subjected to various aspects of standardization, including robust determinations of intra- and intervariability and the establishment of reference ranges using a pool of sera from healthy blood donors [374].

Complement methods utilized in this thesis have relied on internationally recognized protocols which in some cases have been locally optimized for commercially available reagents. The leading complement expertise in the Medical Protein Chemistry Group headed by Professor Anna M. Blom has provided methodological support and all protocols, reagents, and laboratory equipment utilized, if not stated otherwise. In some cases, commercially available laboratory kits have been used. Below are short descriptions of used methods. For details on protocols, refer to the reprinted papers appended at the end of the thesis.

Nephelometry

In nephelometry, antigens (unknowns; particles of interest) are mixed with the relevant antibodies (reagents) in concentrations that facilitate the precipitation of small aggregates (immunoprecipitation). These precipitates remain idle in the solution without directly settling to the bottom. Nephelometry takes advantage of the principle that suspensions of small particles rather scatter than absorb light. Therefore, the amount of light scatter (commonly using a laser) can be measured by collecting the light at an angle. The concentration of the particle of interest is then interpolated using standard curves corresponding to the amount of scatter from known mixtures [375].

In this thesis, levels of C3 and C4 (*paper I*, *II*) were assessed with the appreciated support from staff in the Department of Clinical Chemistry and Pharmacology, using a certified nephelometer in the clinical laboratory (BN Prospec Systems; Siemens Healthineers, Erlangen, Germany).

Enzyme-linked Immunosorbent Assays

In ELISA, antigens of interest are attached to a surface (commonly microtiter plate wells). Then, a matching antibody is applied, thus binding to the antigen. Unbound antibodies are then removed by immunowash. The applied antibody is connected to

an enzyme that produces a detectable signal (commonly a color change) when the enzyme's substrate is added. The absorbance of color is measured, and concentrations are interpolated using the absorbances of standard curves of known antigen concentrations. In 'sandwich' ELISA, the surface is prepared with known quantities of capture antibodies. Then, nonspecific binding sites on the surface are blocked and samples containing the antigen of interest are added. Unbound antigens are then removed by immunowash. Subsequently, another specific (primary) antibody is added (the antigen of interest becomes 'sandwiched' between two antibodies), and a secondary enzyme-linked antibody targeting the primary antibody is added. Finally, a substrate is added which produces a signal as described above [376].

In this thesis, ELISA was used to determine levels of FH-specific antibodies (*paper I*, *II*), levels of FH and FI (*paper II*), levels of sTCC (*paper III*), and levels of sP-selectin and sCD40L (*paper III*; commercial ELISA kits).

Immunoblots

Immunoblotting, also termed western blotting, is a technique to detect the presence of a specified protein. First, samples are boiled to denature present proteins. Then, proteins are separated by size, commonly using SDS-PAGE gel electrophoresis. After electrophoretic separation, the proteins are transferred to a membrane (commonly consisting of polyvinylidene difluoride). Following blocking to prevent non-specific binding, protein-specific (primary) antibodies are added. Subsequently, secondary enzyme-linked antibodies (targeting the primary antibodies) are added. Finally, the membranes are developed using a colorimetric substrate, which reveals the presence of protein [377].

In this thesis, qualitative immunoblot analysis (present/absent) was performed to investigate factor H-related protein 1 (*paper I*, *II*).

Flow Cytometry

In flow cytometry, cells or particles are stained with antibodies targeting the protein of interest. These antibodies are linked to a fluorescence-based indicator (a fluorochrome), such as phycoerythrin. In order to identify the cell populations of interest, different fluorochrome-linked antibodies targeting specific cell populations (e.g., CD4-specific antibodies for T helper cells, CD8-specific antibodies for cytotoxic T cells, and CD20-specific antibodies for B cells) are also added to the solution. Thus, these gating markers enable the post-processing creation of forward and side scatter density plots to identify cell populations of interest and to exclude debris. After incubation with relevant antibodies, erythrocytes are lysed to easier isolate leukocyte subpopulations. Then, the solution is injected into a flow cytometer instrument focused to let cells flow through a laser beam, ideally one at a time. The laser excites the fluorescence-based indicators so light is absorbed and emitted at specific wavelengths. The quantity of emitted light

corresponds to the number of fluorochrome-linked antibodies. Thus, the expression of protein in the different cell populations can be quantified as mean fluorescence intensity [378].

In this thesis, the surface expression of MCP (CD46) was investigated in leukocyte subpopulations to assess whether identified genetic variants in the *CD46* gene were correlated with absent or deficient expression of CD46 on host cells (*paper II*).

Whole Genome Sequencing

High throughput sequencing (HTS), also termed next-generation sequencing, is a sequencing method that has revolutionized veritably all aspects of biological sciences during the last two decades. It utilizes parallel sequencing, at continuously increasing speeds and continuously decreasing costs, thereby outrivalling the considerably slower Sanger sequencing method. Whole genome sequencing (WGS) is a HTS method that sequences the whole genome, including non-coding regions. Hence, WGS can detect large-scale copy number variations, and relevant intronic changes in splice sites and promoter regions [379]. Illumina (San Diego, CA, USA) is an extensively used HTS platform. Briefly, the Illumina workflow consists of four steps. First, a germline DNA sample is randomly fragmented followed by ligating specialized adapters to each end of the DNA fragments. This is called *library preparation*. Secondly, the library is loaded into a flow cell and the fragments are hybridized to the flow cell surface. Thereafter, bound fragments are amplified through bridge amplification in a process termed *cluster amplification*. Thirdly, sequencing reagents are added, including fluorophore-labeled nucleotides, which incorporate the first base. The emission from each cluster is recorded and emission intensity and wavelength are used to identify the base. Thereafter, the fluorophore is removed from the nucleotide whose three-prime end is regenerated to enable hybridization of the next nucleotide in the template sequence. This is called *sequencing*. Finally, reads are aligned and mapped to a human reference genome using bioinformatics software, thereby allowing for the identification of differences between the reference genome and the sequenced reads. This process is termed *alignment and data analysis*. The post-mapping processing of obtained data includes variant calling and various quality control checks, facilitated by using specific tool kits, such as the GATK analysis software (Broad Institute; Cambridge, MA, USA). Thereafter, specific software applications are used to detect potentially causative variants by defining various criteria, including filtration thresholds based on the variant allele frequency in public genome databases. This is called *variant filtering*.

In this thesis, a predefined gene panel including the following genes was used (*paper II*): i. genes encoding complement factors: *C3*, *CFB*, *CFP*; ii. genes encoding complement inhibitors: *CFI*, *CFH*, *CFHR1/3/5*, *CD46*, *VTN*; and iii. genes directly or indirectly influencing thrombus formation: *PLG*, *THBD*, *DGKE*. Variant calling was performed using the GATK v4.1 analysis software and variant filtering was performed

using The Ingenuity Variant Analysis tool (Qiagen; Hilden, Germany). Of note, variants exhibiting ≥ 5% global MAF in 1000 Genomes Project, the Exome Aggregation Consortium, the Genome Aggregation Database or the National Heart Lung and Blood Institute Exome Sequencing Project were excluded. However, in *paper II*, part of the manual ACMG guidelines-compliant assessments of detected genetic variants included the use of the highest MAF regardless of ethnic origin (Hi_Freq MAF) for MAF-related variant classification criteria. Therefore, variants exhibiting a MAF < 5% in the global population (thereby included according to the automated filtering process) could demonstrate a Hi_Freq MAF ≥ 5% which indeed would be very relevant for the classification of the specific variant. Thus, the manual assessments of detected variants were crucial for the final classifications, which is also emphasized by the disease-specific modifications of some classification criteria as previously described.

Statistical Methods

Continuous variables demonstrated non-Gaussian distributions throughout all papers and were descriptively presented as medians with lower and upper quartiles. Qualitative variables were presented with counts and percentages. All *p*-values were two-tailed and values < .05 were considered significant. All analyses were performed using IBM SPSS Statistics (version 24, 25, or 26; IBM, Armonk, NY, USA).

Qualitative variables

Statistical differences for binary variables were evaluated using Fisher's exact test (*paper I*, *II*, *III*).

Quantitative variables

Statistical differences for comparisons of continuous variables between two independent groups were evaluated using the Mann-Whitney U test (*paper I*, *II*, *III*).

Statistical differences for dependent continuous variables were evaluated using the Friedman test followed by a post hoc Dunn's multiple comparisons test. Significant *p*-values were adjusted by the Bonferroni correction for multiple tests (*paper III*).

Bland-Altman plots were used to visualize the agreement between two continuous variables (*paper III*).

A multiple linear regression was performed by the log10 transformation (normalization) of a dependent variable (*paper III*).

Spearman's rank-order correlation tests were used to assess bivariate correlations between variables with non-normally distributed residuals (*paper III*).

Results

In this section, major findings of the included papers are presented. For details, refer to the reprinted papers appended at the end of the thesis.

Paper I/II

Clinical Screening (paper I)

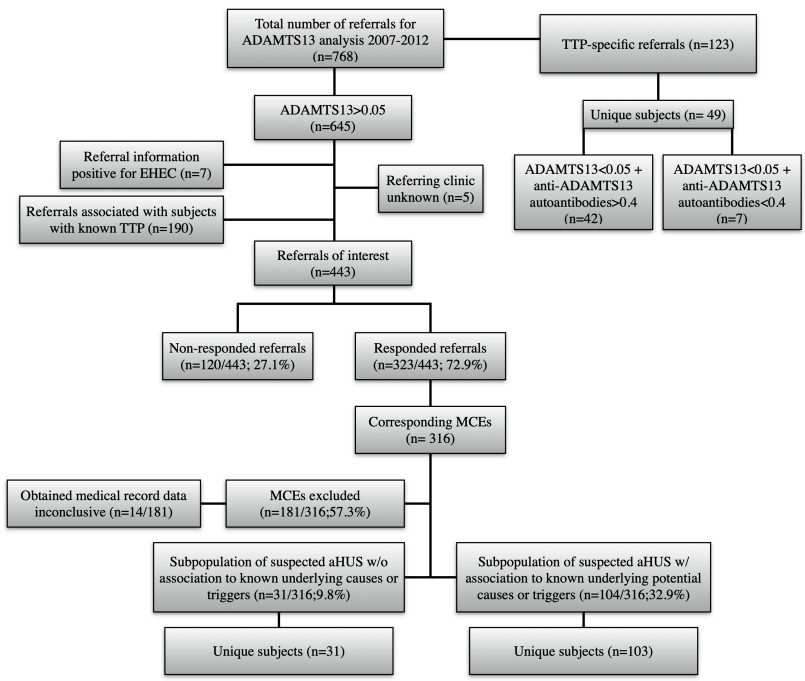

Figure 8. Schematic outline of the study
Total and selected referrals as well as response rate on medical record requisitions are shown. Numbers and percentages of the populations are presented within brackets. The number of medical care episodes (MCE) corresponding to the total number of responded referrals are shown. The MCEs have been screened in line with a clinical report form and distributed into the two populations as defined. Reprinted with permission from John Wiley & Sons, Inc. [380].

One hundred thirty-five (135/316; 43%) screened medical care episodes (MCEs), corresponding to 134 unique subjects, fulfilled the study inclusion criteria. According to documented information available in medical records encompassing the inpatient medical care episode during which samples were sent for ADAMTS13 analysis, one hundred and three subjects were categorized as suspected aHUS associated with potential causes/triggers. Thirty-one subjects were associated with no obvious causes/triggers. A review of identified causes/triggers can be found in *Table 1, paper I* (**Figure 8**).

In summary, the treating physician speculated about a diagnosis of HUS and/or TMA in 44/103 (43%) and 20/31 (65%) cases, respectively. However, in seven and ten cases of suspected aHUS with and without associated causes/triggers, respectively, a histopathological diagnosis of TMA was made. In total, one case of complement-mediated aHUS (deficiency of FHR plasma proteins and autoantibody-positive form of hemolytic uremic syndrome [DEAP-HUS]) was confirmed before discharge.

Laboratory Complement Screening (paper I)

The laboratory investigations consisted of the anonymized assessments of FH-specific antibodies and levels of C3 and C4 for all available samples referred to the Coagulation Unit for analysis of ADAMTS13 from 2007 to 2012. That is, we had no opportunity to only select those who exhibited ADAMTS13 activity > 0.05 AU/L. Therefore, the investigations served as a laboratory screening complementing the clinical screening in the tentative assessment of aHUS prevalence; however, the clinical and laboratory screenings could not be coupled.

In total, 662 samples were available for FH-specific antibody analyses. Twenty-four samples exhibited significant levels of FH-specific antibodies. Six of the 24 (25%) FH-specific antibody-positive samples exhibited homozygous FHR1 deficiency as compared to one of 48 (2%) randomly chosen antibody-negative healthy controls (**Figure 9**). Six hundred twenty-seven samples were available for analysis of C3 and C4. In total, 59 (9%) cases indicated isolated low C3 levels (C3 median: 0.79, range: 0.42–0.85), potentially reflecting alternative pathway consumption. In total, fifty-four FH-specific antibody-negative cases indicated isolated alternative pathway consumption. Five FH-specific antibody-positive cases exhibited isolated low C3 levels. Based on antibody-positive samples and antibody-negative samples exhibiting isolated alternative pathway consumption, a total of 78 cases demonstrated laboratory aberrations consistent with complement dysregulation.

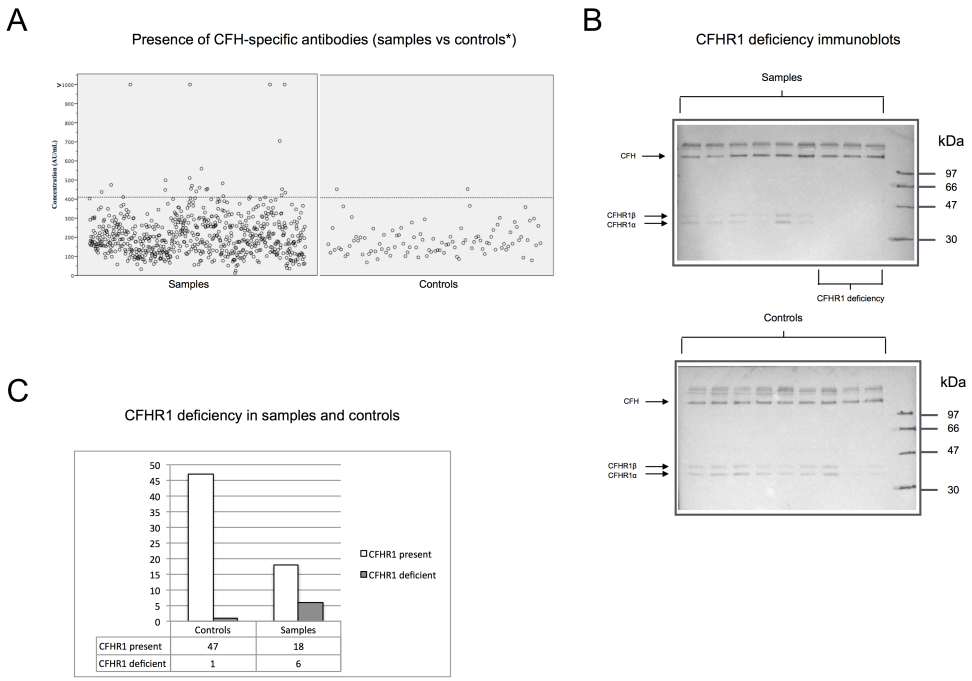

Figure 9. Laboratory screening: presence of FH-specific antibodies and deficiency of FHR1
Samples include all available samples referred to the Coagulation Unit for ADAMTS13 analysis between the years 2007 - 2012. (a) Levels of FH-specific antibodies in samples (n = 662) and healthy controls (n = 100) were assessed by ELISA. Samples exhibited significantly higher levels of FH-specific antibodies and included four outliers with very high levels. Cutoff for antibody-positive samples was determined by $mean_{controls}$ + 3SD. (b) Results from FHR1 immunoblots are illustrated. Complete lack of FHR1 bands corresponds to homozygous deficiency. (c) Homozygous deficiency of FHR1 in FH-specific antibody-positive samples (n = 6/24) and FH-specific antibody-negative controls (n = 1/48) are presented. The proportions are illustrative of the association between FHR1 deficiency and the formation of FH-specific antibodies, as observed in DEAP-HUS. Reprinted with permission from John Wiley & Sons, Inc. [380].

In summary, in 64 of totally 134 (48%) cases, the treating physician speculated about a diagnosis of HUS and/or TMA. In seventeen of these cases, a renal biopsy confirmed TMA. The definite diagnosis of complement-mediated aHUS was only made in one case before discharge. The laboratory screening revealed 78 cases consistent with complement dysregulation. Consequently, at the time of discharge, there was a discrepancy between confirmed (one case) and highly probable diagnoses (17 cases with TMA-positive renal biopsies) and the 78 laboratory cases consistent with complement dysregulation. This suggests a possible underdiagnosis of complement-mediated aHUS (at least in association with discharge); particularly so, as medical records only revealed samples being sent for genetic analyses in totally four cases. Evidently, this does not exclude genetic analyses being sent subsequently during management in the outpatient department. Therefore, a follow-up study was needed.

Clinical Follow-Up (paper II)

The study was conducted in median 63 months (q1, q3: 46, 77) after the inpatient medical care episode during which samples were sent for ADAMTS13 analyses. At the time of study inclusion, 52% (n = 54/103) and 19% (n = 6/31) of patients in the initial cohort of clinically suspected aHUS with and without potential causes/triggers, respectively, were deceased. Thus, almost half (45%) of the total eligible subjects were deceased.

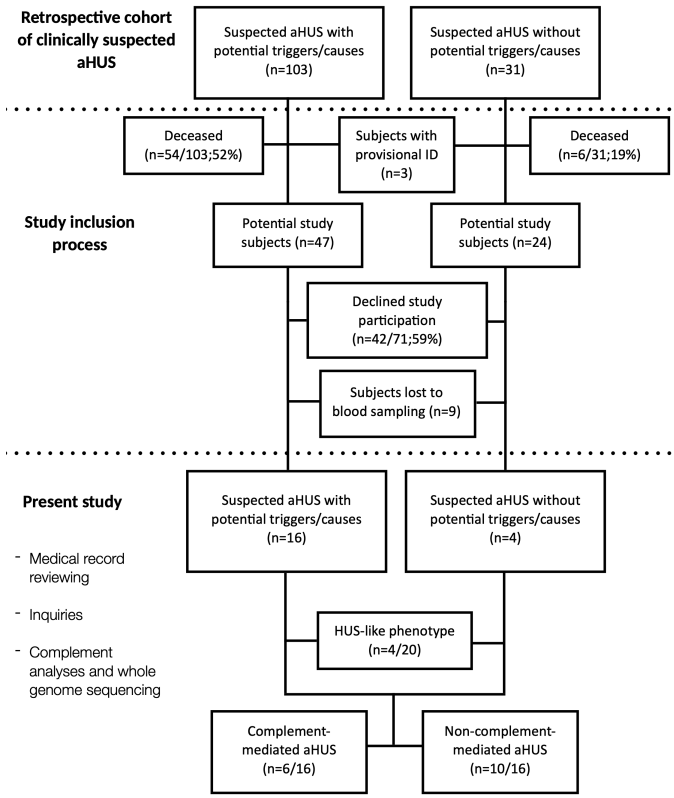

Figure 10. Study inclusion process
Numbers are presented within brackets. Additional percentages are presented with reference to deceased subjects per group and the proportion of potential study subjects that declined study participation. By means of combining clinical and whole genome sequencing data, the study cohort was partitioned into three groups as presented. Reprinted from [381]. © 2021 the Authors.

In total, 20 subjects accepted study inclusion. Six subjects were categorized as definite or highly suspected complement-mediated aHUS. Ten subjects were categorized as noncomplement-mediated aHUS. The remaining four subjects were determined to have an HUS-like phenotype (**Figure 10**).

Two of six subjects (33%) in the complement-mediated aHUS group had not been diagnosed with (complement-mediated) aHUS by their treating physician at study entry. However, one of the two had received a tentative diagnosis of TMA (*Table 3, paper II*).

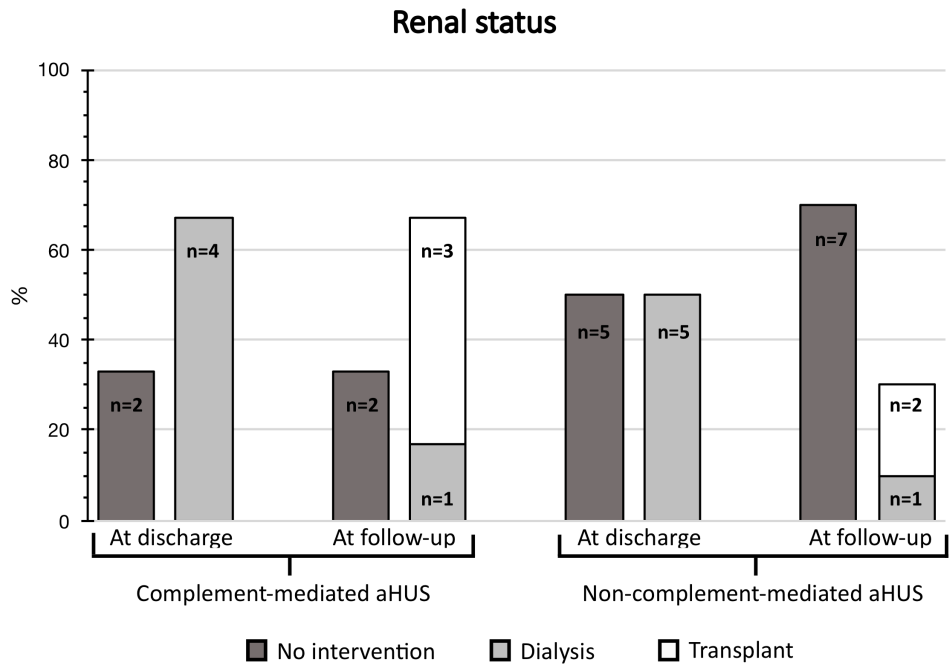

Figure 11. Renal status

Comparisons of the status at discharge and at the time of study. Three of the four dialyzed subjects in the complement-mediated aHUS group had undergone renal transplantation. Two of these subjects were receiving eculizumab maintenance therapy. The two subjects without intervention had been considered for eculizumab but watchful waiting was concluded. Of note, two (40%) of the dialyzed subjects in the noncomplement-mediated aHUS group had been weaned off dialysis without the need for transplantation. Reprinted from [381]. © 2021 the Authors.

Table 4. Acute episode features

VARIABLES	COMPLEMENT-MEDIATED aHUS (n=6)	NONCOMPLEMENT-MEDIATED aHUS (n=10)	*p*-value
General characteristics			
Sex, female - no. (%)	3 (50)	5 (50)	1
Age, years - median (q1-q3)	46 (37-48)	42 (23-59)	.79
Symptoms/clinical manifestations			
Macroscopic hematuria - no. (%)	3 (50)	1 (10)	.12
Abdominal symptoms/manifestations - no. (%)	6 (100)	2 (20)	.23
Routine laboratory results			
Hemoglobin (g/L), min - median (q1-q3)	79 (71-80)	73 (67-85)	.64
Creatinine (µmol/L), max - median (q1-q3)	904 (448-1507)	343 (190-681)	**.03**
Platelet count (x10^9/L), min - median (q1-q3)	69 (35-92)	29 (15-56)	.18
Lactate dehydrogenase (µkat/L), max - median (q1-q3)	23 (15-36)	13 (9-18)	.12
Haptoglobin <0.1 g/L - no (%)	5 (83)	7 (70)	1
Diagnostics and treatment			
Inpatient empirical PEX/PI - no. (%)	5 (83)	3 (30)	.12
Inpatient renal biopsy - no. (%; histology)	3 (50; TMA x3)	2 (20; TMA x1,IgAN x1)	.3
Dialysis at discharge - no. (%)	4 (67)	5 (50)	.63
Scheduled follow-up for suspected/confirmed TMA - no. (%)	4 (67)	4 (40)	.61

Adapted from [381]. © 2021 the Authors.
(IgAN: Immunoglobulin A nephritis; PEX/PI: plasma exchange therapy/plasma infusion; TMA: thrombotic microangiopathy)

In summary, two cases of complement-mediated aHUS (according to study definitions) had not yet received a correct diagnosis during clinical routine follow-up. Differences in renal outcome between complement-mediated and noncomplement-mediated aHUS suggested a qualitatively worse outcome in the former group (**Figure 11**). The worse renal outcome in the complement-mediated aHUS group did also correspond to a significantly higher peak concentration of creatinine in association with the acute episode (during which samples for ADAMTS13 analysis were sent) (**Table 4**).

Genetic Analyses (paper II)

Whole genome sequencing revealed 20 unique genetic variants in 14 subjects. All variants were heterozygous. In total, one (disease-contributing) pathogenic and four (likely disease-contributing) VUS were identified and distributed among five of the six subjects in the complement-mediated aHUS group. One genetic *C3* variant (variant ID 1; c.193A>C,p. K65Q) corresponded to decreased plasma levels of C3 at follow-up (i.e., during a calm state of disease activity). Another genetic variant, in the *CFI* gene (variant ID 12; c.982G >A,p.G328R), corresponded to decreased plasma levels of FI (*Figure 2*, *paper II)*. Finally, a novel likely disease-contributing C-terminal missense variant in the *CFH* gene (variant ID 10; c.3450A>G, p.I1150M) was identified (**Table 5**).

Table 5. Variant type specifics and classification

VARIANT ID†	SUBJECT (#)	GENE (transcript variant)	VARIATION TYPE	TRANS-LATIONAL EFFECT	PROTEIN DOMAIN	PROTEIN VARIANT	IN-SILICO PREDICTIONS	ACMG CLASS-IFICATION
1	A, B	*C3* (c.193A>C)	SNV	Missense	MG 1	p.K65Q	Damaging	Pathogenic
2	F	*C3* (c.4030-4C>T)	SNV	Splice acceptor site	-	-	Benign	Likely benign
3	J	*C3* (c.4850+3G>A)	SNV	Splice donor site	-	-	Benign	Likely benign
4	C, K	*CD46* (c.1058C>T)	SNV	Missense	TM	p.A353V	Benign	Benign
5	D	*CFB* (c.1697A>C)	SNV	Missense	SP	p.E566A	Benign	Likely benign
6	G ,M	*CFH* (c.-307C>T)	SNV	Promoter	-	-	N/A	Likely benign
7	B, L	*CFH* (c.2634C>T)	SNV	Syno-nymous	CCP 15	p.H878H	N/A	Likely benign
8	J	*CFH* (c.3133+8G>T)	SNV	Splice donor site	-	-	Benign	Likely benign
9	E	*CFH* (c.3148A>T)	SNV	Missense	CCP 18	p.N1050Y	Conflicting	Likely benign
10	B	*CFH* (c.3450A>G)	SNV	Missense	CCP 19	p.I1150M	Conflicting	VUS‡
11	D	*CFHR5* (c.485_486dupAA)	Insertion	Frameshift	CCP 3	p.E163fs*10	N/A	VUS‡
12	I	*CFI* (c.982G>A)	SNV	Missense	Linker 2	p.G328R	Damaging	VUS‡
13	H	*CFI* (c.1322A>G)	SNV	Missense	SP	p.K441R	Benign	Benign
14	A	*CFI* (c.1534+5G>T)	SNV	Splice donor site	-	-	Conflicting	Likely benign
15	E	*CFI* (c.1547G>T)	SNV	Missense	SP	p.G516V	Damaging	VUS‡
16	F	*DGKE* (c.35C>T)	SNV	Missense	Signal peptide	p.P12L	Conflicting	Likely benign
17	J	*PLG* (c.185+4T>C)	SNV	Intronic	-	-	Conflicting	Benign
18	E	*PLG* (c.266G>A)	SNV	Missense	PAN	p.R89K	Conflicting	Likely benign
19	G	*PLG* (c.1567C>T)	SNV	Missense	Kringle 5	p.R523W	Conflicting	Likely benign
20	S	*PLG* (c.2356C>T)	SNV	Missense	SP	p.R786C	Conflicting	VUS

† All detected variants were heterozygous.

‡ Likely disease-contributing.

In-silico predictions were assessed for missense and splice site variants. Adapted from [381]. © 2021 the Authors. (SNV: single-nucleotide variant; VUS: variant of unknown significance)

In summary, given the ultra-rarity of disease, the proposed diagnostic scheme for the assessment of potential cases with complement-mediated aHUS, including genetic variant classifications, showed a good ability to identify cases with complement-mediated aHUS in a retrospective, semi-selected material. The four cases who had received a complement-mediated aHUS diagnosis in the clinical setting, were also categorized as complement-mediated aHUS according to a priori-defined study criteria.

Paper III

Correlations between Biomarkers and the CCI

Forty-three transfused and ten nontransfused subjects were included. A total of 54 transfusions were administered. To evaluate correlations between CCI and levels of sTCC, sP- selectin and sCD40L, respectively, biomarkers were assessed in the following settings for groups 1–4 combined:

a. *In vitro* in the transfused concentrates, potentially reflecting lost viability during storage.

b. *In vivo* before transfusion, potentially reflecting pretransfusion activation as an intrinsic factor contributing to an insufficient post-transfusion platelet response.

c. *In vivo* after completed transfusion to determine the 1 h post-transfusion Δconcentration (1 h post-transfusion - pretransfusion), potentially reflecting transfusion-mediated complement activation.

The *in vitro* concentration of sTCC, sP-selectin and sCD40L, respectively, did not reveal any significant correlation with the CCI(1 h). Neither the concentrations of sTCC, sP-selectin or sCD40L before transfusion nor the corresponding 1 h post-transfusion Δconcentrations showed any significant correlations with the CCI(1 h) (**Figure 12**). The corresponding correlations tested for CCI(24 h) were also without significant results. Additionally, subgroup analyses, only including cases with actual concentrations at 1 h after transfusion > calculated post-transfusion concentrations (see Bland-Altman plots in *Figure 2*, *paper III*), for sTCC (n = 35) and sP-selectin (n = 17), did not reveal any significant correlations with the CCI(1 h) or CCI (24 h). Finally, subjects with CCI(1 h) < 5000 (n = 5), i.e., by definition transfusion-refractory, were compared to subjects with CCI(1 h) ≥ 5000 without significant differences observed for levels of biomarkers in the settings examined above.

In summary, no obvious associations between investigated biomarkers and the CCI could be identified in any of the assessed scenarios.

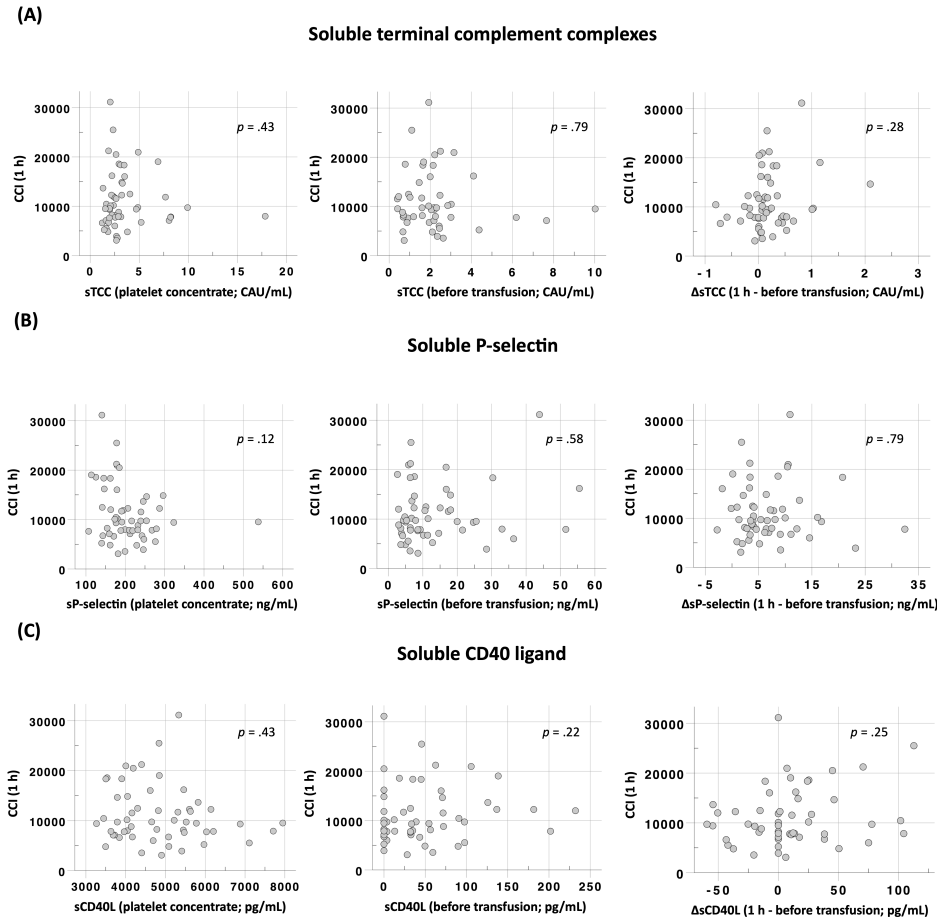

Figure 12. Correlations between investigated biomarkers and the corrected count increment
Data encompassed all 54 transfusion occasions. Scatter plots to the left represent correlations between concentrations in the platelet concentrates (*in vitro*) and the corrected count increment (CCI) at 1 h after transfusion. Scatter plots in the center represent correlations between the *in vivo* concentrations before transfusion and the CCI(1 h). Scatter plots to the right represent correlations between the *in vivo* Δconcentrations at 1 h after transfusion. Reprinted from [382]. © 2021 the Authors.

Clinical and Laboratory Comparisons between Study Groups

The concentration of sTCC before transfusion was significantly higher (p = .04) in group 4 (2.5 [2.2, 5.7] CAU/ mL) compared to groups 1–3 (1.9 [0.9, 2.5] CAU/mL). The 1 h post-transfusion Δconcentration of sTCC was not significantly different comparing groups 1-4 to controls, thus suggesting that the significant trend observed for sTCC levels post-transfusion (groups 1-4 aggregated, *Figure 2*, *paper III*) was clinically negligible. The concentration of sP-selectin before transfusion was significantly higher (p = .02) in group 4 (18.4 [6.8, 34.9] ng/mL) compared to groups

1–3 (7.7 [5.2, 13.3] ng/mL). However, the 1 h post-transfusion Δconcentration of sP-selectin was not different comparing groups 1–3 to group 4 (**Figure 13**).

A routine laboratory assessment revealed significantly higher platelet and white blood cell counts in group 4 compared to groups 1-3 prior to transfusion. No other relevant differences for routine laboratory and clinical characteristics were identified (*Table 1*, *paper III*).

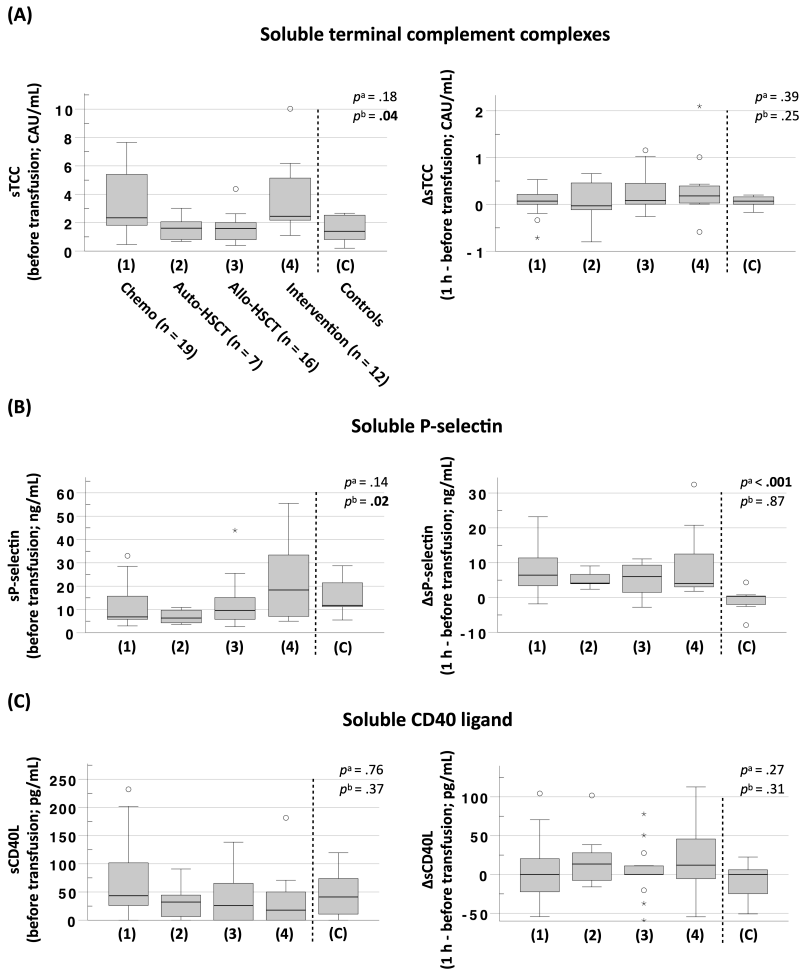

Figure 13. Differences in levels of biomarkers between clinical groups

Data encompassed all 54 transfusion occasions and are presented according to study groups. Box plots to the left represent comparisons of the *in vivo* concentrations before transfusion. Boxplots to the right represent comparisons of the *in vivo* Δconcentrations at 1 h after transfusion (1 h post-transfusion - pretransfusion). The dashed lines separate the control subjects (C) from the remaining cohort. Controls (n = 10) were nontransfused hematological patients with a morning platelet count of 10 - 20 x 10^9/L, who were sampled in accordance with the study protocol to account for nontransfusion-related temporal variations of the investigated biomarkers. Reprinted from [382]. © 2021 the Authors.
p^a: groups 1-4 (transfused) versus controls (nontransfused).
p^b: groups 1-3 (hypo-proliferative) versus group 4 (nonhypo-proliferative).

To estimate the effect of the platelet and white blood cell counts on the *in vivo* concentration of sP-selectin before transfusion, a multiple linear regression for all subjects was performed. The analysis indicated a positive effect of the platelet count (p = .01); however, the contribution of the white blood cell count was not significant (p = .52).

In summary, the significantly greater platelet count observed in group 4 compared to groups 1-3 likely contributed to the significantly higher sP-selectin levels associated with the same group before transfusion. Because of the association between complement and platelet activation, the higher sP-selectin levels demonstrated in group 4 may also have contributed to the significantly higher sTCC levels observed in the same group before transfusion. Of note, sP-selectin levels in group 4 were, however, still considerably lower compared to the reference range in a healthy population.

Discussion and Conclusions

Paper I/II

As previously described, complement-mediated aHUS is an ultra-rare disease associated with multiple challenges. Effective treatment does exist which necessitates a resolute assessment. Phenotypical overlaps, including routine laboratory investigations, obscure a straightforward diagnosis. Indeed, laboratory parameters cannot differentiate between complement-mediated aHUS and other types of phenotypically similar conditions [298]. Moreover, in cases with milder presentations, the full laboratory tetrad is not always fulfilled [266,275]. An essential part of the diagnostic workup consists of genetic analyses and interpretations. However, given the general lack of functional analyses, identified genetic variants are not always easily classified. Thus, complement genetic expertise is generally recommended to guide the interpretation of genetic findings. In Sweden, one clinical expert genetic laboratory exists [383]. The aHUS project was launched by my supervisors at a time (year 2013) during which the availability of WGS still was limited and associated with high costs. Moreover, the nomenclature in the scientific aHUS community experienced a shift of focus at the time, which likely contributed to some confusion concerning clinical assessments of patients with phenotypical HUS. Thus, it was hypothesized that cases of complement-mediated aHUS potentially were underdiagnosed. As the Coagulation Unit in Malmö disposed of a large number of samples previously referred for ADAMTS13 analysis, it was hypothesized that these samples were enriched with cases of complement-mediated aHUS. Given the ultra-low prevalence, this provided an unprecedented opportunity to investigate the potential presence of complement-mediated and noncomplement-mediated aHUS in a Swedish setting. However, since then, the field of research has developed immensely, the costs of genetic analyses have decreased, and the awareness of aHUS among physicians and awareness of available guidance by expert laboratories have increased. In fairness, the two papers constituting the aHUS project (*paper I* and *II*) do not provide solid confirmation of the initial hypothesis of underdiagnosis. Results in *paper I* are seriously flawed by the retrospective study design, relying on medical record documentation, thereby introducing an obvious physician-mediated information selection bias. The study design was also limited by the inability to couple the clinical and laboratory screening processes. However, the results were consistent with a possible underdiagnosis at the time of discharge, although it revealed nothing

about diagnoses being made during the subsequent clinical outpatient management. Only information on four samples being sent for genetic analyses was present in medical records, which may be indicative of inconclusive assessments.

Additionally, the laboratory analyses served as a blunt screening tool, only identifying FH-specific antibody-positive cases and cases revealing alternative pathway consumption. Far from all cases of complement-mediated aHUS demonstrate low C3 levels. Hence, the laboratory screening process may have underestimated the number of potential cases.

In conclusion, *paper I* does, to the best of my knowledge, however, represent a first staggering attempt trying to investigate complement-mediated aHUS on a large scale in a Swedish setting.

In *paper II*, it was revealed that almost half of the eligible subjects were deceased at the time of follow-up. Of note, it was not only individuals of high age who were deceased, which is illustrative of the overall high mortality rate of diseases associated with MAHA. Due to the low sample size, results from *paper II* did not provide any firm evidence of underdiagnosis. Two of the six subjects who had been categorized as complement-mediated aHUS according to study definitions had not received such a diagnosis in the clinical setting. However, one of the two had received a diagnosis of TMA, which suggests clinical awareness of the associated disease panorama. Interestingly, the one patient who had not received a diagnosis at all suffered from a previous episode of phenotypical HUS 15 years earlier. Genetic sequencing was not performed and consequently, no diagnosis of complement-mediated aHUS was made. However, the individual suffered from a nonlethal ischemic stroke during the relapse, which may have impacted clinical priorities.

Evidently, *paper II* was flawed by an obvious selection bias as patients who had received an aHUS diagnosis in the clinical setting likely were more inclined to accept study participation. This may have contributed to an underestimation of underdiagnosed cases.

Additionally, genetic analyses in *paper II* were based solely on the identification of rare genetic variants by WGS. However, the contribution of complex genomic rearrangements commonly observed in the CFH-CFHR genomic region has been identified in five to ten percent of the total cases in previous studies [114,280]. The complexity of these genetic alterations is not easily recognized by WGS. These investigations often require other methodological approaches, such as multiplex ligation-dependent probe amplification [285]. Thus, there may be unidentified cases of complement-mediated aHUS caused/facilitated by disease-contributing large copy number variations and/or non-allelic homologous recombinations among the included subjects. However, as the prevalence in previous studies is relatively low, it would likely

not significantly increase the number of patients categorized as complement-mediated aHUS in our study cohort.

Method descriptions and study inclusion criteria are generally vaguely defined in clinically oriented aHUS studies. Thus, the main contribution of *paper II* is the systematic presentation of a diagnostic scheme for the assessment of potential cases with complement-mediated aHUS, including the disease-specific modifications made to the ACMG classification criteria. The phenotype-genotype correlations described in *paper II* also nicely illustrate the heterogeneity of disease and the contribution of complement-triggering events during flare-ups. However, the findings also raise some relevant questions. In the two subjects harboring the pathogenic *C3* variant (subject A and B, *paper II*) low C3 levels indicated an ongoing, subclinical consumption by the alternative complement pathway. These samples were obtained during follow-up, i.e., during a calm state of disease activity. To what extent (if any) subclinical complement activation contributes to long-term renal outcome is not yet explored. This is indeed an important area of investigation as it may guide indications for complement-inhibiting maintenance therapy.

In conclusion, to the best of my knowledge, assessments of aggregated clinical and genetic data in cases of suspected aHUS have not been previously described in a Swedish setting. Evidently, *paper II* was strongly limited by the small cohort size. However, considering the ultra-low disease prevalence, we managed to identify and systematically categorize cases of suspected aHUS by applying a proposed diagnostic scheme to a cohort previously referred for ADAMTS13 analysis in the clinical setting.

Paper III

As described in a previous section, the relevance of complement activation in immune PTR has been indicated in several studies. However, the potential role of complement activation in nonimmune PTR has not been explored. Moreover, no previous studies have investigated the effect of *in vitro* complement activation during platelet product storage on the *in vivo* post-transfusion response. Hence, the present study was designed, including a predefined subset of a study previously published by some of the co-authors [384]. As previously described, a mutual influence between platelet and complement activation exists. Consequently, platelet activation biomarkers were also investigated. Our results could not confirm a significant correlation between the corrected count increment and complement and platelet activation biomarkers, respectively, in any of the three predefined settings (described in the previous section). To the best of my knowledge, this is the first study to investigate these correlations prospectively and for sequential time points. However, as study sample size estimations had been performed with respect to the aim of the initial study, our laboratory investigations may have been

underpowered to reveal true differences. This is obviously a central limitation of the study design. Given the lack of previous studies, our investigations provide some novelty, serving as a steppingstone for confirmation of results in larger cohorts. For instance, several *in vitro* investigations concerning platelet product constituents, including platelet and complement activation biomarkers, have been previously published [359,385,386], demonstrating correlations between levels of biomarkers and platelet product storage time. Thus, it has been proposed that there may be an association between increased biomarker levels *in vitro* and a poor *in vivo* post-transfusion response. Attempts testing this hypothesis have not been published before our own study.

Another limitation of the study was the heterogeneity of included diseases and that differences in characteristics of the transfused platelet concentrates may have influenced the post-transfusion response in unanticipated ways. Thus, although platelet concentrates were randomly distributed among the study subjects, concentrate characteristics theoretically may have impacted clinical study groups disproportionally given the small sample sizes.

Finally, on account of the study aim to investigate PTR in nonalloimmunized patients, another limitation was that the prevalence of HLA- and HPA-mediated alloimmunization was assessed according to clinical routine and not for all participating subjects. However, this limitation would have been more problematic in the event of positive study results.

In conclusion, complement and platelet activation do tentatively not appear relevant for the post-transfusion response in hematological patients receiving prophylactic platelet transfusions. However, our study results must be interpreted cautiously, and the study should primarily be regarded as a pilot study, providing results that can be utilized in power calculations of future studies.

Future Perspectives

This thesis has described different aspects of complement activation in relation to thrombocytopenic disorders. A general limitation of the individual projects that constituted this thesis was the lack of sample size estimations. The rarity of investigated conditions and the hypothesis-generating approach, respectively, made it practically impossible to adhere to this fundamental aspect of applied research. Moreover, the work of this thesis has been carried out in a center internationally renowned for excellent hemophilia research; however, without specific in-house competence related to complement-related disorders in general and complement-mediated aHUS in particular. This too has presented several challenges and I am truly grateful for the kind support provided by the Medical Protein Chemistry Group headed by Professor Anna M. Blom in the making of this thesis.

The number of diseases caused, contributed, or exacerbated by complement activation is continuously growing. As previously described, the phenotypical landscape is vast and the presence of identical or similar genetic alterations may, for yet unknown reasons, result in very different clinical manifestations. The question of disease penetrance and manifested phenotypes in relation to coincident complement dysregulation needs further exploration.

Moreover, a better understanding of the dynamics between physiological complement activation and mechanisms of immunomodulation will probably shed valuable light on various autoimmune and autoinflammatory diseases. The potential importance of complement activation in thromboinflammatory disorders has recently been emphasized by the ongoing global pandemic of the coronavirus disease 2019 (COVID-19) [387,388]. Several associations between complement activation, endothelial injuries, and severe systemic inflammatory responses have been proposed [389]. Additionally, manifestations of renal thrombotic microangiopathy in case series of COVID-19 patients have been presented [390,391]. This suggests the contribution of an exaggerated complement response, particularly affecting the intrinsically complement-susceptible renal microcirculation. Indications of complement activation in COVID-19 have further resulted in the launching of several trials investigating the effects of complement-inhibiting therapy [167,392-394]. So far, these studies have presented ambiguous results, thus indicating a multifactorial model where disease-contributing complement activation may be relevant for the pathogenesis in a subset of

COVID-19 patients. Still, much more work needs to be done to clarify relevant pathogenetic mechanisms in this area. Evidently, the dynamics between the intravascular plasma cascade systems, platelet activation, and the acquired immune response are extremely complex *in vivo*.

Regarding the disorders investigated in this thesis, several specific challenges lie ahead.

An accessible, swift, and easily interpreted diagnostic gold standard would be helpful in diagnosing complement-mediated aHUS in the clinical setting. In this regard, several plasma-dependent tests have been proposed, although none of them obviously can take into consideration genetic variants encoding membrane-bound complement regulators. However, factor H is the major complement regulator also responsible for most of the membrane-bound complement inhibition in the renal microcirculation. Therefore, future diagnostic tests of complement dysregulation may ideally focus on the level of plasma-derived complement inhibition on test cells (e.g., modified endothelial cells) in *in vitro* test settings. Another possible solution would be to assess total complement activation in the native setting using patient biopsy material *ex vivo*. However, performing renal biopsies in the acute setting of disease is practically restricted due to generally low platelet counts. Therefore, myself [254] as well as others have speculated about the possible surrogate use of skin biopsies in the assessment of clinically relevant complement activation. Interestingly, one small study found that skin biopsies from nine and 13 patients with and without aHUS, respectively, demonstrated extensive depositions of TCC by immunofluorescence methods in the former group [395]. Obviously, confirmation of results is needed in larger studies. Moreover, future study designs should use a thorough combined assessment of phenotype and genotype as a posteriori diagnosis criteria instead of relying on clinical criteria for study group classifications. In order to reliably investigate whether biopsy material can differentiate complement-mediated from noncomplement-mediated disease, the definite or highly probable diagnoses of included patients must be evaluated before correlation analyses between biopsy findings and concluded diseases are performed. This is important as some systemic diseases manifesting with (noncomplement-mediated) aHUS may contribute to 'second hit' complement activation, which potentially also could manifest with increased complement staining in biopsies. In other words, a lack of stringency in study group classification can potentially forfeit the study purpose of diagnostic differentiation.

The differential diagnostic challenge of finding a single laboratory test to determine the probability of complement-mediated aHUS is likely not easily resolved. Therefore, simultaneous attempts to improve the tiered weight of evidence approach (**Figure** 7) are important. In light of the difficulty of interpretation, which is partly prevalent due to the lack of functional analyses for most of the identified rare genetic variants, important steps have been taken by setting up international complement genetics databases [284]. These joint international collaborations will likely prove significant for

future aHUS research as well as for the interpretations of genetic variants in the clinical setting.

Regarding PTR, complement activation likely contributes to immune PTR, at least in a subset of patients. Future research should focus on what characteristics of anti-HLA antibodies that make platelets susceptible to coincident complement depositions. As previously described, studies do indeed suggest that the C1q-based compared to the IgG-based SAB assay more accurately predicts the post-transfusion platelet response in alloimmunized patients. This suggests different immunogenic profiles for different alloantibodies. Consequently, complement-inhibiting therapy may be of potential benefit, particularly in persistently transfusion-refractory patients with high bleeding risks and a positive C1q-based SAB assay result. Evidently, this area needs much more exploration.

The potential impact of complement activation in nonimmune PTR, and as a biomarker of platelet concentrate viability, has not been previously explored. Our study did not confirm any contribution of complement activation, neither in nonimmune PTR nor as a biomarker of platelet concentrate viability. However, apart from our own investigations, studies assessing the potential role of complement activation in these settings are nonexistent Many known and unknown factors may contribute to increased platelet consumption. It is very likely that complement activation to some extent is involved in the pathogenesis of PTR in some of these cases, e.g., in patients coincidently suffering from systemic inflammation. However, that complement activation may be a general driver of nonimmune PTR is not supported by our study results. Consequently, it would be a clinical challenge to identify cases in which complement activation potentially contributes to nonimmune PTR. Further, it would potentially make more sense to treat the complement-triggering condition rather than focusing on therapeutic complement inhibition in these cases. The distinction between complement activation as a driver and as a contributor of disease is important. While complement activation is suggested to act as a driver of disease in a subset of patients with immune PTR, this does, so far, not seem to be the case for the heterogeneous panorama of nonimmune-related factors. Evidently, more studies are needed to confirm this suspicion.

Errata

In the abstract of **paper II,** it reads "*Subjects were investigated with […] nephelometry for complement components three of four.*"

It should read: "*[…] complement components 3/4*".

In the printed version of **paper III**, the final portion of figure text for *Figure 2* is missing.

It should read: "*[…] the temporal concentrations. The Bland-Altman plot indicated a median difference of -183 (-233, -144) pg/mL between the actual and calculated concentration. The 95% LoA ranged from -369 to -53 pg/mL.*"

In the printed version of **paper III**, Nadler's equation for the estimation of the total blood volume is missing for female gender on *page 3*.

It should read:

$$TBV(L)_{female} = 0.3561 \times [height(m)]^3 + 0.03308 \times weight(kg) + 0.1833$$

In the printed version of **paper III**, the formula for the estimation of residual plasma volume per concentrate is missing on *page 3*.

It should read:

$$RP(mL)_{concentrate} = \frac{volume(mL)_{concentrate} \times\ plasma\ fraction(\%)_{concentrate}}{100}$$

Of note, corrections for the presented errors of **paper III** were submitted during review of manuscript proof before publication; however, the publisher did not manage to correct it.

Populärvetenskaplig sammanfattning

Komplementsystemet utgör en uråldrig del av vårt medfödda (ospecifika) immunförsvar. Till största del produceras komplementsystemets proteiner i levern och cirkulerar fritt i blodomloppet. Till mindre del produceras de lokalt och finns uttryckta som förankrade ytproteiner på våra cellers höljen (cellmembran). Komplementsystemets proteiner har många olika egenskaper, och ny kunskap om deras funktioner upptäcks kontinuerligt. De senaste trettio åren har flera funktioner bortom deras roll i vårt immunförsvar avslöjats. Till exempel har komplementproteiner visat sig viktiga för utveckling och reparation av vårt nervsystem, men också för cellomvandling och ämnesomsättning i många andra vävnadstyper. Den mest vedertagna och etablerade rollen är dock fortsatt som del i vårt immunförsvar. Komplementsystemet hjälper till i kroppens försvar mot mikroorganismer samt hjälper till att städa bort döda kroppsegna celler som annars skulle kunna bidra till inflammation och utveckling av autoimmun sjukdom. De flesta komplementproteiner cirkulerar som inaktiva proteiner (så kallade prekursorer) i blodomloppet. Det är först när de klyvs av proteiner med enzymaktivitet (så kallade proteaser) som de omvandlas till aktiva proteiner. Aktiva komplementproteiner har i de flesta fall egen potential (proteasaktivitet) att klyva inaktiva komplementproteiner. Således skapas en kaskadreaktion där aktiva komplementproteiner klyver inaktiva komplementproteiner i en självförstärkande process. Därför refererar man ibland till komplementsystemets huvudsakliga funktion som komplementkaskaden. Komplementkaskaden kan startas via tre olika aktiveringsvägar: den klassiska vägen, lektinvägen och den alternativa vägen. Aktiverade komplementproteiner har flera olika funktioner i immunförsvaret. De ökar inflammation, rekryterar vita blodkroppar samt hjälper till att identifiera och markera mikroorganismer så att de lättare kan slukas av immunförsvarets celler (fagocytos). Det sista steget i komplementkaskaden utgörs utav bildandet av det så kallade membranattack-komplexet, även benämnt det terminala komplement-komplexet. Detta proteinkomplex är sammansatt av flera komplementproteiner som i serie fäster och monteras på ytan utav invaderande mikroorganismer vilket leder till att det skapas ett hål i mikroorganismens cellmembran. Vid komplementaktivering monteras oräkneliga membranattack-komplex på en invaderande mikroorganisms cellmembran. Detta medför att flertalet hål skapas vilket i sin tur leder till kraftiga förändringar i koncentrationerna av olika (så kallade osmotiskt aktiva) molekyler i mikroorganismens inre. Upprätthållandet av så kallat osmotiskt tryck är livsviktigt för alla cellers funktion.

Därför medför de kraftiga förändringar av osmotiska krafter som membranattack-komplexen bidrar till att cellmembranet sprängs sönder (lyserar) och att mikroorganismen därmed dör.

Komplementsystemets destruktiva potential, framför allt reflekterat utav membranatack-komplexets lyserande egenskaper, medför att det är av största vikt att komplementkaskaden ej aktiveras på kroppsegna cellers ytor. Därför består en del av komplementsystemet utav proteiner med komplementhämmande aktivitet. Dessa cirkulerar delvis fritt som proteiner i blodomloppet men de finns också uttryckta på kroppsegna cellers ytor för att förhindra komplementaktivering. Flera sjukdomar har förknippats med bristande komplementhämning. Ofta beror detta på nedärvda förändringar i gener som kodar för komplementhämmande proteiner. Dessa genetiska förändringar bidrar till att vissa komplementhämmande proteiner ej kan produceras alls, produceras i reducerad mängd, eller med total, eller delvis, avsaknad utav normal funktion. Medfödda eller förvärvade brister i den komplementhämmande förmågan är kända för att bidra till ovanliga men livsfarliga njursjukdomar, bland annat så kallat komplement-orsakat atypiskt hemolytiskt uremiskt syndrom. Detta är en mycket ovanlig sjukdom som drabbar knappt en individ per två miljoner individer årligen. Ofta leder sjukdomen till oåterkalleliga njurskador som gör att den drabbade personen behöver njurdialys eller -transplantation om inte effektiv behandling direkt påbörjas. Utmaningen med detta är att sjukdomen manifesterar sig på liknande sätt som andra allvarliga sjukdomar som kräver annan typ av behandling. Dessa sjukdomar har följande gemensamt: så kallad hemolyserande blodbrist, brist på blodplättar och njursvikt. I skrivande stund finns det inte något enkelt test eller blodprov som kan avslöja att det rör sig om komplement-orsakat atypiskt hemolytiskt uremiskt syndrom. Diagnosen ställs efter noggrann analys av flera olika komplementprover och involverar genetiska analyser vars svar ofta dröjer. Därför är det tänkbart att komplement-orsakat atypiskt hemolytiskt uremiskt syndrom är ett underdiagnostiserat tillstånd. Detta har tidigare ej undersökts på stor skala bland svenska patienter med misstänkt sjukdom.

Även ett normalfungerande komplementsystem, utan medfödda eller förvärvade brister, har visat sig utgöra en viktig orsak till vissa autoimmuna och så kallade autoinflammatoriska tillstånd. Därför har komplementhämmande behandling inte bara godkänts för sjukdomar som beror på brister i komplementhämmande förmåga, som i ovan exempel med komplement-orsakat atypiskt hemolytiskt uremiskt syndrom. Komplementhämmande behandling utgör nu också en del av behandlingsarsenalen för andra sjukdomar som inte karakteriseras av fel i kroppens komplementhämmande förmåga. För dessa sjukdomar verkar ett normalfungerande komplementsystem underhålla och/eller förstärka de sjukdomsalstrande mekanismer som bidrar till tillståndet. Ett exempel är den neuromuskulära sjukdomen generaliserad mystenia gravis.

Vidare har flera tidigare studier visat på ett samband mellan aktivering av blodplättar och komplemenaktivering. Blodplättar (trombocyter) är en blodcellstyp som är avgörande för kroppens blodstillningsförmåga (så kallade hemostas). Bristande blodplättsantal medför ökad risk för blödning. Aktivering av blodplättar är viktigt för hemostasen men ökad aktivering medför också att blodplättar i högre utsträckning rensas bort från blodomloppet med ett sjunkande blodplättsantal som följd. Tidigare studier har visat att komplementaktivering är en möjlig orsak till bristande blodplättsökning hos patienter som genomgår blodplättstransfusioner på grund av sjukdomar eller behandlingar som ger ett lågt blodplättsantal (så kallad trombocytopeni). En bristande förväntad ökning av blodplättsantalet efter avslutad transfusion brukar benämnas transfusionsrefraktär trombocytopeni. Sambandet mellan komplementaktivering och transfusionsrefraktär trombocytopeni har huvudsakligen identifierats hos patienter som bildar antikroppar mot vissa proteiner som bland annat är uttryckta på blodplättarnas cellmembran (alloimmunisering). Upprepade blod- och/eller blodplättstransfusioner medför ökad risk att utveckla dessa så kallade alloantikroppar. Något samband mellan komplementaktivering och transfusionsrefraktär trombocytopeni har hittills inte studerats hos trombocytopena patienter utan alloantikroppar. Det är därför oklart om komplementaktivering bidrar till bristande blodplättsökning efter blodplättstransfusion hos dessa patienter.

I denna avhandling har dels förekomsten av atypiskt hemolytiskt uremiskt syndrom undersökts i en svensk population. Dels har komplementaktiverings eventuella roll vid bristande blodplättsökning efter blödningsförebyggande blodplättstransfusioner undersökts hos trombocytopena patienter som vårdas på hematologisk vårdavdelning. Originalartiklarna finns bifogade som bilagor i avhandlingens slut.

I *paper I* undersöktes förekomsten av misstänkt atypiskt hemolytiskt uremiskt syndrom (ej genetiskt bekräftade fall) i en patientpopulation där blodprover från hela landet har skickats till koagulationslaboratoriet i Malmö för analys av ett enzym (ADAMTS13) som man har brist på vid ett sjukdomstillstånd (trombotisk trombocytopen purpura) som presenterar sig på samma sätt som atypiskt hemolytiskt uremiskt syndrom. Med tanke på den låga förekomsten av komplement-orsakat atypiskt hemolytiskt uremiskt syndrom ville vi därför undersöka om detta material var berikat med misstänkta fall utav sjukdomen. Under tidsperioden 2007 till 2012 hade 768 prover skickats till koagulationslaboratoriet för analys av ADAMTS13. Av dessa var 443 av intresse för vidare granskning efter att ha tillämpat uppsatta exklusionskriterier. Svarsfrekvensen på journalrekvirering var 73%. I slutändan identifierades 134 unika individer som efter journalgranskning uppfyllde inklusionskriterierna. I samband med vården på sjukhus då blodprov skickats för analys av ADAMTS13 hade endast en individ erhållit diagnosen komplement-orsakat atypiskt hemolytiskt uremiskt syndrom. Ytterligare 17 individer hade genomgått njurbiopsi med resultat som var förenligt med sjukdomen. Alla tillgängliga ADAMTS13-prover under ovan angiven period analyserades också för

laborativa avvikelser förenliga med ett dysreglerat komplementsystem. I slutändan identifierades 78 prover som var förenliga med diagnosen komplement-orsakat atypiskt hemolytiskt uremiskt syndrom. Således förelåg det en diskrepans mellan de totalt 18 fallen av bekräftad eller misstänkt klinisk sjukdom och de 78 blodprover som var förenliga med samma sjukdom. Detta skulle kunna tala för att komplement-orsakat atypiskt hemolytiskt uremiskt syndrom var ett underdiagnostiserat tillstånd i den undersökta patientpopulationen. För att bekräfta förekomsten av sjukdomen krävs i de flesta fall dock genetiska analyser. Därför erbjöds individer som inkluderats i den första studien att delta i studien för *paper II* som bland annat innefattade genetiska analyser.

Studieinlusion till *paper II* genomfördes i mediantid 63 månader efter de ursprungliga analyserna av ADAMTS13. Vid denna tidpunkt hade ca 45% av samtliga individer av intresse avlidit vilket speglar den överdödlighet som är associerad med det sjukdomspanorama som komplement-orsakat atypiskt hemolytiskt uremiskt syndrom tillhör. I slutändan rekryterades 20 av 71 (28%) möjliga individer för uppföljande analyser. Efter analys av kliniska data och genetiska avvikelser kategoriserades sex individer som komplement-orsakat atypiskt hemolytiskt uremiskt syndrom, tio individer som icke-komplement-orsakat atypiskt hemolytiskt uremiskt syndrom och fyra individer exkluderades då detaljerad genomgång av deras kliniska presentation gjorde att hemolytiskt uremiskt syndrom ej bedömdes vara en kliniskt sannolik diagnos. Av de sex individer som kategoriserades som komplement-orsakat atypiskt hemolytiskt uremiskt syndrom hade fyra erhållit motsvarande diagnos i samband med det kliniska rutinomhändertagandet. En individ hade erhållit en snarlik diagnos (trombotisk mikroangiopati) och en individ hade inte blivit diagnostiserad med motsvarande eller snarlik diagnos överhuvudtaget. *Paper II* var starkt begränsad av två faktorer: dels det låga antalet inkluderade patienter, dels det selektionsbias som introducerades av att patienter som erhållit diagnosen sannolikt var mer benägna att vilja delta i en studie om sjukdomen. Således kunde *paper II* ej säkert bekräfta att komplement-orsakat atypiskt hemolytiskt uremiskt syndrom skulle vara ett underdiagnostiserat tillstånd. Kliniska studier om atypiskt hemolytiskt uremiskt syndrom lider ofta av otydlighet kring inklusionskriterier. Vår studie påvisade möjligheten att använda tydligt uppsatta inklusionskriterier, inklusive tolkning av genetiska avvikelser, för att kategorisera atypiskt hemolytiskt uremiskt syndrom. Vidare identifierade vi en tidigare ej beskriven genetisk avvikelse som sannolikt bidrar till komplement-orsakad sjukdom.

I *paper III* undersöktes huruvida komplement- och/eller blodplättsaktivering var förenat med en sämre blodplättsökning efter blodplättstransfusion hos patienter som vårdades på hematologisk avdelning, led av trombocytopeni och som inte var kända bärare av så kallade alloantikroppar. Fyrtiotre individer inkluderades och totalt genomfördes 54 blödningsförebyggande blodplättstransfusioner. Transfusionsresponsen (blodplättsökning efter transfusion korrigerad för individuella faktorer) undersöktes i förhållande till komplement- och blodplättsaktivering i tre olika

scenarion: i. komplement- och/eller blodplättsaktivering i blodplättstransfusaten, ii. komplement- och/eller blodplättsaktivering hos transfunderade individer innan transfusion, och iii. transfusions-orsakad komplement- och/eller blodplättsaktivering. Inget samband identifierades mellan komplement- respektive blodplättsaktivering och transfusionsresponsen efter avslutad blodplättstransfusion i någotdera scenario. Studien begränsades av att vi inte visste huruvida studiedeltagarna var tillräckligt många för att statistiskt avfärda att något samband förelåg. Med andra ord finns det en risk att studieresultatet är falskt negativt. Således krävs större studier för att bekräfta våra resultat.

Sammantaget har denna avhandling belyst aspekter av, och bidragit till ökad kunskap om, komplementaktivering vid trombocytopena tillstånd. En genomgående begränsning för avhandlingens inkluderade delar har varit att sjukdomarnas ovanlighet, samt det hypotesgenererande tillvägagångssättet, starkt begränsat studiedesign och förmågan att dra slutsatser utifrån materialet. De enskilda studierna inkluderade i denna avhandling kan förhoppningsvis fungera som språngbrädor för större studier i framtiden.

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Paper I

NEPHROLOGY

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Original Article

Indications of underdiagnosis of atypical haemolytic uraemic syndrome in a cohort referred to the Coagulation Unit in Malmo, Sweden, for analysis of ADAMTS13 2007–2012

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SUMMARY AT A GLANCE

Complement-mediated atypical haemolytic uraemic syndrome (aHUS) is a rare disease and might be under diagnosed due to manifestations overlap with thrombotic thrombocytopenic purpura. Examination of serum C3 and C4, complement factor H (CFH)-specific antibodies and associated deficiency in CFH-related proteins may facilitate the diagnosis of aHUS.

ABSTRACT:

Aim: **Complement-mediated atypical haemolytic uraemic syndrome (aHUS) is a rare disease with high mortality and morbidity if left untreated. The diagnostic work-up is complicated and the manifestations overlap with other conditions. Therefore, we hypothesize that complement-mediated aHUS is an under diagnosed disease.**

Methods: **A cohort of 768 referrals referred to the Coagulation Unit in Malmo, Sweden, for analysis of a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13 (ADAMTS13), 2007–2012, were retrospectively reviewed. Subjects were included on the basis of presence of haemolytic anaemia, thrombocytopaenia, renal failure and ADAMTS13 $>$ 0.05. They were excluded if tested positive for *Escherichia coli*. Included subjects were categorized as "suspected HUS" with and without potential causes and triggers. Levels of C3 and C4, presence of complement factor H (CFH)-specific antibodies and associated deficiency in complement factor H related protein 1 (CFHR1) were analyzed on frozen samples.**

Results: **In total, 134/316 (42%) unique subjects fulfilled inclusion criteria; 103 were categorized as "suspected HUS associated with potential causes/triggers" and 31 subjects categorized as "suspected HUS" without such association. One case of complement-mediated aHUS had been confirmed during the treatment period. Laboratory analyses performed showed that in total 78 cases had findings consistent with complement-mediated aHUS: 24 cases indicated presence of CFH-specific antibodies whereof five cases had isolated low C3 titres and six cases had deficiency of CFHR1. Additionally 54 cases indicated isolated alternative pathway consumption.**

Conclusion: **The results suggest that the presence of complement-mediated aHUS was under diagnosed in this cohort calling for improvement of diagnostic availability.**

Complement-mediated atypical haemolytic uraemic syndrome (aHUS) is associated with an excessive activation of the alternative complement pathway. It is characterized by microangiopathic haemolytic anaemia (MAHA), thrombocytopaenia and acute renal failure.[1] Histopathologic examination of a kidney biopsy typically shows thrombotic microangiopathy (TMA); a histopathological manifestation affecting the endothelial structures in the microvasculature of end-organ arterioles and capillary beds. In complement-mediated aHUS it partly results from excessive deposition of membrane attack complexes (MAC) on the host cells.[2] There is no firmly defined appliance of terminology in the field of study why aHUS to some extent may also be referred to with other mechanisms of action in mind. Consequently, we will refer to complement-mediated disease as complement-mediated aHUS.

Complement-mediated aHUS has historically been treated with plasma therapy. Although the overall response rate is approximately 70%[3] the proportion of long-term renal recovery is low.[3,4] In 2011 an alternative treatment for complement-mediated aHUS was approved by the European Medicines Agency (EMA) and the US Food and Drug Administration (FDA) following successful single-arm clinical trials.[4–6] Eculizumab (Soliris;

Alexion Pharmaceuticals, Cheshire, CT, USA) is a recombinant, monoclonal IgG targeting complement component C5. It is associated with a time-dependent improvement of renal function. As the great majority of conditions acutely presenting with MAHA are life-threatening and specific and effective treatment targeting excessive complement activation is now available, the underlying condition should be diagnosed promptly.

In several studies only 5–10% of the total HUS cases are classified as complement-mediated.[6,7] This may be due to the fact that verification of disease is complicated, particularly in adults where a multitude of differential diagnoses must be considered. Different genetic aberrations as well as presence of antibodies account for diverse severity of disease. It is suggested that the presence of a rare genetic mutation, a common at-risk genetic variant (e.g. single nucleotide polymorphisms and haplotype blocks) and an extrinsic factor (e.g. infection) triggering a complement response may be necessary for manifestation of disease.[8,9] Without proper treatment, the complement-mediated aHUS is associated with a 50% rate of progression to end-stage renal disease and a mortality-rate of approximately 25% in the acute phase.[7]

To shorten the time to diagnosis, schemes of clinical prediction have been proposed, but these do not accurately differentiate aHUS from thrombotic thrombocytopenic purpura (TTP) in the acute phase.[10] Due to the present lack of easily accessible laboratory gold standard and robust clinical definitions, no solid numbers on incidence of complement-mediated aHUS are available in the Swedish population. In some cohorts[1,2] the suggested incidence is 1–2/1 000 000 annually, which translated into Swedish conditions would correspond to approximately 10–20 people being afflicted yearly. Most likely however, the prevalence of complement-mediated aHUS is underestimated.

In this study we aimed to evaluate the prevalence of suspected complement-mediated aHUS with or without potential triggering events and known underlying causes in a retrospective Swedish cohort referred to the Coagulation Unit in Malmo (Department of Laboratory Medicine, Skane) on the clinical suspicion of TTP for analyses of a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13 (ADAMTS13) between the years of 2007–2012. We show that a significant part of these patients may potentially have been diagnosed with complement-mediated aHUS if accurate diagnostic work up had been available.

METHODS

Clinical material screening

In total, 768 referrals were submitted for analysis of ADAMTS13, 2007–2012. With the aim of identifying subjects with a possible diagnosis of complement-mediated aHUS, subjects with levels of ADAMTS13 < 0.05 were excluded since they by definition were diagnosed with TTP. Referred subjects previously associated with laboratory confirmed TTP were excluded regardless of a present result of ADAMTS13 analysis. Subjects with confirmed presence of enterohaemorrhagic *Escherichia coli* (*E. coli*) in microbiological analyses (polymerase chain reaction and/or stool cultures) were excluded.

A clinical data acquisition report form (CRF) was designed for medical record screening purposes. Inclusion criteria were presence of: (i) haemolytic anaemia, (ii) thrombocytopaenia, and (iii) renal impairment (elevated creatinine and/or cystatine C). The presence of haemolysis was defined as fulfilment of more than one of the following criteria: low plasma/serum haptoglobin, indirect hyperbilirubinaemia, high serum lactate dehydrogenase and blood reticulocytosis. Data were interpreted in accordance with stated normal ranges of references of the local laboratories.

Study populations were defined as (i) suspected HUS with or (ii) without association to potential causes or triggers, respectively (Fig. 1). In cases where more than one potential trigger or cause was possible, the probable critical cause of disease development was defined based on temporal pattern and accessible clinical data. Suspected causative and/or triggering mechanisms were determined by such precedent established factors in the field of study.[2] Presence of a diagnosis of HUS and/or TMA, initiation of plasma therapy and results from complement analyses were noted.

Laboratory material analyses

Stored plasma samples from the referred subjects were screened for complement consumption and presence of complement factor H (CFH)-specific antibodies. However, blood samples had not been collected prospectively. Consequently the results of laboratory analyses performed could not be linked to individual patients and related to clinical symptoms.

ADAMTS13 analysis

ADAMTS13 analyses were performed using the FRETS-VWF73 method.[11,12] With this method severe ADAMTS13 deficiency is defined as $<5\%$ of normal levels (100% equals to 1.0 arbitrary units/L). Severe deficiency is regarded specific for TTP.

Complement component 3 and 4 analyses

Levels of C3 and C4 were measured by nephelometry using BN Prospec Systems (Siemens Healthcare, Erlangen, Germany). The threshold for lower range protein detection is 10 mg/L. Normal range of references based on healthy controls are stated as follows: (i) C3 0.86–1.73 g/L, (ii) C4 0.13–0.31 g/L. Low titres of C3 combined with normal titres of C4 were interpreted as isolated consumption in the alternative complement pathway.

Anti-complement factor H immunoassay

Samples and controls were screened for the presence of CFH-specific antibodies using enzyme-linked immunosorbent assay

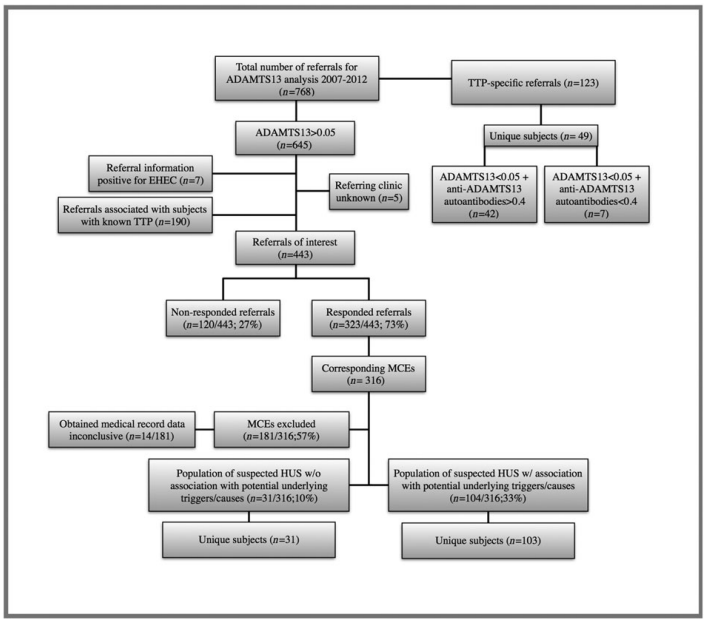

Fig. 1 Schematic outline of study. Total and selected referrals as well as response rate on medical record requisitions are shown. Numbers and percentages of the populations are presented in brackets. The number of MCEs corresponding to total number of responded referrals is shown. The MCEs have been screened in line with a CRF and distributed into the two populations as defined.[11]

(ELISA). 100 unmatched controls were selected from a Swedish cohort of healthy subjects. The immunoassay has been reproducibly established by independent research groups.[13–16] All samples and controls were analyzed in duplicates. Final concentrations were calculated as means from the two independent assays.

Complement factor H was previously purified from plasma and passed through protein A-Sepharose HiTRAP (GE Healthcare, Uppsala, Sweden) to remove possibly contaminating human immunoglobulins.[13] Microtiter plates (Maxisorp; Nunc, Roskilde, Denmark) were coated overnight with purified complement CFH diluted to 1 µg/mL in 75 mM sodium carbonate buffer, pH 9.6. The plates were washed in immunowash buffer (50 mM Tris- HCl, 150 mM NaCl, 0.1% Tween20; pH 8.0) and blocked with PBS/1% BSA for 1 h at 37 °C. Plasma samples and controls diluted 1:50 in PBS/1% BSA/0.05% Tween20 were added to the plates and incubated for 1 h at 37 °C. CFH-specific antibodies were detected with polyclonal rabbit anti-human IgG antibodies (DakoCytomation, Glostrup, Denmark) diluted 1:4000 and incubated for 1 h at 37 °C. The plates were then incubated with polyclonal swine anti-rabbit antibodies conjugated with horseradish peroxidase (HRP) (DakoCytomation) diluted 1:2000 for 1 h at 37 °C.

The plates were developed with *o*-phenylenediamine (OPD) substrate (DakoCytomation) and the absorbance was measured at 490 nm (Varian 50 MPR Microplate Reader; Varian Medical Systems, CA, USA). Polyclonal rabbit anti-human CFH antibodies (H- 300; Santa Cruz Biotechnology, TX, USA) were used as standards at two-fold dilution series starting from 1:50. Concentrations of CFH-specific antibodies were calculated relative to a logarithmic standard curve, first point set at 10 arbitrary units (AU)/mL using non-linear regression sigmoidal dose-response (variable slope) in GraphPad Prism (version 6.0; GraphPad Software, CA, USA). Sample concentrations were interpolated from the estimated standard curves and multiplied with the dilution factor. Samples with levels above the mean plus three standard deviations (SD) of those in the controls were considered positive. The cut off was calculated to 410 AU/mL.

Complement factor H related protein 1 immunoblots

Twenty-four CFH-specific antibody positive samples were analyzed. Controls for the CFHR1 immunoblots were 48 randomly selected CFH-specific antibody negative samples (AU/L <3SD). Samples and controls were diluted 1:100 and separated under non-reducing conditions using 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were transferred onto polyvinylidene fluoride (PVDF) membranes, blocked with quenching solution (washing buffer: 50 mM Tris-HCl, 150 mM NaCl, 0.1% (v/v) Tween 20, pH 7.5 with 0.3% fish gelatin (Norland Products, Cranbury, NJ, USA))

and incubated with mouse monoclonal anti-CFH (C18/3; Santa Cruz Biotechnology, Santa Cruz, CA, USA) that identifies the conserved C-terminus of FH (150 kDa) and the two differentially glycosylated forms of CFHR1α and CFHR1β (37 and 42 kDa). Bound antibodies were detected with a polyclonal anti-mouse IgG antibody conjugated with HRP (DakoCytomation). The blots were developed using 3,3′- diaminobenzidine tetrahydrochloride (Sigma-Aldrich, St Louis, MO, USA) colorimetric substrate system as previously described.[16] A previous study confirmed the capacity of the immunoblot technique to reliably identify CFHR1 deletion by comparing study samples tested by both western blot and genetic analysis using Mutiplex Ligation-dependent Probe Amplification (MLPA) (MRC-Holland, Amsterdam, the Netherlands).[13] A good correlation has been shown between the genetic deficiency of CFHR1/3 deletions and the deficiency of CFHR1 protein detected by immunoblotting in samples of *De*ficiency of CFHR plasma proteins and *a*utoantibody-*p*ositive form of HUS (DEAP-HUS) patients.[17,18]

Statistical analyses

Differences between non-normally distributed continuous variables were evaluated using the Mann–Whitney *U*-test. Differences between categorical variables (2 × 2 contingency tables) were tested with Fishers exact test.

Ethical approval

The study was performed in conformance with the Declaration of Helsinki. The Regional Ethics Committee at Lund University (diary number: 2013/514) approved the study.

RESULTS

Clinical material screening

Out of in total 768 referrals 443 (58%) were of interest (Fig. 1). Medical records were requested from local clinics and the requisition response rate was 73% (323/443). Fourteen received medical care episodes (MCE) had to be excluded due to insufficient medical data. Seven patients were tested positive for EHEC. Clinical information was available for 316 MCEs. One hundred thirty-five (135/316; 43%) screened MCEs corresponding to 134 unique subjects did meet the laboratory inclusion criteria. Thirty-one out of 134 subjects that met the criteria for potential HUS had no association to potential

Table 1 Cohort of suspected HUS with potential causes/triggers

Category of primary cause/trigger (*n*)	Median age; range (years)	Confirmation of HUS/TMA in medical records	Blood smear indicating schistocytosis	Renal biopsy indicating TMA	Dialysis	Plasma therapy initiated	Deceased in conjunction with MCE of referral
***Solid tumours* (3)**	70; 67–90	1/3	2/3	N/A ×3	1/3	1/3 (PE)	0/3
***Haematologic malignancies* (6)**	76; 53–85	3/6	2/4 + N/A ×2	N/A ×6	2/6	1/6 (PE)	1/6
***Autoimmune disease other than SLE and APS* (4)**	65; 54–78	1/4	1/2 + N/A ×2	0/1 + N/A ×3	3/4	4/4 (PE ×4)	0/4
***DIC* (28)**	65;17–83	8/28	5/12 + N/A ×16	0/2 + N/A ×26	15/28	6/28 (PE ×6)	8/28
***Cobalamin deficiency* (2)**	69; 63–74	0/2	1/1 + N/A ×1	N/A ×2	0/2	1/2 (PE)	0/2
***APS* (2)**	44; 38–50	0/2	N/A ×2	N/A ×2	0/2	1/2 (PE)	0/2
***Post renal transplant* (6)**	42; 29–62	3/6	1/3 + N/A ×4	1/4 + N/A ×2	2/6	1/6 (PE)	0/6
***Malignant hypertension* (5)**	43; 1–48	4/5	1/2 + N/A ×3	1/1 + N/A ×4	4/5	2/5 (PE ×1, PI ×1)	0/6
***HELLP a/o preeclampsia* (9)**	34; 17–43	2/9	2/2 + N/A ×7	1/1 + N/A ×8	5/9	1/9 (PE)	0/9
***Influenzae* (2)**	47; 39–55	1/2	N/A ×2	N/A ×2	1/2	1/2 (PE)	0/2
***SLE* (6)**	20; 17–35	3/6	3/5 + N/A ×1	2/5 + N/A ×1	3/6	2/6 (PE ×2)	0/6
***IgA nephritis* (1)**	25	0/1	N/A ×1	0/1	1/1	0/1	0/1
***Post-partum onset* (2)**	30; 23–37	1/2	1/1 + N/A ×1	0/1 + N/A ×1	2/2	1/2 (PE)	0/2
***Post HSCT* (11)**	44; 9–61	5/11	5/7 + N/A ×4	N/A ×11	5/11	3/11 (PE ×2, PI ×1)	6/11
***Malaria* (1)**	65	0/1	N/A ×1	N/A ×1	1/1	1/1 (PI)	0/1
***HIT/T* (1)**	55	0/1	N/A ×1	0/1	1/1	0/1	0/1
***Anti-neoplastic drug therapy* (8) (*Gemcitabine ×2*, *calcineurin inhibitor ×2*, *mTOR inhibitor ×4*)**	52; 9–69	8/8	6/8	1/1 + N/A ×7	1/8	4/8 (PE ×2, PI ×2)	1/8
***Complement dysregulation*; *CFH-specific antibody positive* (1)**	4	1/1	N/A ×1	N/A ×1	0/1	1/1 (PI)	0/1
***Miscellaneous conditions* (5)**		3/5	1/3	1/2	4/5	1/5	0/5
***Total* (103)**	54 (1–90)	44/103	32/54	7/21	51/103	32/103	16/103

APS, anti-phospholipid syndrome; CFH, complement factor H; DIC, disseminated intravascular coagulation; HELLP, syndrome of haemolysis, elevated liver enzymes and low platelets; HIT/T, heparin induced thrombocytopaenia/thrombosis; HUS, haemolytic uraemic syndrome; HSCT, haematologic stem cell transplantation; PE, plasma exchange therapy; PI, plasma infusion therapy; SLE, systemic lupus erythematosus; TMA: thrombotic microangiopathy.

underlying triggers/causes. Potential causes/triggers could be identified in 103/134 subjects. Identified potential causes/triggers and associated data are shown in Table 1 and (Table S4). Presentation of cases where no known potential trigger/cause was present is shown in Tables S5 and S6. The treating physician assessed a diagnosis of HUS and/or TMA in 20/31 (65%) and 44/103 (43%) cases respectively (*P*-value: 0.04). Plasma therapy was initiated in 18/31 (58%) and 32/103 (31%) cases, respectively (*P*-value: 0.01) (Table 2). In 11/134 (8%) cases there were information with regard to performed complement analyses (C3/C4). Two cases indicated alternative pathway consumption. There were in total only one subject with confirmation of complement-mediated aHUS (DEAP-HUS) in the MCE of referral.

Laboratory material analyses

For analyses of C3 and C4 levels, 627 samples out of the total 768 referrals were of sufficient volume for analysis. In total 59/627 (9%) cases indicated isolated consumption in the alternative complement pathway (C3 median: 0.79, range: 0.42–0.85). Seventy-nine out of 627 (13%) cases were associated with a combination of low C3 and C4 levels (C3 median: 0.63, range: 0.85–0.16; C4 median: 0.08, range: 0.02–0.12). For analyses of CFH-specific antibodies there were 662/768 samples of sufficient volume for analysis. Titres of CFH-specific antibodies were significantly higher in samples (24/662) compared to controls (2/100) (*P*-value: <0.05) (Fig. 2a). Six out of 24 CFH-specific antibody positive samples were shown to lack expression of CFHR1 compared to 1/48 in the controls (Fig. 2b,c). In total, nine CFH-specific antibody positive samples had low titres of C3, including three cases with simultaneous CFHR1 deficiency. Four of the nine cases had coincident low C4 titres (Table 3).

Table 2 Comparison of characteristics

Characteristics	Suspected HUS w/o potential causes/ triggers ($n = 31$)	Suspected HUS with potential causes/ triggers ($n = 103$)	*P*-value
Age	54 (0–81)	54 (1–90)	0.33
Female sex	12/31 (39%)	56/103 (54%)	0.15
Diagnosis of HUS/TMA	20/31 (65%)	44/103 (43%)	0.04
Plasma therapy initiated	18/31 (58%)	32/103 (31%)	0.01
Schistocytosis in peripheral blood smears	12/18 (67%)	32/54 (59%)	0.78
TMA in renal biopsies	10/12 (83%)	7/21 (33%)	0.01

For non-normally distributed continuous variables (age), median and range are presented. Count and percentage are presented for categorical variables. HUS, haemolytic uraemic syndrome; TMA, thrombotic microangiopathy.

DISCUSSION

In the clinically screened material 134 unique subjects were determined to have clinical and routine laboratory parameters consistent with complement-mediated aHUS. A diagnosis of HUS/TMA was settled significantly more frequent in the population of suspected HUS without potential causes and triggers. This may be explained by the combined result of two factors: (i) The underlying conditions potentially causing or triggering the manifestation of suspected HUS was determined as primary diagnosis regardless of being an actual trigger (of e.g. excessive complement activation) or a native cause. (ii) The population of suspected HUS without potential causes and/or triggers are enriched in pure cases of aHUS; complement-mediated mechanisms as well as other undetermined mechanisms. Plasma therapy was significantly more frequently initiated in the population of suspected HUS without potential causes and triggers. This further strengthens the suspicion of enrichment of true cases of complement-mediated aHUS in this cohort. However, remarkably only one case was confirmed with this diagnosis in the time period where the patient was evaluated for the disease. There were four cases with information on blood samples being sent for mutational analyses, although results of analyses were not available at the end of the MCEs.

In the clinically screened material 11/134 cases had been assessed with respect to C3 and C4 levels. Two of these had levels consistent with alternative pathway consumption. In the laboratory assessed material there were 59/627 cases with isolated low C3 levels: five out of 59 cases were accompanied by CFH-specific antibodies. In light of the clinical suspicion of MAHA, thrombocytopaenia and renal impairment needed for referral, the cases of isolated alternative pathway consumption suggest potential presence of underlying complement-mediated aHUS. Furthermore, it is important to stress that several mutations resulting in complement-mediated aHUS do not necessarily present with low titres of C3. Moreover, simultaneous acute phase reactions drive renewed synthesis of C3, thus potentially masking its consumption. Therefore the presence of normal titres of C3 does not necessarily reflect an absence of complement-mediated aHUS. The proportion of true cases thus may be higher.

There were 24 CFH antibody positive cases in the laboratory material. Six out of these 24 cases were accompanied by a deficiency of CFHR1, suggesting the presence of DEAP-HUS.[14] In the clinically reviewed material, there was one confirmed case of DEAP-HUS. This suggests that at least five cases of DEAP-HUS and additional 18 cases of CFH antibody positive subjects were not identified during the time the patients were hospitalized.

We hypothesized that true cases of aHUS are enriched and under diagnosed in this semi-selected retrospective cohort. Considering the semi-selection, the prevalence of e.g. DEAP-HUS in the laboratory material are lower than in pure aHUS cohorts.[19] Our clinical screening included 323/443 referrals of interest. 134 unique subjects fulfilled inclusion criteria. Sixty-

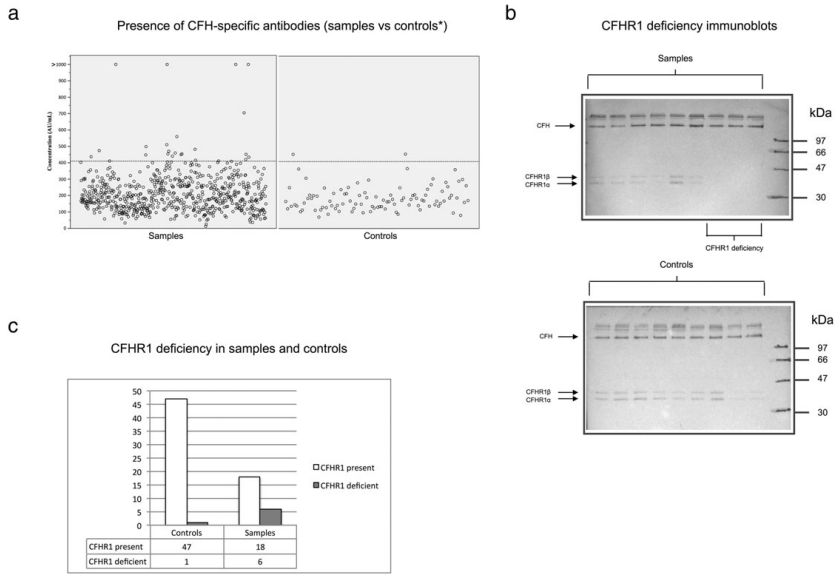

Fig. 2 Presence of CFH-specific antibodies and deficiency of CFHR1. (a) Distribution of samples and controls in CFH-specific enzyme-linked immunosorbent assays. Plasma samples ($n = 662$) and healthy controls ($n = 100$) were analyzed for binding of IgG autoantibodies on immobilized purified CFH. Samples with levels above the mean plus three standard deviations of those in the controls (≈410 AU/mL) were considered positive. The dotted line represent cut-off: 2/100 controls and 24/662 samples were positive. Four samples had an estimated concentration of >1000 AU/mL. Statistical significance of differences between titres in samples compared to controls was calculated using the Mann–Whitney *U*-test. $P < 0.05$ were considered statistically significant. *$P < 0.05$. (b) Plasma samples of patients and healthy controls were separated on 12% SDS-PAGE, and analyzed by immunoblotting using a mouse monoclonal CFH-specific antibody (C18/3) identifying CFH (150 kDa) and the two differently glycosylated forms of CFHR1α and CFHR1β (37 and 42 kDa). Developed PVDF membranes illustrate representation for samples and controls respectively. (c) Presence of homozygous deficiency of CFHR1 in CFH-specific antibody positive samples ($n = 6/24$) and CFH-specific antibody negative controls ($n = 1/48$).[11]

four out of our 134 cases were accompanied by a diagnosis of HUS/TMA, by their treating physician; however, there was only one confirmed subject with complement-mediated aHUS. Our laboratory assessed cases revealed a mismatch between the number of confirmed and suspected aHUS cases in our clinically screened material on the one hand and the larger number of laboratory screened cases indicating alternative complement dysregulation consistent with complement-mediated aHUS on the other.

Our study has the major weakness that laboratory results could not be directly coupled to the phenotype of individual patients. However, it does provide support to the notion that complement-mediated aHUS is diagnosed less frequently than its actual occurrence. Considering the fact that the underlying diseases are rare as well as the current lack of a Swedish TMA registry, we have had the opportunity to clinically and laboratory screen an unusually extensive cohort where the majority of referrals have been sent as part of a work up of clinically suspected TMA. Moreover, our retrospective study design limits us to the decisions of our subjects' treating physicians in terms of what laboratory analyses being performed in the actual clinical setting as well as their selection of information for

Table 3 Laboratory data summary

	Isolated low C3 (*n*)	Combined low C3 and C4 (*n*)	Normal C3 levels (*n*)	Total (CFH-antibody analyses; *n*)
CFH-antibody positive samples with CFHR1 deficiency	2	1	3	6
CFH-antibody positive samples without CFHR1 deficiency	3	3	12	18
CFH-antibody negative samples	54	75	474	638
Total (C3/C4 analyses; n)	*59*	*79*	*489*	*627*; 662

C3/4, complement component 3/4; CFH, complement factor H.

medical record documentation. Therefore our collected data, however collected in a uniform matter, is biased in this regard.

To conclude, in our reviewed cohort 135/316 MCEs fulfilled clinical criteria for aHUS. If proper laboratory evaluation had been performed the diagnosis of complement-mediated aHUS may had been confirmed to a greater extent. Treatment for complement-mediated aHUS has up to recently solely relied on empirical plasma therapy. We are likely still in a transitional phase where physicians to some extent still rely on plasma therapy and where further investigation of underlying mechanisms are not always initiated due to ambiguity with regard to overlapping clinical manifestations. Our clinical screening also indicates an inconsistent appliance of terminology in the clinical setting, which may also influence diagnostic follow-up negatively. This calls for uniformity in nomenclature. Our results provide the foundation for further investigation with genetic analyses and follow-up clinical data in our identified populations to reveal a more precise estimate of the true incidences and associated data on clinical characteristics of complement-mediated aHUS in a Swedish population.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

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AUTHORS CONTRIBUTIONS

A.Å. collected all the clinical data, performed all the laboratory analyses and wrote the draft. J.K. and E.Z. designed the study and supervised the process. A.B. and K.S. contributed essential laboratory reagents and tools and offered valuable input with regard to laboratory analyses in particular and the field of study in general. All authors reviewed and contributed to the paper.

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SUPPORTING INFORMATION

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

Table S4. Miscellaneous conditions in cohort of suspected HUS with potential causes/triggers
Table S5. Cohort of suspected HUS without potential causes/triggers
Table S6. Clinical specifics of cohort of suspected HUS without potential causes/triggers

Indications of underdiagnosis of atypical haemolytic uraemic syndrome in a cohort referred to the Coagulation Unit in Malmo, Sweden, for analysis of ADAMTS13 2007–2012

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SUPPORTING INFORMATION

Supplementary tables

Table S4. Miscellaneous conditions in cohort of suspected HUS w/ potential causes/triggers
Table S5. Cohort of suspected HUS w/o potential causes/triggers
Table S6. Clinical specifics of cohort of suspected HUS w/o potential causes/triggers

Table S4. Miscellaneous conditions in cohort of suspected HUS w/ potential causes/triggers

Miscellaneous conditions	Age (years)	Confirmation of HUS/TMA in medical records	Blood smear indicating schistocytosis	Renal biopsy indicating TMA	Dialysis	Plasma therapy initiated	Deceased in conjunction with MCE of referral	Comments
Methotrexate-mediated renal failure	62	No	No	N/A	Yes	No	No	/
NSAID-mediated renal failure	34	Yes	Yes	No	Yes	No	No	PE not initiated due to hypo-natremia. Renal biopsy indicating ATN
Rituximab therapy	19	Yes	No	Yes	Yes	No	No	SLE patient with mabthera therapy. Speculations of mabthera-induced TMA
AFLP	25	No	N/A	N/A	No	No	No	/
Antibiotics-mediated HUS	57	Yes	N/A	N/A	Yes	Yes (PI)	No	Speculation of presentation of HUS due to piperacillin/ tazobactam or trimethoprim -sulfa

HUS: haemolytic uraemic syndrome; TMA: thrombotic microangiopathy; PE: plasma exchange therapy; PI: plasma infusion therapy; ATN: acute tubular necrosis; MCE: medical care episode; AFLP: acute fatty liver disease

Table S5. Cohort of suspected HUS w/o potential causes/triggers

Case (#)	Age	Confirmation of HUS/TMA in medical records	Blood smear indicating schistocytosis	Renal biopsy indicating TMA	Dialysis	Plasma therapy initiated	Deceased in conjunction with MCE of referral
1.	71	No	N/A	N/A	Yes	No	No
2.	68	Yes	N/A	Yes	Yes	Yes (PI)	No
3.	68	Yes	Yes	No	Yes	No	No
4.	64	Yes	No	N/A	Yes	Yes (PE)	No
5.	65	Yes	Yes	N/A	Yes	Yes (PE)	No
6.	67	No	No	N/A	Yes	No	Yes
7.	64	Yes	Yes	N/A	Yes	Yes (PE)	No
8.	55	Yes	Yes	Yes	Yes	Yes (PE)	Yes
9.	58	No	N/A	N/A	No	Yes (PE)	No
10.	54	Yes	Yes	N/A	Yes	Yes (PE)	No
11.	51	Yes	Yes	N/A	Yes	No	No
12.	49	No	N/A	N/A	No	No	No
13.	46	Yes	N/A	Yes	Yes	Yes (PI)	No
14.	44	Yes	N/A	Yes	Yes	No	No
15.	45	Yes	N/A	Yes	No	No	No
16.	36	Yes	N/A	Yes	Yes	Yes (PE)	No
17.	20	Yes	Yes	N/A	No	Yes (PE)	No
18.	1	Yes	Yes	N/A	No	Yes (PI)	No
19.	0	Yes	N/A	N/A	Yes	Yes (PE)	No
20.	81	No	N/A	N/A	No	No	No
21.	70	No	No	N/A	Yes	No	Yes
22.	67	No	N/A	N/A	No	No	No
23.	52	No	No	N/A	Yes	No	Yes
24.	44	No	N/A	No	Yes	No	No
25.	42	No	Yes	N/A	Yes	Yes (PE)	Yes
26.	79	Yes	No	Yes	Yes	Yes (PI)	Yes
27.	60	Yes	N/A	N/A	Yes	Yes (PE)	No
28.	54	Yes	Yes	N/A	Yes	Yes (PE)	No
29.	72	No	N/A	Yes	Yes	No	No
30.	31	Yes	Yes	Yes	Yes	Yes (PE)	No
31.	25	Yes	Yes	Yes	No	Yes (PE)	No

HUS: haemolytic uraemic syndrome; TMA: thrombotic microangiopathy; MCE: medical record episode; PI: plasma infusion therapy; PE: plasma exchange therapy

Table S6. Clinical specifics of cohort of suspected HUS w/o potential causes/triggers

Case (#)	Patient specifics
1.	Thrombocytopenia, haemolysis and renal failure post erysipelas. No sign of systemic infection. HUS suspected. Improves on haemodialysis alone.
2.	Onset with suspected MAHA. No underlying cause found. No complement analyses performed. Condition improves on PI.
3.	Onset with suspected HUS, Renal biopsy indicating ATN, however performed late in acute phase of disease (dialysis and steroids already administered). Autoimmune antibody titers negative (ANA, RF, GBM, ANCA, SSA, cardiolipin).
4.	Seizure. PE initiated on basis of suspected TTP. Condition improves.
5.	Onset post renal PTA. Condition improves on PE.
6.	Patient with methotrexate therapy on basis of RA. Onset with dyspnea. Methotrexate-mediated renal failure suspected. Cardiac arrest. Deceased in critical care.
7.	Onset post renal PTA. Condition improves on PE.
8.	Patient with known HUS (no data on complement or genetic analyses). Intermittent PE. Dies of femoral aneurysm.
9.	Initially suspected haemolysis on basis of LVAD and unknown renal failure. ANCA titers low. LVAD function normal, not considered cause of haemolysis. PE initiated.
10.	Nausea, fatigue. Autoimmune antibody-titers low. HUS suspected. PE initiated. Condition stabilized. No data on complement or genetic analyses.
11.	Prior travel to Egypt. Cultures and PCR negative for EHEC. No data on complement or genetic analyses.
12.	Frontotemporal ischemic CVI. Suspected TTP. Prepared for PE, however, condition improves spontaneously.
13.	Vomiting, nausea. Prior to admission bitten by dog. Administered amoxicillin/clavulanic acid and diTeebooster. PI improves condition.
14.	Chronic renal failure. Titters of C3, C4 and FB normal.
15.	Prior travel to Egypt. Cultures and PCR negative for EHEC. Possible case of typical HUS because of administration of ciprofloxacin prior to onset. However, ciprofloxacin-mediated HUS may also be the case. Normal titers of C3 and C4.
16.	Nausea, emesis. Titers of ANA and c/p-ANCA low. Improves on PE. Medical record notes suggest ”post infectious HUS”. Cultures and PCR are negative. No data on complement.
17.	Haemorrhagic CVI. Suspected TTP. PE initiated. Stabilized post PE. No data on complement. Medical record notes suggest blood samples to be analysed with respect to complement mutations.
18.	Fever, cough. Titers of C4 low. No data on C3. FB and FH titers normal. Blood samples sent for genetic analyses.
19.	Fever, emesis. General consumption of complement. Medical record notes suggest ”post infectious HUS”.
20.	Fatigue. No convincing hemolysis. Uncertain cause of condition. Hypothyroidism, Addisons and sepsis excluded.
21.	Fatigue. Acute onset of renal failure. Autoimmune antibody titers low. Circulatory collapse. Dies in critical care.
22.	Dyspnea. Improves on steroid therapy. Suspicion of autoimmune hemolytic anemia, though no data on DAT.
23.	Femoral ischemia and thrombosis. Circulatory instability. Haemolytic anaemia does not respond to steroids. Circulatory collapse. Deceased in critical care.
24.	Liver failure. Hepatorenal syndrome. Discretely elevated LD, no other sign of haemolysis.
25.	Titers of ANCA and ANA normal. NASH. Hepatorenal syndrome. Deceased in critical care. Portal hypertension may explain presence of schistocytosis.
26.	Symptoms of acute renal failure. Malignant hypertension. Condition worsened despite PI and dialysis. Deceased in critical care.
27.	Several episodes of suspected TMA in patient history. PE initiated. Condition improved. No data on complement or genetic analyses.
28.	Stomach pain, headache. Acute renal failure. Malignant hypertension. HUS suspected. PE initiated. No data on complement or genetic analyses.
29.	Fever, muscle ache, throat pain. Renal biopsy indicates TMA and poststreptococcus glomerulonephritis. Discretely elevated LD, no other sign of haemolysis. Haemodialysis normalizes laboratory parameters.
30.	Autoimmune antibody titers low. Extensive viral panel negative. Condition improved on PE therapy. Blood samples sent for genetic analyses.
31.	Renal transplant. Acute renal failure. Suspected to be triggered by hypertension. Genetic analyses normal with regard to FH, FI, MCP, C3 and FB. Genetic polymorphism found. PE improves condition.

HUS: haemolytic uraemic syndrome; TMA: thrombotic microangiopathy; PI: plasma infusion therapy; PE: plasma exchange therapy; FH: factor H; FI: factor I; FB: factor B; MCP: membrane cofactor protein; C3: complement component 3; LD: lactate dyhydrogenase; ANA: anti-nuclear antibody; ANCA: Anti-neutrophil cytoplasmic antibody; NASH: non-alcoholic steatohepatitis; PCR: polymerase chain reaction; TTP: thrombotic thrombocytopenic purpura; CVI: cerebral vascular insult; LVAD: left ventricular assistance device; EHEC: enterohaemorrhagic Escherica coli; RA: rheumatoid arthritis; RF: rhematoid factor; GBM: glomerular basement antibody; SSA: Sjögrens syndrome-related antigen A; PTA: percutaneous transluminal angioplasty; DAT: direct antiglobulin test

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Clinical characterization and identification of rare genetic variants in atypical hemolytic uremic syndrome: A Swedish retrospective observational study

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Abstract

Complement-mediated atypical hemolytic uremic syndrome (aHUS) is an ultra-rare renal disease primarily caused by genetic alterations in complement proteins. The genetic work-up required for confirmation of diagnosis is complicated and not always logistically accessible. The aim of the present study was to apply a diagnostic scheme compliant with the American College of Medical Genetics and Genomics guidelines to investigate the prevalence of complement-mediated aHUS among subjects formerly included in a retrospective cohort of clinically suspected aHUS. Clinical outcomes and genetic correlations to complement analyses were assessed. Subjects were investigated with medical record reviewing, inquiries, and laboratory analyses composed of whole genome sequencing; enzyme-linked immunosorbent assays for factor I, factor H, and factor H-specific antibodies; nephelometry for complement components three of four; flow cytometry for CD46 surface expression and immunoblotting for the presence of factor H-related protein 1. In total, 45% ($n = 60/134$) of the subjects were deceased at the time of study. Twenty of the eligible subjects consented to study participation. Based on genetic sequencing and clinical characteristics, six were categorized as definite/highly suspected complement-mediated aHUS, 10 as non-complement-mediated aHUS and four as having an HUS-like phenotype. In the complement-mediated aHUS group, two subjects had not received an aHUS diagnosis during the routine clinical management. Disease-contributing/likely disease-contributing genetic variants were identified in five subjects, including a novel missense variant in the *complement factor H* gene (c.3450A>G, p.I1150M). This study illustrates the risk for misdiagnosis in the management of patients with complement-mediated aHUS and the importance of a comprehensive assessment of both phenotype and genotype to reach a diagnosis.

KEYWORDS

atypical hemolytic uremic syndrome, complement pathway, alternative, mutation, renal dialysis, thrombotic microangiopathies

1 | INTRODUCTION

Complement-mediated atypical hemolytic uremic syndrome (aHUS) is an ultra-rare disease caused by a dysregulated activation of the alternative complement pathway. It approximately afflicts one individual per two million person-years [1] and it predominantly occurs in a relapsing fashion. If not promptly treated, aHUS is associated with a high degree of renal morbidity and overall mortality [2]. However, the disease penetrance and severity of renal outcome rest on a complex synergy of several factors: (1) coinciding complement-amplifying conditions (e.g., concomitant autoimmune diseases or infections), (2) present risk haplotypes and polymorphisms, (3) copy number variations and complex genomic rearrangements, (4) the potential presence of factor H-specific antibodies, and (5) rare variants residing in genes encoding complement activators, inhibitors, and thrombosis-associated proteins [3]. Consequently, manifestation of disease is generally conditioned by the specific combination of predisposing factors. Rather than being causative, the rare genetic variants mainly appear to be risk factors for clinical breakthrough [4] which illustrates the genetic heterogeneity of disease.

The phenotypical resemblance with more prevalent diseases that manifest with microangiopathic hemolytic anemia, thrombocytopenia, and acute renal failure makes the diagnostic assessment ambiguous and time to diagnosis is often delayed. These diseases are primarily typical HUS (caused by enterohemorrhagic *Escherichia coli* or *Shigella dysenteriae* type 1) and thrombotic thrombocytopenic purpura (confirmed by the finding of ADAMTS13 activity <5%) [1]. Furthermore, several conditions causing non-complement-mediated aHUS have been described and these too must be tentatively ruled out before genetic testing is conducted [5]. In addition, some of the latter conditions have been suggested to potentially contribute to flare-ups of complement-mediated aHUS by means of triggering a complement response already genetically prone to dysregulation. Ultimately, this renders the diagnostic work-up difficult without the genetic testing [6]. However, the genetic work-up is complicated, time-consuming and not always accessible in the routine clinical setting. Moreover, it is recommended that the genetic results are interpreted by a laboratory with specific expertise in aHUS [1]. In Sweden, one clinical expert genetic laboratory is established [7].

In this study, we present a diagnostic scheme for the assessment of potential cases with complement-mediated aHUS that is compliant with the American College of Medical Genetics and Genomics (ACMG) guidelines for the interpretation of sequence variants [8]. We applied it to 20 subjects, originally identified in a previously described Swedish retrospective inpatient cohort of clinically suspected, but unconfirmed aHUS patients [9]. We aimed to investigate characteristics and outcomes of subjects with clinically suspected aHUS and potential differences in clinical features between cases of complement-mediated versus non-complement-mediated disease. We hypothesized that less severe or less frequently relapsing cases of complement-mediated aHUS were not subjected to genetic work-up in the routine clinical setting and that they consequently remained undiagnosed.

2 | SUBJECTS AND METHODS

2.1 | Study population

The inclusion process was performed as previously described [9]. Briefly, subjects referred nationwide to the Coagulation Unit, Skane University Hospital, Sweden, during the years 2007–2012, with a confirmed ADAMTS13 activity of >5% and routine laboratory findings of (1) hemolytic anemia (increased lactate dehydrogenase *and* hypohaptoglobinemia *and/or* reticulocytosis *and/or* unconjugated hyperbilirubinemia), (2) thrombocytopenia, and (3) renal failure (increased creatinine *and/or* cystatin C) were included in a retrospective cohort of clinically suspected aHUS patients. Subjects with a positive microbiological testing for enterohemorrhagic *Escherichia coli* were excluded. Results from peripheral blood smears and direct antigen tests (DAT) were not required for inclusion. Included subjects were categorized into two clinical groups: suspected aHUS with (n = 103), respectively, without (n = 31) potential triggers or causes. The prior study indicated that complement-mediated aHUS potentially was underdiagnosed in the cohort at the time of discharge [9].

Subjects fulfilling the inclusion criteria of the prior study were asked for participation in the present investigation which was composed of blood sampling (whole blood and EDTA plasma) for whole genome sequencing and complement analyses, medical record reviewing and inquiries regarding current diagnoses and renal status.

2.2 | Laboratory investigations

2.2.1 | Nephelometry

Complement component 3 (C3) and 4 (C4) concentrations were determined using BN Prospec Systems (Siemens Healthineers). Reference range based on healthy controls was 0.86–1.73 g/L for C3 and 0.13–0.31 g/L for C4. The threshold for lower range protein detection was 10 mg/L.

2.2.2 | Enzyme-linked immunosorbent assay

Concentrations of factor I (FI), factor H (FH), and FH-specific antibodies were determined using enzyme-linked immunosorbent assays (ELISA). Factor I and FH-specific antibodies were determined as previously described [10,11]. Details on the FH ELISA are provided in Appendix S1.

Fifty-three unmatched control subjects were randomly selected in a Swedish cohort of healthy subjects. Concentrations were calculated relative to a logarithmic standard curve using a nonlinear regression sigmoidal model. Samples and controls were analyzed in duplicates and the final concentration was calculated as the mean of the two independent assays.

The FI and FH concentrations were expressed as the percentage of $\text{mean}_{Controls}$ (normal range 100 ± 30%). The concentration of FH-specific antibodies was considered significantly increased if titers were > ($\text{mean}_{Controls}$ + 3 SD). The cut-off was calculated to 289 AU/mL.

2.2.3 | Immunoblotting

Presence of factor H-related protein 1 (FHR1) was determined as previously described [9].

2.2.4 | Flow cytometric determination of CD46 surface expression

The surface expression of CD46 (membrane cofactor protein) on monocytes, lymphocytes, and neutrophils was determined to investigate whether genetic variants identified in the *CD46* gene were correlated with absent or deficient expression of CD46 on host cells. Normal expression was defined as mean fluorescence intensity ±3 SD of 18 healthy control subjects. Details are provided in Appendix S1.

2.2.5 | Whole genome sequencing

The following genes were screened for rare genetic variants: (1) genes encoding complement factors: *C3*, *CFB*, *Properdin* (*CFP*; enhancer of the alternative pathway); (2) complement inhibitors: *CFI, CFH, CFHR1/3/5, CD46, Vitronectin* (*VTN*), and (3) genes directly or indirectly influencing thrombus formation: *Plasminogen* (*PLG*), *Thrombomodulin* (*THBD*), *Diacylglycerol kinase ϵ* (*DGKE*).

Genomic DNA from whole blood was purified using AllPrep DNA/RNA Mini Kit (Qiagen) and library prepared using Nextera DNA Flex Library Prep Kit (Illumina). Paired-end whole genome sequencing was performed on the NovaSeq6000 sequencer (Illumina). Raw fastq files were aligned to the human reference genome (hg19). Quality thresholds for sequencing were ≥ 30-fold average sequencing depth and ≥98% of the genome sequenced at minimum 10-fold. Alignment file pre-processing and germline variant calling was performed by GATK v4.1 analysis software (Broad Institute). The Ingenuity Variant Analysis tool (Qiagen) was used to identify potentially causative variants. Variants were kept for further analysis if variant call quality was ≥20 and if variants were outside the top 5% most exonically variable 100-base windows in healthy public genomes. Variants which had ≥5% allele frequency in 1000 Genomes Project, the Exome Aggregation Consortium, the Genome Aggregation Database (gnomAD) or the National Heart Lung and Blood Institute Exome Sequencing Project were excluded. Variants that were exonic or no more than 10 bases into intron as well as predicted pathogenic or likely pathogenic according to the CentoMD or the Human Gene Mutation Database were kept. Finally, missense variants and variants that were associated with a loss-of-function of a gene were kept, that is, frameshifts, inframe insertion/deletions (InDel), or start/stop codon changes.

In addition, c.3572C>T,p.S1191L and c.3590T>C,p.V1197A are frequent pathogenic variants residing in the *CFH* gene. They arose through gene conversion between *CFH* and *CFHR1* [12] and are at risk of aligning to *CFHR1* in bioinformatics. Thus, exon 23 of *CFH* and exon 6 of *CFHR1* were manually curated in the Integrative Genomics Viewer software [13] and a p.S1191L/p.V1197A positive control was provided for reference in the assessment.

2.3 | Variant classification

The detected variants were classified in compliance with the established ACMG guidelines with modifications to allele frequency-related criteria and some exceptions to *the Rules for Combining Criteria*.

2.3.1 | In-silico predictions

For missense variants the established SIFT [14], PROVEAN [15], PolyPhen-2 [16], and CADD [17] prediction tools were applied. Cutoffs for damaging predictions were set to alignment score < 0.05, alignment score < −2.5, Naïve Bayes posterior probability score > 0.15 and c-score > 15, respectively. For splicing defects, we applied CADD, NNSplice [18], dbscSNV [19], and MaxEntScan [20]. Cutoffs for

damaging predictions were set to c-score > 15, mutation ÷ wildtype score < 0.85, RF score > 0.6, and mutation ÷ wildtype score < 0.80, respectively. The output of the tools needed to be unanimous to provide *supporting evidence* of pathogenic or benign impact.

2.3.2 | Variant allele frequencies

Data were retrieved from the gnomAD [21]. The minor allele frequencies (MAF) for detected variants in the non-Finnish European population, the global population, and the highest MAF regardless of ethnic origin (Hi_Freq) were collected.

MAF-related criterion for benign variant classification

(1) 0.1% ≤ Hi_Freq MAF < 1% provided *supporting evidence* of benign impact, (2) 1% ≤ Hi_Freq MAF < 5% provided *strong evidence* of benign impact, (3) Hi_Freq MAF ≥ 5% provided *stand-alone* evidence of benign impact.

MAF-related criterion for pathogenic variant classification

(1) Hi_Freq 0.01% ≤ MAF < 0.1% provided *supporting evidence* of pathogenic impact for single-nucleotide variants (SNV) in *CFH, CFI, CD46, C3, DGKE*, and *VTN* [22], (2) Hi_Freq MAF < 0.01% provided *moderate evidence* of pathogenic impact for SNVs and InDel variants in *CFH, CFI, CD46, C3*, and *DGKE* [23].

2.3.3 | Exceptions to the ACMG rules for combining criteria

Reclassification of variants of unknown significance (VUS)

If the MAF-related criterion constituted the only provided evidence in favor of pathogenic impact, variants with conflicting evidence (both benign and pathogenic criteria) were classified as likely benign.

Reclassification of VUS for CFB, CFP, and C3 variants

Gain-of-function alterations in the *CFB*, *CFP*, and *C3* genes result in an excessive complement activation. However, in-silico predictions potentially do not recognize gain-of-function alterations as damaging. Moreover, loss-of-function alterations are potentially predicted as damaging in-silico although the absence of *CFB, CFP*, and *C3* gene products in principle does not result in an excessive complement response [24]. Due to the lack of functional evaluations, classifications heavily rely on in-silico predictions. Therefore, null variants (loss-of-function) were classified as likely benign if (1) pathogenic in-silico prediction output resulted in conflicting evidence or if (2) available evidence was insufficient to determine a classification according to the *Rules for Combining Criteria to Classify Sequence Variants*. However, if the criterion for *functional studies supportive of pathogenic impact* (unlikely) was met, the variant was defaulted to VUS granted that it was unqualified for a likely pathogenic/pathogenic classification. Non-null variants with conflicting evidence were regarded VUS without exception, that is, including cases where the MAF-related criterion constituted the only provided evidence in favor of pathogenic impact.

2.3.4 | Clinical classification

Definite complement-mediated aHUS

(1) The subject harbored ≥1 disease-contributing genetic variant (pathogenic or likely pathogenic) and/or (2) the subject featured significant titers of FH-specific antibodies.

Highly suspected complement-mediated aHUS

(1) The subject harbored ≥1 likely disease-contributing genetic variant (VUS located in a mutational hotspot and/or a critical functional domain) and/or (2) the subject featured distinct alternative pathway complement consumption during the acute episode and thrombotic microangiopathy (TMA) was confirmed in a renal biopsy.

Non-complement-mediated aHUS

(1) The subject harbored no disease-contributing/likely disease-contributing genetic variants and (2) the subject was afflicted by a condition acknowledged to cause phenotypical HUS by means of other mechanisms (Table 1).

HUS-like phenotype

(1) The subject harbored no disease-contributing/likely disease-contributing genetic variants and (2) the clinical presentation and/or additional laboratory results deemed aHUS unlikely, for example, if the hemolysis criterion in a subject presenting with septic shock was met due to increased lactate dehydrogenase and slightly elevated unconjugated hyperbilirubinemia alone, without further evidence of microangiopathic hemolytic anemia.

2.4 | Statement of ethics

The study was conducted in accordance with the Declaration of Helsinki. The Regional Ethics Committee at Lund University approved the study (diary number: 2013/514)

TABLE 1 Triggers and causes of atypical hemolytic uremic syndrome

Infections		Drugs		Malignancy	Autoimmune disease
Neuraminidase-mediated aHUS	Bacteria	Calcineurin inhibitors	Antibiotics and antiparasitics	Metastatic microvascular neoplasms	Systemic lupus erythematosus
Influenza A	*Haemophilus influenzae*	Cyclosporine	Ciprofloxacin	Lymphoma	Anti-phospholipid syndrome
Streptococcus pneumoniae	*Bordetella pertussis*	Tacrolimus	Quinine	HUS phenotypes have also been reported	Renal scleroderma crisis
	Clostridium difficile	VEGF inhibitors	Platelet inhibitors	in association with the following malignancies	Dermatomyositis
Viruses	*Campylobacter upsaliensis*	Bevacizumab	Clopidogrel		
HHV6, VZV, CMV, EBV	*Fusobacterium necrophorum*	Sunitimib	Interferon alpha/beta	Prostatic	
HCV, HAV		mTOR inhibitors	Contraceptives	Gastric	
HIV	Parasites	Sirolimus	Illicit drugs	Hepatic	
Coxsackie B virus	*Plasmodium falciparum*	Everolimus	Heroin	Pancreatic	
Parvovirus B19		CD52 inhibitors	Ecstasy	Breast	
Dengue virus		Alemtuzumab	Cocaine	Ovarian	
Norovirus		General cytotoxics		Lung	
	Potentially all infections resulting in sepsis with MOF and DIC	Mitomycin C		Colon	
		Cisplatin			
		Vincristine			
		Gemcitabine			

Syndromes of HSCT	Pregnancy-mediated	Vasculitis	Renal cortical necrosis	Unspecified glomerular disease	Malignant hypertension
Radiation	Pregnancy	Polyarteritis nodosa	Regardless cause	Regardless cause	Regardless cause
Graft-versus-host disease	Postpartum period	Infectious vasculitis (e.g. *Rickettsia rickettsii*)			
CMV infections	Preeclampsia				
Associated drugs	HELLP syndrome				

Heparin-induced thrombocytopenia and thrombosis	Paroxysmal nocturnal hemoglobinuria	Pancreatitis	Combined methylmalonic aciduria and homocystinuria	Disseminated intravascular coagulopathy	Hepatitis B vaccination

Notes: The table is adapted and modified from Akesson et al. [25]. The conditions presented may be triggers of complement-mediated aHUS, causes of non-complement-mediated aHUS or both.

Abbreviations: CMV, cytomegalovirus; DIC, disseminated intravascular coagulation; EBV, Epstein–Barr virus; HAV, hepatitis A virus; HCV, hepatitis C virus; HELLP, hemolysis, elevated liver enzymes, low platelets syndrome; HHV6, human herpes virus 6; HIV, human immunodeficiency virus; HSCT, hematopoietic stem cell transplantation; MOF, multiple organ failure; mTOR, mammalian target of rapamycin; VEGF, vascular endothelial growth factor; VZV: varicella zoster virus.

and the use of healthy control samples for the laboratory assays (diary number: 2017/582). Written informed consent was obtained from all subjects (or by proxy legal guardians).

2.5 | Statistical analyses

Continuous variables with a non-Gaussian distribution were descriptively presented with medians and lower and upper quartiles (q1–q3). Statistical differences for continuous non-normally distributed variables between two groups were evaluated using the Mann–Whitney *U* test. Statistical differences between categorical variables were evaluated using Fisher's exact test. All tests were exact and all *p*-values were two-tailed. The *p*-values <0.05 were considered significant. Statistical analyses were performed using IBM SPSS Statistics version 25.0 (IBM). The ELISA curve fits were executed using GraphPad Prism version 8.0.0 (GraphPad Software).

3 | RESULTS

3.1 | Mortality ratio and study inclusion

The present study was conducted in median 63 months (q1–q3: 46–77) subsequently to the acute episode onset. At the time of study inclusion, 52% ($n = 54/103$) and 19% ($n = 6/31$) of the subjects of the initial cohort of clinically suspected aHUS patients with respectively without potential triggers or causes were deceased.

Three subjects were excluded due to untraceable provisional identification numbers and 59% ($n = 42/71$) of the remaining subjects declined or did not respond to the study participation inquiry. Nine additional subjects were lost due to nonresponse after request of rebleed following incomplete blood sampling by local laboratories. Ultimately, 20 subjects were included in the present study for the assessment of cases with complement-mediated aHUS: those with ($n = 16$) and those without ($n = 4$) potential triggers or causes. Three out of the four subjects

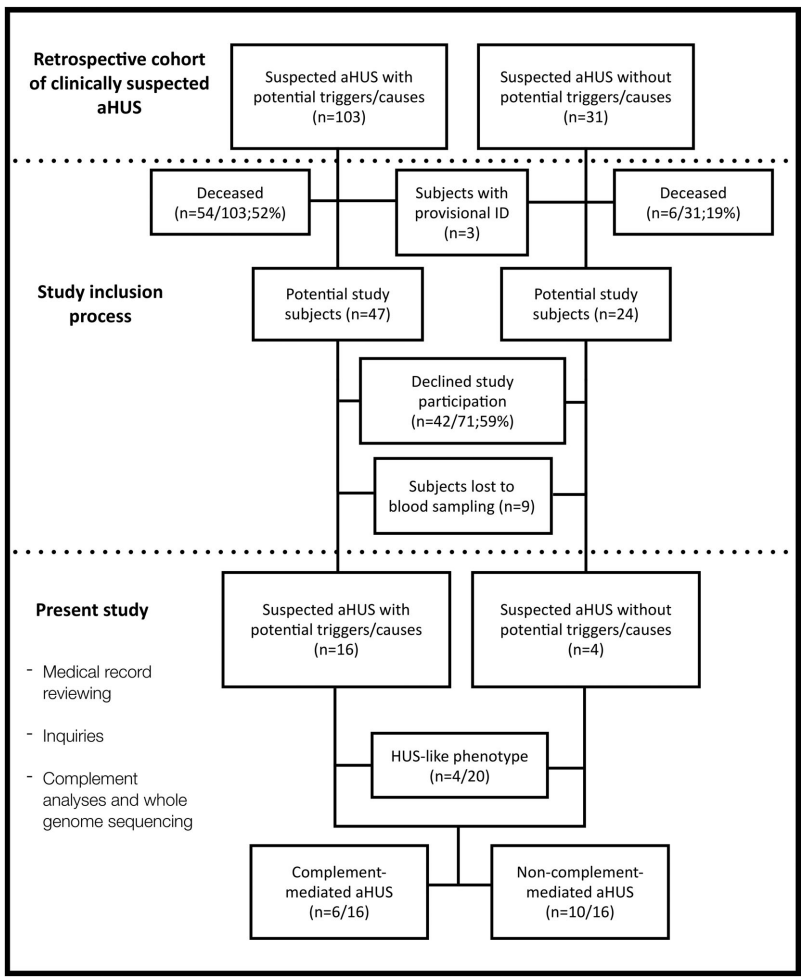

FIGURE 1 Study inclusion process. Numbers are presented within brackets. Additional percentages are presented with reference to deceased subjects per group and the proportion of potential study subjects that declined study participation. By means of combining clinical and whole genome sequencing data, the study cohort was partitioned into three groups as presented

without association to potential triggers or causes were categorized as definite or highly suspected complement-mediated aHUS. Thrombotic thrombocytopenic purpura was confirmed for the fourth subject subsequently to discharge. Thus, it was categorized as an HUS-like phenotype. In total, four subjects were attributed an HUS-like phenotype, 10 were categorized as non-complement-mediated aHUS, and six were categorized as definite or highly suspected complement-mediated aHUS (Figure 1).

3.2 | Variant classifications

Twenty unique genetic variants were identified in 14 subjects. All variants were heterozygous and missense variants were the most prevailing (Table 2). Additional information on variant specifics, reference database identifications, MAFs and the ACMG classifications are provided in Tables S1, S2, S3, and S4. In total, one (disease-contributing) pathogenic and four (likely disease-contributing) VUS were identified and distributed among five out of six subjects in the complement-mediated aHUS group (Table 3).

3.3 | Complement analyses

No subjects had significant titers of FH-specific antibodies. All subjects showed expression of FHR1. Nineteen subjects, including subject C and K who harbored a benign *CD46* variant (variant ID 4, Table 2), exhibited normal CD46 surface expression. The flow cytometric

TABLE 2 Variant type specifics and classification

Variant ID[a]	Subject (#)	Gene (transcript variant)	Variation type	Translation effect	Protein domain	Protein variant	In-silico predictions	ACMG classification
1	A, B	*C3* (c.193A>C)	SNV	Missense	MG 1	p.K65Q	Damaging	Pathogenic
2	F	*C3* (c.4030-4C>T)	SNV	Splice acceptor site	—	—	Benign	Likely benign
3	J	*C3* (c.4850+3G>A)	SNV	Splice donor site	—	—	Benign	Likely benign
4	C, K	*CD46* (c.1058C>T)	SNV	Missense	TM	p.A353V	Benign	Benign
5	D	*CFB* (c.1697A>C)	SNV	Missense	SP	p.E566A	Benign	Likely benign
6	G, M	*CFH* (c.-307CT)	SNV	Promoter	—	—	N/A	Likely benign
7	B, L	*CFH* (c.2634C>T)	SNV	Synonymous	CCP 15	p.H878H	N/A	Likely benign
8	J	*CFH* (c.3133+8G>T)	SNV	Splice donor site	—	—	Benign	Likely benign
9	E	*CFH* (c.3148A>T)	SNV	Missense	CCP 18	p.N1050Y	Conflicting	Likely benign
10	B	*CFH* (c.3450A>G)	SNV	Missense	CCP 19	p.I1150M	Conflicting	VUS[b]
11	D	*CFHR5* (c.485_486dupAA)	Insertion	Frameshift	CCP 3	p.E163fs*10	N/A	VUS[b]
12	I	*CFI* (c.982G>A)	SNV	Missense	Linker 2	p.G328R	Damaging	VUS[b]
13	H	*CFI* (c.1322A > G)	SNV	Missense	SP	p.K441R	Benign	Benign
14	A	*CFI* (c.1534+5G>T)	SNV	Splice donor site	—	—	Conflicting	Likely benign
15	E	*CFI* (c.1547G>T)	SNV	Missense	SP	p.G516V	Damaging	VUS[b]
16	F	*DGKE* (c.35C>T)	SNV	Missense	Signal peptide	p.P12L	Conflicting	Likely benign
17	J	*PLG* (c.185+4T>C)	SNV	Intronic	—	—	Conflicting	Benign
18	E	*PLG* (c.266G>A)	SNV	Missense	PAN	p.R89K	Conflicting	Likely benign
19	G	*PLG* (c.1567C>T)	SNV	Missense	Kringle 5	p.R523W	Conflicting	Likely benign
20	S	*PLG* (c.2356C>T)	SNV	Missense	SP	p.R786C	Conflicting	VUS

Notes: In-silico predictions were assessed for missense and splice site variants only. The prediction was conflicting if the output of the implemented annotation tools were not unanimous.

Abbreviations: SNV, single-nucleotide variant; VUS: variant of unknown significance.

[a]All detected variants were heterozygous.

[b]Likely disease-contributing.

TABLE 3 Subject characteristics

Subject (#)	Variant ID (classification)	Time to study (months)	Dialysis or transplant	Diagnosis	Medication and interventions
HUS-like phenotype					
P	—	94	—	TTP	Plasmapheresis, rituximab
T	—	65	—	SLE	Azathioprine, hydroxychloroquine, prednisone
J	3 (LB), 8 (LB), 17 (B)	87	—	SLE	Azathioprine, prednisone
M	6 (LB)	49	—	—	—
Non-complement-mediated aHUS					
N	—	60	Transplant	T1D	Mycophenolate mofetil, tacrolimus
C	4 (B)	33	—	HSCT r/t T-ALL	Irrelevant
O	—	75	Transplant	IgA nephropathy	Mycophenolate mofetil, tacrolimus
F	2 (LB), 16 (LB)	88	—	—	—
G	6 (LB), 19 (LB)	40	—	—	—
Q	—	52	Dialysis	CKD	Irrelevant
R	—	38	—	PCV	Irrelevant (no IFN-α)
S	20 (VUS)	74	—	—	—
K	4 (B)	48	—	GPA	Azathioprine, prednisone
L	7 (LB)	91	—	—	—
Complement-mediated aHUS					
A	1(P), 14 (LB)	45	—	aHUS	—[a]
B	1 (P), 7 (LB), 10 (VUS)	55	Dialysis	aHUS	—[a]
D	5 (LB), 11 (VUS)	40	—	—	—
E	9 (LB), 15 (VUS), 18 (LB)	72	Transplant	aHUS	Eculizumab, mycophenolate mofetil, prednisone, tacrolimus
H	13(B)	72	Transplant	TMA (unspecified)	Ciclosporin, mycophenolate mofetil, prednisone
I	12(VUS)	78	Transplant	aHUS	Eculizumab, leflunomide, prednisone, tacrolimus

Notes: Median time to study was 63 months (q1-q3: 46–77) after the ADAMTS13 referral. Subject A and B were categorized as definite complement-mediated aHUS. The remaining subjects in the complement-mediated group were categorized as highly suspected complement-mediated aHUS.
Abbreviations: ADAMTS13, a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 1; aHUS, atypical hemolytic uremic syndrome; B, benign; CKD: chronic kidney disease; HSCT: hematopoietic stem cell transplantation; IFN-α: interferon alpha; LB, likely benign; MOF: multiorgan failure; P, pathogenic; PCV: polycythemia vera; SLE: systemic lupus erythematosus; T-ALL: T-cell acute lymphoblastic leukemia; TTP: thrombotic thrombocytopenic purpura; T1D: diabetes mellitus type 1; VUS: variant of unknown significance.
[a]Watchful waiting. No therapeutic complement inhibition.

analysis for subject E was inconclusive as the sample indicated >90% apoptotic cells. Results for the analyses of C3 and C4, and CFI and CFH, respectively, are provided in Figure 2.

3.4 | Clinical setting for diagnosis

Two out of six subjects (33%) in the complement-mediated aHUS group had not been diagnosed with (complement-mediated) aHUS by their treating physician prior to study entry. Subject H had received a tentative diagnosis of thrombotic microangiopathy and subject D had not been diagnosed at all (Table 3).

3.5 | Acute episode features

Differences in acute episode clinical presentations were assessed after the post hoc categorization of the study group into complement-mediated and non-complement-mediated disease. There was no significant difference in

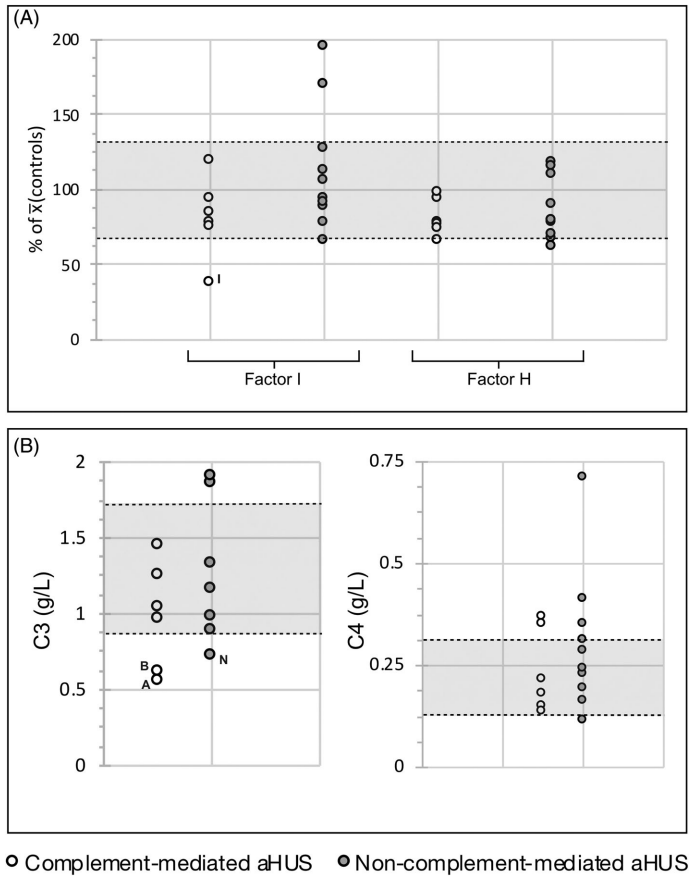

FIGURE 2 Complement analyses. Analyses were performed at the time of study. Normal range is illustrated by the accentuated fields. (a) Concentrations of factors I and H are expressed as percentage of $mean_{Controls}$. Subject I is indicated by the bold letter and harbored a heterozygous missense variant in exon nine of *CFI* (variant ID 12; c.982G >A,p.G328R). (b) Concentrations of C3 and C4. Subject A, B, and N are indicated by bold letters. Subjects A and B harbored a pathogenic heterozygous missense variant in exon two of *C3* (variant ID 1; c.193A>C,p.K65Q). Neither subject was treated with eculizumab. No variants were detected in subject N

age ($P = 0.79$) or sex ($P = 1$) between the complement-mediated and non-complement-mediated aHUS group. General symptoms were dominant in both groups, although specific renal and gastrointestinal symptoms were slightly more prevalent in the former. During inpatient care, peak creatinine levels were significantly higher ($P = 0.03$) in the complement-mediated aHUS group, although individual overlaps with the upper quartile of the non-complement-mediated aHUS group were present for two subjects with complement-mediated aHUS. No other significant differences for routine laboratory variables were identified between the groups (Table 4). Inferentially, one cannot reliably utilize the clinical presentation or routine laboratory results for differential diagnostic guidance during the acute episode.

3.6 | Renal outcome

Four out of the six subjects (67%) in the complement-mediated aHUS group were subjected to dialysis at discharge as compared to five out of 10 (50%) in the non-complement-mediated aHUS group. At the time of study, three out of four (75%) dialyzed subjects in the complement-mediated aHUS group had undergone renal transplantation compared to two out of five (40%) in the non-complement-mediated aHUS group. Of note, two (40%) of the dialyzed subjects in the latter group had been weaned off dialysis without the need for transplantation (Figure 3). Consequently, the complement-mediated aHUS group had a qualitatively worse renal outcome at follow-up (study entry) compared to subjects with non-complement-mediated disease.

TABLE 4 Acute episode features

	Complement-mediated aHUS ($n = 6$)	Non-complement-mediated aHUS ($n = 10$)	*P*-value
General characteristics			
Sex, female - no. (%)	3 (50)	5 (50)	1
Age, years - median (q1-q3)	46 (37–48)	42 (23–59)	0.79
Symptoms/clinical manifestations			
Macroscopic hematuria - no. (%)	3 (50)	1 (10)	0.12
Abdominal symptoms/manifestations[a] - no. (%)	6 (100)	2 (20)	0.23
Routine laboratory results			
Hemoglobin (g/L), min - median (q1–q3)	79 (71–80)	73 (67–85)	0.64
Creatinine (μmol/L), max - median (q1–q3)	904 (448–1507)	343 (190–681)	**0.03**
Platelet count (x10^9/L), min - median (q1–q3)	69 (35–92)	29 (15–56)	0.18
Lactate dehydrogenase (μkat/L), max - median (q1–q3)	23 (15–36)	13 (9–18)	0.12
Haptoglobin <0.1 g/L - no (%)	5 (83)	7 (70)	1
Diagnostics and treatment			
Inpatient empirical PEX/PI - no. (%)	5 (83)	3 (30)	0.12
Inpatient renal biopsy - no. (%; histology)	3 (50; TMA x3)	2 (20; TMA x1, IgAN x 1)	0.3
Dialysis at discharge - no. (%)	4 (67)	5 (50)	0.63
Scheduled follow-up for suspected/ confirmed TMA - no. (%)	4 (67)	4 (40)	0.61

Abbreviations: IgAN, Immunoglobulin A nephritis; PEX/PI, plasma exchange therapy/plasma infusion; TMA, thrombotic microangiopathy.
[a]Diarrhea, abdominal pain, emesis and/or nausea.

4 | DISCUSSION

In this study, we present a diagnostic scheme for the assessment of potential cases with complement-mediated aHUS that relies on clinical characteristics and the ACMG guidelines, with minor disease-related modifications regarding the assessment of MAF and the significance of *CFB, CFP*, and *C3* gene products.

To our knowledge, previous efforts of analyzing aggregated clinical and genetic data in patients with suspected aHUS are lacking in a Swedish context. Although our study was strongly limited by the small cohort size, and considering the ultra-low prevalence, we managed to identify and systematically categorize cases of suspected aHUS by applying the diagnostic scheme to a cohort referred for ADAMTS13 analysis in the clinical setting. This systematic approach is often lacking in clinically oriented aHUS studies. In total, six subjects with definite or highly suspected complement-mediated aHUS were identified. Two of these subjects had not been diagnosed with complement-mediated aHUS during the routine clinical management. In addition, for the first time, a likely disease-contributing missense VUS in *CFH* (variant ID 10; c.3450A>G,p.I1150M) was detected.

Subjects A and B illustrate the general requisite of a complement-triggering event to develop flare-ups of clinical disease in spite of the presence of disease-contributing genetic variants. Both subjects harbored a pathogenic missense variant in the *C3* gene (variant ID 1; c.193A>C,p.K65Q). Subject A harbored no additional disease-contributing/likely disease-contributing variants but was infected with influenza A which increases the risk of complement dysregulation by the neuraminidase-mediated desialylation of cell surface glycans [26], potentially resulting in weaker FH binding alongside surface-deposited C3b [27]. In light of the reduced binding of FH to C3b also associated with p.K65Q [28], this may have severely hampered the inhibitory function of FH. Subject B harbored an additional likely disease-contributing novel missense VUS in the *CFH* gene (variant ID 10; c.3450A>G,p.I1150M) corresponding to the C-terminal complement control protein (CCP) 19 domain of FH. This is a confirmed mutational hotspot, as alterations in the C-terminus disrupt the cell surface-binding

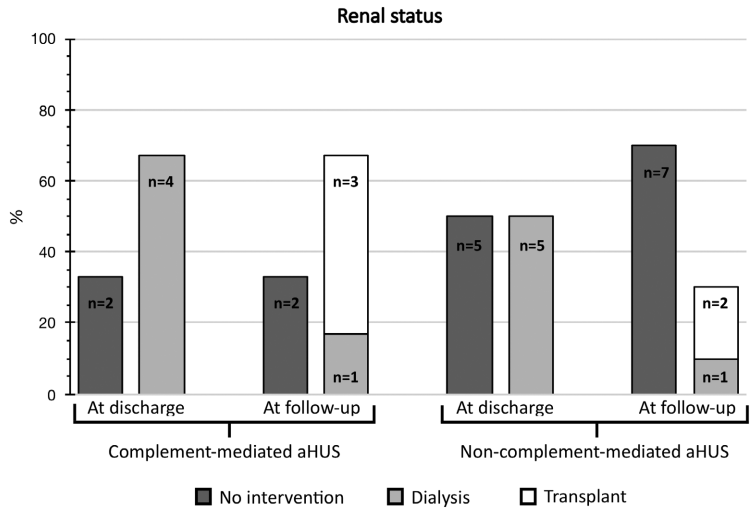

FIGURE 3 Renal status. Comparison of the status at discharge and at the time of study. Three of the four dialyzed subjects in the complement-mediated aHUS group had undergone renal transplantation. Two of these subjects were receiving eculizumab maintenance therapy. The two subjects without intervention had been considered for eculizumab but watchful waiting was concluded

capability of FH [23]. Nevertheless, a complement-amplifying gastrointestinal infection was required to trigger a dysregulated complement response (Table S5). Interestingly, at the time of study neither subject was treated with eculizumab, nor had they suffered from subsequent relapses. Patients with complement-mediated aHUS due to pathogenic *C3* variants have an approximated 50% risk of disease relapse [25] and a native end-stage renal disease risk of 40%–70% [25,29], generally developing within two years of disease presentation [1]. However, only subject B suffered from persistent renal failure and was subjected to continued intermittent hemodialysis after discharge. Neither subject had been subjected to renal transplantation at the time of study more than three years after discharge. Of note, the *C3* variant was heterozygous and both subjects exhibited decreased C3 levels at the time of study, suggesting that heterozygosity was sufficient to increase the complement drive during stable disease. The consumption of C3 and the fact that neither of them had yet suffered from relapses illustrate a need to investigate to what extent subclinical complement dysregulation potentially affects renal function over time. Detailed accounts of the genotype/phenotype correlations for the remaining subjects in the complement-mediated aHUS group are provided in Appendix S1.

Since many conditions may both trigger flare-ups of complement-mediated aHUS and cause non-complement-mediated disease, one cannot rely on clinical features and routine laboratory tests for guidance. Although renal involvement is more severe in complement-mediated aHUS at group level [5,30], it is of little help in the management of a specific case. However, in non-complement-mediated aHUS the symptoms generally improve alongside with the management of the predisposing conditions. This is illustrated by subject N who was admitted due to an HUS phenotype eventually attributed to the use of gemcitabine and/or interferon alpha (Table S5). The condition was improved upon the discontinuation of the medications, both of which are associated with the risk of developing drug-induced non-complement-mediated aHUS [29]. To conclude, the management of patients with suspected aHUS must seek to eliminate potential causes of non-complement-mediated disease and swiftly run complement tests before the initiation of empirical plasma therapy [1]. In due course, genetic testing is necessary to reach a diagnosis.

During the 2017 aHUS and C3 Glomerulopathy KDIGO Controversies Conference, a suggestion for the categorization of potentially aHUS-related genetic variants was presented [1]. However, the criteria, although sought to be harmonized with the ACMG *Criteria for Classifying Sequence Variants*, were equally weighted in terms of their significance for the *Rules for Combining Criteria to Classify Sequence Variants*. The authors did point out that the categorization was insufficient and that the opinion of an expert laboratory still was needed. The genetic knowledge base in aHUS is comparatively limited but it is continuously growing by the addition of genetic variants to global aHUS databases [23]. However, the number of expert laboratories providing the service of interpreting genetic results are scarce. In addition,

complement laboratory tests are important alongside information on disease presentation and empirical therapy response, in order to provide phenotypical support for the assessment of significance regarding novel or restrictedly documented genetic variants. Our suggested diagnostic scheme is concretely compliant with the ACMG guidelines, and even though it by no means aims to replace the assistance of experts, it may provide some guidance for the treating physician.

Our study was restricted to the identification of rare sequencing variants that potentially contribute to complement-mediated aHUS. However, a comprehensive genetic investigation must include copy number variations and non-allelic homologous recombination events in the *CFH-CFHRs* genomic region [1]. Several studies have reported a 5%–10% prevalence of copy number variations and homozygosity for complex genomic rearrangements in larger aHUS cohorts [2,31]. The absence of FHR1 by immunoblot technique has reliably been correlated to *CFHR1* deletions detected using multiplex ligation-dependent probe amplification [11]. Moreover, a good correlation has been shown between combined *CFHR1/3* deletions and the absence of FHR1 proteins [32]. Our FHR1 immunoblot investigations did not show absence of FHR1 in any subject. Therefore, it is unlikely that any subject was afflicted by homozygous complex genomic rearrangements resulting in *CFHR1* or *CFHR1/3* deletions. Evidently though, we cannot exclude that some subject harbored an infrequent disease-contributing copy number variation or complex genomic rearrangement. Thus, it makes our categorization tentative and the number of undiagnosed cases with complement-mediated disease may potentially be higher.

5 | CONCLUSIONS

We present a diagnostic scheme with which we identified six cases of definite or highly suspected complement-mediated atypical hemolytic uremic syndrome (aHUS). At the time of study, two subjects had not received a diagnosis in the routine clinical setting. Moreover, our genetic investigations revealed a novel and likely disease-contributing C-terminal missense variant in the *CFH* gene. In addition, the need for triggering events despite the presence of pathogenic variants, have illustrated the multi-level complexity of complement-mediated aHUS.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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Clinical characterization and identification of rare genetic variants in atypical hemolytic uremic syndrome: a Swedish retrospective observational study

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Supplementary information

Supplementary methods

Enzyme-linked immunosorbent assay of factor H concentration

Flow cytometric determination of CD46 surface expression

Supplementary discussion

Complement-mediated aHUS: genotype/phenotype correlations

Supplementary tables

References

Enzyme-linked immunosorbent assay (ELISA) of factor H concentration

Maxisorp microtiter plates (Nunc, Roskilde, Denmark) were coated with sheep anti-human factor H (FH) antibody (#ab8842; Abcam plc, Cambridge, UK) in coating buffer (50 mM sodium carbonate, pH 9.6) at 4 °C overnight. Between each step the plates were washed with immunowash (50 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20, pH 8.0). The subsequent incubations were performed in quench (immunowash, 3% fish gelatin) at 37 °C for 1 h. Purified factor H was used as standard. Factor H was detected using mouse anti-human factor H antibody (MRC OX24; home-made) followed by goat anti-mouse antibody conjugated with horseradish peroxidase (Dako, Glostrup, Denmark). O-phenylenediamine dihydrochloride (Dako) was used as substrate and the absorbance was measured with a Varian 50 MPR Microplate Reader (Varian Medical Systems, CA, USA) at 490 nm.

Flow cytometric determination of CD46 surface expression

Membrane expression of complement inhibitor CD46 on neutrophils, monocytes and lymphocytes from patients and control subjects was assessed by flow cytometry. Cells were stained with phycoerythrin (PE)-labeled mouse anti-human CD46 antibody (#352402; BioLegend, San Diego, CA, USA). T helper cells were gated with fluorescein isothiocyanate-labeled mouse anti-human CD4 antibody (#F0766; Dako), cytotoxic T-cells with PE-Texas Red-labeled mouse anti-human CD8 antibody (#737659; Beckman Coulter, Brea, CA, USA), B-lymphocytes with PE-Cy5.5-labeled mouse anti-human CD20 antibody (#B23134; Beckman Coulter) and monocytes with PE-Cy7-labeled mouse anti-human CD14 antibody (#A22331; Beckman Coulter). After 10 min incubation at room temperature, erythrocytes were lysed, and samples were immediately analyzed using Navios or Gallios flow cytometer (Beckman Coulter). Apoptotic and dead cells were excluded by forward/site scatter characteristics. Leukocyte subpopulations were identified according to the applied gating markers and the expression of CD46 was quantified as *mean fluorescence intensity* (MFI). Normal CD46 expression was defined as mean MFI $\pm$ 3 SD of 18 control subjects for each leukocyte subpopulation.

Complement-mediated aHUS: genotype/phenotype correlations

Subject I harbored a likely disease-contributing missense variant of unknown significance (VUS) in the *CFI* gene (variant ID 12; c.982G>A,p.G328R). The variant affects the amino acid sequence of the linker 2 domain between the LDLR 2 module and the serine protease domain. The reduced factor I (FI) levels assessed by ELISA indicate that the variant results in misfolding and degradation of the protein. It has previously been reported in patients with advanced macular degeneration (1), but to our knowledge not in the context of phenotypical hemolytic uremic syndrome (HUS).

Subject E harbored a missense VUS in the *CFI* gene (variant ID 15; c.1547G>T,p.G516V) affecting the serine protease domain of FI. Circulating levels of FI assessed by ELISA were normal. The variant has previously been described in patients with C3 glomerulopathy (2). Moreover, a polymorphism in the *CFH* gene (variant ID 9; c.3148A>T,p.N1050Y) corresponding to the CCP 18 domain in FH was identified. It has previously been reported in numerous patients with complement-mediated atypical HUS (aHUS) (3-5) suggesting that it may be a common susceptibility variant.

Subject D is the only subject in the cohort that had suffered from a previous episode of phenotypical HUS (15 years earlier). Genetic sequencing had never been performed in the clinical work-up and consequently no diagnosis was concluded. However, the subject suffered from a nonlethal ischemic stroke during the relapse, which may have had an impact on the clinical priorities. Our investigations revealed a missense polymorphism in the *CFB* gene (variant ID 5; c.1697A>C,p.E566A) and a frameshift variant in *CFHR5* (variant ID 11; c.485_486dupAA,p.E163fs*10). The former variant corresponds to the serine protease domain of factor B and has previously been identified in several patients with complement-mediated aHUS and C3 glomerulonephritis (6, 7). Remarkably, it was the only variant detected in a pediatric eculizumab-responding patient with phenotypical HUS and membranoproliferative glomerulonephritis in the renal biopsy (8). However, the 3% South Asian minor allele frequency (MAF) suggests that it is a common susceptibility variant. The latter variant generates a reading frameshift leading to loss-of-function either through protein truncation or nonsense-mediated mRNA decay. The association to complement-

mediated renal diseases has been previously reported (9, 10). Interestingly, it is associated with a 4% Finnish European MAF, strongly implying that heterozygosity for this variant is not sufficient to cause disease. No apparent triggering condition was however recognized during hospitalization, thus suggesting that the phenotype depended on a subclinical trigger of complement activation in addition to a synergetic effect of the coincident *CFB* variant or the presence of unidentified copy number variations or complex genomic rearrangements.

Subject H harbored no disease-contributing/likely disease-contributing genetic variants. However, the acute episode was associated with distinct alternative pathway complement consumption. No coincident causes of non-complement-mediated aHUS were identified. Moreover, histopathological investigation of a renal biopsy confirmed thrombotic microangiopathy and the subject subsequently received a renal transplant based on a diagnosis of unspecified thrombotic microangiopathy.

Table S1. Variant type genetic properties and location

Subject/variant ID	Gene	Protein	Variation type	Sample allele	Allele fraction (%)	Transcript variant (cDNA)	Protein variant	Translation effect	Cytogenetic location	Gene region	Chromosome position (hg19)
A/1	*C3*	C3	SNV	G	42.31	c.193A>C	p.K65Q	Missense	19p13.3	Exon 2	6719296
A/14	*CFI*	FI	SNV	A	55.88	c.1534+5G>T	-	-	4q25	Splice site	110663642
B/1	*C3*	C3	SNV	G	54.43	c.193A>C	p.K65Q	Missense	19p13.3	Exon 2	6719296
B/7	*CFH*	FH	SNV	T	51.61	c.2634C>T	p.H878H	Synonymous	1q31.3	Exon 17	196706642
B/10	*CFH*	FH	SNV	G	56.76	c.3450A>G	p.I1150M	Missense	1q31.3	Exon 21	196715086
C/4	*CD46*	MCP	SNV	T	40.00	c.1058C>T	p.A353V	Missense	1q32.2	Exon 11	207958446
D/5	*CFB*	FB	SNV	C	51.72	c.1697A>C	p.E566A	Missense	6p21.33	Exon 13	31918468
D/11	*CFHR5*	FHR5	Insertion	AA	44.83	c.485_486dupAA	p.E163fs*10	Frameshift	1q31.3	Exon 4	196963258
E/9	*CFH*	FH	SNV	T	47.37	c.3148A>T	p.N1050Y	Missense	1q31.3	Exon 20	196712596
E/15	*CFI*	FI	SNV	A	37.25	c.1547G>T	p.G516V	Missense	4q25	Exon 13	110662254
E/18	*PLG*	PLG	SNV	A	46.51	c.266G>A	p.R89K	Missense	6q26	Exon 3	161128812
F/2	*C3*	C3	SNV	A	50.00	c.4030-4C>T	-	-	19p13.3	Splice site	6684665
F/16	*DGKE*	DGKE	SNV	T	43.84	c.35C>T	p.P12L	Missense	17q22	Exon 2	54912191
G/6	*CFH*	FH	SNV	T	61.54	c.-307C>T	-	-	1q31.3	Promoter	196620941
G/19	*PLG*	PLG	SNV	T	48.65	c.1567C>T	p.R523W	Missense	6q26	Exon 12	161152905
H/13	*CFI*	FI	SNV	C	42.22	c.1322A>G	p.K441R	Missense	4q25	Exon 11	110667485
I/12	*CFI*	FI	SNV	T	52.94	c.982G>A	p.G328R	Missense	4q25	Exon 9	110670717
J/3	*C3*	C3	SNV	T	56.98	c.4850+3G>A	-	-	1q31.3	Splice site	6678160
J/8	*CFH*	FH	SNV	T	52.70	c.3133+8G>T	-	-	1q31.3	Splice site	196711189
J/17	*PLG*	PLG	SNV	C	59.52	c.185+4T>C	-	-	6q26	Intronic	161127578
K/4	*CD46*	MCP	SNV	T	48.89	c.1058C>T	p.A353V	Missense	1q32.2	Exon 11	207958446
L/7	*CFH*	FH	SNV	T	34.38	c.2634C>T	p.H878H	Synonymous	1q31.3	Exon 17	196706642
M/6	*CFH*	FH	SNV	T	48.78	c.-307C>T	-	-	1q31.3	Promoter	196620941
S/20	*PLG*	PLG	SNV	T	46.88	c.2356C>T	p.R786C	Missense	6q26	Exon 19	161174016

Table S2. Variant database identification and allele frequency

Gene/variant ID	HGVS ID (hg19)	dbSNP ID	Minor allele frequency		
			gnomAD (NFE)	gnomAD (Global)	gnomAD (Hi_Freq)
C3/1	NC_000019.9(NM_000064.4):c.193A>C	rs539992721	0	0.000004	0.00005 (EAS)
C3/2	NC_000019.9:g.6684665G>A	rs372612816	0.0005	0.0002	0.0005 (NFE)
C3/3	NC_000019.9:g.6678160C>T	rs185428134	0	0.0003	0.004 (EAS)
CD46/4	NC_000001.10(NM_002389.4):c.1058C>T	rs35366573	0.020	0.015	0.06 (FE)
CFB/5	NC_000006.11(NM_001710.5):c.1697A>C	rs45484591	0.010	0.011	0.03 (SAS)
CFH/6	NC_000001.10:g.196620941C>T	rs74842824	0.011	0.007	0.04 (AJ)
CFH/7	NC_000001.10(NM_000186.3):c.2634C>T	rs35292876	0.010	0.010	0.03 (SAS)
CFH/8	NC_000001.10:g.196711189G>T	rs142718541	0	0.001	0.02 (EAS)
CFH/9	NC_000001.10(NM_000186.3):c.3148A>T	rs35274867	0.020	0.015	0.03 (A)
CFH/10	NC_000001.10(NM_000186.3):c.3450A>G	ND	0	0	0
CFHR5/11	NC_000001.10(NM_030787.3):c.485_486dup	rs565457964	0.006	0.007	0.04 (FE)
CFI/12	NC_000004.11(NM_000204.4):c.982G>A	rs144164794	0.0001	0.00006	0.0001 (NFE)
CFI/13	NC_000004.11(NM_000204.4):c.1322A>G	rs41278047	0.002	0.004	0.05 (AJ)
CFI/14	NC_000004.11:g.110663642C>A	rs114013791	0.016	0.007	0.02 (NFE)
CFI/15	NC_000004.11(NM_000204.4):c.1547G>T	rs764347930	0	0.00004	0.0005 (EAS)
DGKE/16	NC_000017.10(NM_003647.3):c.35C>T	rs146866423	0.008	0.005	0.008 (NFE)
PLG/17	NC_000006.11:g.161127578T>C	rs117192504	0.00005	0.004	0.06 (EAS)
PLG/18	NC_000006.11(NM_000301.3):c.266G>A	rs143079629	0.01	0.006	0.01 (NFE)
PLG/19	NC_000006.11(NM_000301.3):c.1567C>T	rs4252129	0.01	0.007	0.01 (NFE)
PLG/20	NC_000006.11(NM_000301.3):c.2356C>T	rs200857824	0.0002	0.0001	0.0002 (NFE)

Hi_Freq: the population in gnomAD with the highest minor allele frequency regardless of ethnic origin.
A: African; AJ: Ashkenazi Jewish; EAS: East Asian; FE: Finnish European; NFE: Non-Finnish European; SAS: South Asian; ND: not detected.

Table S3. Splicing defect predictions

Variant ID	dbSNP ID	CADD	NNSplice	dbscSNV	MaxEntScan
2	rs372612816	Benign (10.25)	Benign (0.92)	Benign (0.006)	Benign (0.95)
3	rs185428134	Benign (13.85)	Benign (0.96)	Benign (0.004)	Benign (0.91)
8	rs142718541	Benign (4.58)	Benign (0.89)	Benign (0)	Benign (0)
14	rs114013791	Damaging (17.84)	Benign (0.87)	Benign (0.622)	Damaging (0.79)
17	rs117192504	Benign (7.34)	Damaging (0.82)	Benign (0.06)	Benign (1.09)

Cutoffs for damaging predictions: CADD scaled c-score >15, NNSplice mutation÷wildtype score <0.85, dbSNV RF score >0.6 and MaxEntScan mutation÷wildtype score <0.80.

Table S4. Variant classification specifics

Variant ID	Gene (transcript variant)	Translation effect	In-silico prediction (score)				NCBI ClinVar[†] (submission year)	Functional evaluation	ACMG classification	References (PMID)
			SIFT	PROVEAN	PolyPhen-2	CADD				
1	*C3* (c.193A>C)	Missense	Damaging (0.04)	Deleterious (-2.98)	Probably damaging (0.98)	Damaging (23.70)	Likely pathogenic (2017)	The variant results in decreased C3b binding to CFH by ELISA. Suggests impaired C3b inactivation in vivo (11).	Pathogenic	25608561, 23307876, 29500241, 22669319, 25899302, 26541438, 28025630
2	*C3* (c.4030-4C>T)	Splice acceptor site	N/A	N/A	N/A	Table S4	Likely benign (2016)	-	Likely benign	-
3	*C3* (c.4850+3G>A)	Splice donor site	N/A	N/A	N/A	Table S4	-	-	Likely benign	[‡]
4	*CD46* (c.1058C>T)	Missense	Tolerated (0.42)	Neutral (0.00)	Benign (0.003)	Benign (0.001)	Likely benign (2016)	Expressed in CHO cells, the variant indicates a normal phenotype by western blot and normal C3b/C4b binding by ELISA (12).	Benign	19373492, 20652818, 22171659, 23508668, 26613026, 20974643, 28980667, 19373492, 23431077, 29500241, 16621965
5	*CFB* (c.1697A>C)	Missense	Tolerated (0.50)	Neutral (-1.21)	Benign (0.00)	Benign (2.85)	Likely benign (2016)	-	Likely benign	23847193, 28056875, 25400666, 28210841, 25400666, 25532781, 24009284
6	*CFH* (c.-307C>T)	Promoter	N/A	N/A	N/A	N/A	-	-	Likely benign	-
7	*CFH* (c.2634C>T)	Synonymous	N/A	N/A	N/A	N/A	Likely benign (2016)	-	Likely benign	30377230, 12960213
8	*CFH* (c.3133+8G>T)	Splice donor site	N/A	N/A	N/A	Table S4	Likely benign (2016)	-	Likely benign	-
9	*CFH* (c.3148A>T)	Missense	Damaging (0.04)	Deleterious (-3.10)	Probably damaging (0.84)	Benign (13.58)	Likely benign (2016)		Likely benign	28056875, 15661753, 20059470, 18268093, 22171659, 16299065, 12960213, 11170896, 30377230, 29515306, 21215749, 29142942

10	*CFH* (c.3450A>G)	Missense	Damaging (0.005)	Neutral (-2.37)	Probably damaging (0.975)	Damaging (16.50)	-	-	VUS	-
11	*CFHR5* (c.485_486dupAA)	Frameshift	N/A	N/A	N/A	N/A	VUS (2016)	-	VUS	22503529, 25260719, 17000000, 28522344
12	*CFI* (c.982G>A)	Missense	Damaging (0.00)	Damaging (-7.23)	Probably damaging (1.0)	Damaging (25.00)	-	-	VUS	25788521
13	*CFI* (c.1322A>G)	Missense	Tolerated (0.32)	Neutral (-0.73)	Benign (0.007)	Benign (0.12)	Likely benign (2016)	-	Benign	30377230, 29329521, 28941939, 29500241, 21445332, 22903728
14	*CFI* (c.1534+5G>T)	Splice donor site	N/A	N/A	N/A	Table S4	Likely benign (2016)	-	Likely benign	28509134, 16621965, 28750931, 30377230, 20513133, 22250080, 17599974, 17089378, 27268256, 29500241
15	*CFI* (c.1547G>T)	Missense	Damaging (0.00)	Deleterious (-8.47)	Probably damaging (0.96)	Damaging (28.2)	-	-	VUS	29566171
16	*DGKE* (c.35C>T)	Missense	Damaging (0.04)	Neutral (-0.76)	Benign (0.09)	Damaging (22.4)	Likely benign (2016)	-	Likely benign	29500241 [§]
17	*PLG* (c.185+4T>C)	Intronic	N/A	N/A	N/A	Table S4	-	-	Benign	-
18	*PLG* (c.266G>A)	Missense	Tolerated (0.59)	Neutral (-0.52)	Benign (0.173)	Damaging (16.92)	-	-	Likely benign	29500241
19	*PLG* (c.1567C>T)	Missense	Tolerated (0.09)	Neutral (-2.13)	Benign (0.056)	Damaging (16.75)	-	-	Likely benign	25208887, 30131343, 29500241
20	*PLG* (c.2356C>T)	Missense	Tolerated (0.17)	Deleterious (-3.07)	Benign (0.058)	Damaging (19.00)	-	-	VUS	-

[†] Interpretations provided by e.g. Illumina Clinical Services Laboratory, adding *supporting evidence* of benign respectively pathogenic impact.

[‡] One report outside the NCBI search engine identified. ISTH Academy. Pongphitcha P. Reported Presentation, Treatment, Mutation Analysis and Outcome of Two Children with Atypical Hemolytic Uremic Syndrome. Available at: https://academy.isth.org/isth/2019/melbourne/264582/pongpak.pongphitcha.reported.presentation.treatment.mutation.analysis.and.html. [Last accessed: 2020 Sept 9]

[§] One report outside the NCBI search engine identified. *Rheumatology*, Volume 58, Issue Supplement 2, March 2019. Nikolai Bulanov et al. Atypical hemolytic uremic syndrome associated with diacylglycerol kinase-E mutation in a patient with microscopic polyangiitis: a possible relationship via complement activation? Available at: https://doi.org/10.1093/rheumatology/kez063.052. [Last accessed: 2020 Sept 9]

Table S5. Acute episode characteristics

Subject (#)	Sex (♂/♀)	Age (yr)	Prior HUS-like episode	Comorbidities	Clinical features	Related event[†]	DAT	Schisto-cytosis	Renal biopsy	Complement analyses[‡]	Hb min (g/L)	LD[§] max (µkat/L)	PLC min (x 10^9/L)	Crea max (µmol/L)	PEX / PI	Dialysis at discharge
HUS-like phenotype																
P	♀	20	No	No	Purpura	TTP episode[¶]	Neg	Yes	-	-	98	6.2	25	136	PEX	No
T	♀	17	No	No	Arthralgia, pyrexia	SLE nephritis	Pos	No	LN class V	C3 0.23 g/L C4 0.04 g/L	84	14	59	271	PEX	No
J	♀	26	No	No	Arthralgia, macroscopic hematuria	SLE nephritis	Pos	No	LN class IV	C3 0.15 g/L C4 0.19 g/L	93	8.6	34	223	PEX	No
M	♂	59	No	Abdominal abscesses r/t colon disease	Abdominal pain, pyrexia	Neutropenic sepsis w/ MOF	-	No	-	-	75	68.7	25	329	-	No
Non-complement-mediated aHUS																
N	♀		No	Chronic nephropathy r/t DM I	Abdominal pain, headache, nausea	Malignant hypertension	Neg	Yes	-	-	58	9.1	64	750	PEX	Yes (PD)
C	♂	9	No	T-ALL	Malaise	HSCT (discontinued immuno-suppression)	-	Yes	-	-	76	3.9	15	67[#]	-	No
O	♂	25	No	No	Fatigue, nausea	IgA nephropathy	Neg	Yes	IgAN	C3 0.72 g/L C4 0.26 g/L C3d 10.1 mg/L CFH 115% CFB 128% CFI 106%	62	10.5	98	1270	PEX	Yes (IHD)
F	♂	17	No	No	Pyrexia, jaundice, sore throat	Streptococcal sepsis w/ MOF	-	Yes	-	-	105	5.3	36	292	-	No
G	♀	31	No	Pregnancy (gestation week 37)	Ague, pyrexia	Streptococcal sepsis w/ MOF	Neg	Yes	-	-	67	15	9	661	-	Yes (IHD)
Q	♂	71	No	Postrenal AKI, relieved by JJ stent	Ague, pyrexia	Enterococcal sepsis w/ MOF	Neg	Yes	-	-	68	60	15	597	PEX	Yes (IHD)
R	♂	54	No	Pancreatic cancer, PCV	Fatigue	Gemcitabine, IFN-α	Neg	Yes	TMA	C3 0.95 g/L C4 0.28 g/L	86	17.9	15	344	-	Yes (IHD)
S	♀	38	No	Pregnancy (gestation week 34)	Fatigue, headache	HELLP syndrome	Neg	Yes	-	-	72	19.7	53	338	-	No

K	♀	78	No	No	Macroscopic hematuria, malaise	GPA	Neg	Yes	-	-	74	14	33	216	PEX	No
L	♀	55	No	Orbital pseudotumor (azathioprine, prednisone)	Dyspnea, fatigue, pyrexia	Systemic CMV infection	Neg	Yes	-	-	85	12.6	24	127	PI	No
Complement-mediated aHUS																
A	♀	39	No	No	Emesis, pyrexia, macroscopic hematuria, muscle pain, non-bloody diarrhea	Influenza A virus	Neg	Yes	-	C3 0.51 g/L C4 0.32 g/L	76	7.3	7	342	PEX	No
B	♂	45	No	No	Pyrexia, non-bloody diarrhea	GI tract infection (traveler's diarrhea)	Neg	Yes	TMA	-	70	9.3	126	1994	-	Yes (IHD)
D	♀	46	Yes (yr 1997)	No	Abdominal pain, emesis, macroscopic hematuria	-	Neg	Yes	-	-	81	39	44	506	PEX	No
E	♂	31	No	No	Abdominal pain, chest pain, headache	-	Neg	Yes	TMA	C3 0.80 g/L C4 0.30 g/L C3d 8.3 mg/L	54	35	60	860	PEX	Yes (IHD)
H	♀	46	No	No	Abdominal pain, emesis, malaise	-	Neg	No	TMA	C3 0,17 g/L C4 0.18 g/L C3d 12.2 mg/L	68	22	78	992	PI	Yes (IHD)
I	♂	54	No	No	Abdominal pain, confusion, headache, macroscopic hematuria, nausea	Malignant hypertension	Neg	Yes	-	-	80	20.6	101	1344	PEX	Yes (IHD)

[†] Triggering or causative of the clinical presentation.

[‡] Analyzed during the inpatient clinical management.

[§] All subjects also had hypohaptoglobinemia and/or reticulocytosis and/or unconjugated hyperbilirubinemia.

[¶] The ADAMTS13 activity was 8% according to results obtained during inpatient care. Subsequently repeatedly tested <5% and associated with elevated ADAMTS13-specific antibodies.

[#] Pediatric reference range (7-11 years): 28 - 57 µmol/L.

aHUS: atypical hemolytic uremic syndrome; AKI: acute kidney injury; CMV: cytomegalovirus; DAT: direct antiglobulin test; DM I: type 1 diabetes mellitus; GI: gastrointestinal; GPA: granulomatosis with polyangiitis; HELLP: hemolysis, elevated liver enzymes, and a low platelet count; HSCT: hematopoietic stem cell transplantation; IFN-α: interferon alpha; IgAN: IgA nephritis; IHD: intermittent hemodialysis; LN: lupus nephritis; MOF: multiple organ failure; PCV: polycythemia vera; PD: peritoneal dialysis; PEX/PI: plasma exchange/plasma infusion; SLE: systemic lupus erythematosus; T-ALL: T-cell acute lymphoblastic leukemia; TMA: thrombotic microangiopathy; TTP: thrombotic thrombocytopenic purpura.

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Biomarkers of complement and platelet activation are not correlated with the one or twenty-four hours corrected count increments in prophylactically platelet transfused hematological patients: a prospective cohort study

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Abstract

Platelet transfusion refractoriness is a serious clinical concern that complicates the management of thrombocytopenic patients. Previous studies have suggested a potential role for both complement and platelet activation based on *in vitro* analyses of platelet concentrates. In this study, the post-transfusion platelet response, as indicated by the corrected count increment at 1 and 24 h after prophylactic platelet transfusions, respectively, was correlated with the 1 h post-transfusion Δconcentration (1 h post-transfusion – pretransfusion) of complement and platelet activation biomarkers. The study was registered as a clinical trial at ClinicalTrials.gov (identifier: NCT02601131) and patients were recruited during inpatient care in the hematological department. Soluble terminal complement complexes, soluble P-selectin and soluble CD40 ligand were analyzed. Confirmed alloimmunized patients were excluded. Included subjects were either given platelet transfusions (n = 43) and categorized into four clinical study groups or included in a non-transfused control group (n = 10). In total, 54 transfusions were included. No transfusion-mediated complement activation was observed. The transfusions were associated with a significant increase in the concentration of soluble P-selectin ($p < .001$), primarily corresponding to the passive infusion of soluble P-selectin-containing plasma residuals. The Δconcentration of soluble P-selectin was, however, not significantly correlated with the corrected count increments. Thus, significant correlations between biomarkers of complement and platelet activation and the post-transfusion platelet response could not be demonstrated in this study.

MeSH KEYWORDS

Platelet transfusion, Complement activation, Platelet activation, Thrombocytopenia

History

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Introduction

Platelet transfusion refractoriness is a serious clinical concern that complicates the management of patients in need of platelets. It is associated with adverse clinical outcomes and is frequently related to nonimmune platelet consumption, e.g. secondary to infections, drugs, graft-versus-host disease or splenomegaly [1]. However, alloimmunization against human leukocyte antigens (HLA) and human platelet antigens (HPA) are important immunological causes reported in approximately one third of the cases [2]. In patients recently diagnosed with nonimmune platelet transfusion refractoriness (defined as absence of alloimmunization), it is difficult to single out one definite causative factor, particularly in patients currently not burdened with apparent risk factors and who previously have responded successfully to transfusions.

The risks and benefits of prophylactic platelet transfusions in patients with hypo-proliferative thrombocytopenia have previously been debated [3][4]. Outcomes in more recent randomized trials nevertheless support the use of prophylactic platelet transfusions in severely thrombocytopenic patients (platelet count [PLC] < 10 x 10^9/L) [5][6]. Routine prophylactic platelet transfusions are part of management to reduce the risk of bleeds in the guidelines for therapy-induced hypo-proliferative thrombocytopenia [7].

Platelet concentrate characteristics, such as storage time [8], apheresis *versus* pooled concentrates [9] and ABO incompatibility [10] have been demonstrated to affect the post-transfusion platelet response. Additionally, platelet [11] and complement activation [12] in the platelet concentrates are suggested to potentially affect the post-transfusion response negatively. However, there is a lack of studies assessing the *in vivo* effects. Former studies have reported ambiguous results regarding the effect of platelet activation on the post-transfusion responsiveness [13–15].

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While previous studies have shown increased complement activation in the presence of HLA-specific antibodies [16,17], no studies have yet prospectively investigated the correlation between complement activation and the post-transfusion response in non-alloimmunized patients.

Therefore, we designed this prospective cohort study in hematological patients with the aim to investigate biomarkers of complement and platelet activation *in vitro* (in the platelet concentrates) as well as *in vivo* at defined time points before and shortly after prophylactic platelet transfusions. We hypothesized that biomarkers of complement and/or platelet activation would be correlated with the post-transfusion platelet response as indicated by the corrected count increment (CCI) at 1 and 24 h after transfusion, respectively.

Methods

The present investigation included a predefined subset of a study previously published by some of the coauthors. The initial study reported a linear decline in the trend for the CCI in patients receiving prophylactic platelet transfusions during inpatient care in the hematological department at Skåne University Hospital in Lund, Sweden [18].

Written informed consent was obtained from all participating subjects prior to inclusion. The Regional Ethics Review Board in Lund approved the study (diary number: 2015/628) and it was a priori registered as a clinical trial at ClinicalTrials.gov (identifier: NCT02601131). The study was conducted in compliance with the Declaration of Helsinki and the manuscript was prepared according to the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guidelines for observational studies [19].

Patient Recruitment

Patients hospitalized in the hematological department between February 12th and September 28th 2016 were eligible for inclusion. Inclusion criteria were patient age $\geq$ 18 years, presence of a central venous catheter (with the exception of subjects recruited to study group 4, described below) and an identified need for prophylactic platelet transfusion during office hours. Exclusion criteria were the presence of clinically evident bleeds and coincidental conditions and treatments associated with a substantial demand for platelet substitution, including a diagnosis of acute promyelocytic leukemia and the administration of anti-thymocyte globulin or amphotericin B therapy < 3 days prior to transfusion. Additionally, the confirmed presence of HLA- or HPA-specific antibodies, thus requiring HLA- and HPA-matched transfusions, served as an exclusion criterion. The presence of HLA-specific antibodies was routinely investigated in all patients undergoing allogeneic hematopoietic stem-cell transplantation (HSCT). For the remaining patients, however, it was investigated at the discretion of the treating physician. The presence of HPA-specific antibodies was investigated at the discretion of the treating physician for all patients. Included subjects who demonstrated platelet transfusion refractoriness during the study were not excluded a posteriori.

Study Design

Eligible subjects were prospectively recruited into the study. Blood samples within 1 h before transfusion and at 1 and 8 h after transfusion, respectively, were arbitrary chosen to investigate the temporal development of the levels *in vivo* for each biomarker. The platelet count was measured within 1 h before transfusion, and at 1 and 24 h after transfusion, respectively, to account for immediate and delayed effects on the post-transfusion response.

To investigate potential differences between clinical categories of patients, the participating subjects were categorized into four transfused study groups and one non-transfused control group:

Group 1 (Chemo): hypo-proliferative thrombocytopenia due to induction or consolidation chemotherapy.

Group 2 (Auto-HSCT): hypo-proliferative thrombocytopenia due to autologous HSCT.

Group 3 (Allo-HSCT): hypo-proliferative thrombocytopenia due to allogeneic HSCT.

Group 4 (Intervention): scheduled insertion of a central venous catheter and a PLC < 50 x 10^9/L.

Controls: non-transfused hematological control subjects with a morning PLC of 10– 20 x 10^9/L, who were sampled in accordance with the study protocol to account for non-transfusion-related temporal variations in the levels of investigated biomarkers.

Further details and clinical characteristics for the study groups are provided in Table 1.

According to a local protocol, a morning PLC < 10 x 10^9/L (applicable for groups 1–3) or a PLC < 50 x 10^9/L prior to a scheduled minor intervention (applicable for group 4), served as a general trigger to consider a patient for prophylactic platelet transfusion. All platelet transfusions were administered at the discretion of the treating physician. Eligible subjects could be recruited into the study for a maximum of two transfusion occasions.

Corrected Count Increment

Post-transfusion platelet response is commonly evaluated using formulas assessing the increase in PLC at defined time points after transfusion, adjusted by the estimated number of transfused platelets and individual characteristics such as body surface area (BSA). One of the most established formulas are CCI [10,20].

In this study the CCI formula was applied for the ΔPLC of the 1 and 24 h post-transfusion time point, respectively (1 or 24 h post-transfusion – pretransfusion):

$$CCI = \frac{\Delta PLC\left(10^9 \cdot L^{-1}\right) \times BSA\left(m^2\right) \times 10^5}{platelets\ transfused\left(10^9\right)}$$

The BSA was calculated using the Mosteller formula [21]:

$$BSA(m^2) = \sqrt{\frac{height(cm) \times weight(kg)}{3600}}$$

In this study, platelet transfusion refractoriness was defined as a CCI < 5000 at 1 h post-transfusion [10].

Platelet Concentrates

The transfusions were either given as a single donor platelet concentrate obtained by apheresis technique or as a pooled platelet concentrate processed from four random donor whole blood components using the buffy-coat method [22]. The approximate total volume of apheresis and pooled platelet concentrates, including the platelet additive solution (T-PAS+; Terumo BCT Inc., Lakewood, CO, USA), was 250 mL and 300 mL, respectively, of which 80 mL and 120 mL were residual plasma.

The concentrates were tested for bacterial contamination by culturing 10 mL samples in an automatic blood culture system (BD BACTEC; Becton-Dickinson, Franklin Lakes, New Jersey, USA). The concentrates were released for clinical use based on

the negative-to-date principle, that is, at the time of issue, the concentrates were negative for bacterial growth. All concentrates were ≤ 7 days old, leukocyte-reduced and ABO-matched. Further methodological details are provided in the previous publication [18].

The PLC in each platelet concentrate was determined by extracting a minimal volume 1 h before transfusion. The approximate number of platelets transfused was calculated using the determined PLC and the volume of the specific concentrate. The remnants were centrifuged, aliquoted into plasma microtubes and stored at −80°C until analyzed.

Calculated Concentrations after Transfusion

To estimate the calculated post-transfusion *in vivo* concentration per biomarker caused by the passive infusion of plasma residuals, the following formulas were applied.

Estimated total blood volume (TBV) was calculated using Nadler's equation [23]:

$$TBV(L)_{male} = 0.3669 \times [height(m)]^3 + 0.03219 \times weht(kg) + 0.6041$$

Estimated plasma volume (PV) was calculated using the following equation:

$$PV(mL)_{concentrate} = \frac{volume(mL)_{concentrate} \times plasmafraction(\%)_{concentrate}}{100}$$

The approximate fraction of residual plasma (RP) in apheresis and pooled concentrates was 32% (80/250 mL) and 40% (120/300 mL), respectively. Therefore, the residual plasma volume per concentrate was estimated using the following formula:
Finally, to estimate the calculated concentration per biomarker after transfusion, the following formula was applied:

$$Calculated\ concentration\left(unit.mL^1\right)_{in\ vivo\ post-transfusion} = \frac{concentration\left(unit.mL^1\right)_{in\ vivo\ posttransfusion} \times PV(mL) + RP(mL)_{concentrate} \times concentration\left(unit.mL^{-1}\right)_{concentrate}}{PV(mL) + RP(mL)_{concentrate}}$$

Laboratory Investigations

Blood Sampling and Assessment of the Platelet Count

Venous blood was obtained from a central venous catheter using a vacutainer system (Becton-Dickinson) and sampled into 3 mL ethylenediaminetetraacetic acid vacuum tubes (#367 838; Becton-Dickinson). Prior to collection, the catheter was flushed with 0.9% saline and the first 10 mL of blood was discarded. For study group 4, the pretransfusion blood sample was obtained via peripheral venous cannulation. The PLC was determined according to accredited routine in the Department of Clinical Chemistry and Pharmacology, primarily by using the Sysmex XN-10 automated hematology system (Sysmex corp., Kobe, Japan). The remaining samples were centrifuged twice at 1.800 x g for 10 min at 5°C, aliquoted into plasma microtubes and stored at −80°C until analyzed.

Soluble Terminal Complement Complexes

The concentration of soluble terminal complement complexes (sTCC) was determined using an enzyme-linked immunosorbent assay (ELISA) protocol developed by Mollnes et al. [24]. A monoclonal C9 neoepitope-specific capture antibody [24] (clone aE11, #HM2167; Hycult Biotech, Uden, The Netherlands) was used together with an in-house biotinylated monoclonal C6-specific antibody [25] (#A219; Quidel, San Diego, CA, USA) followed by incubation with horseradish peroxidase-conjugated streptavidin (#DY998; R&D Systems, Minneapolis, MN, USA). The assay was developed with 1,2-phenylenediamine dihydrochloride tablets (Dako, Glostrup, Denmark) as substrate and the absorbance was measured at 490 nm using a Varian 50 MPR Microplate Reader (Varian Medical Systems, Palo Alto, CA, USA). The reproducibility of the assay has been established and the read-out was given in complement activation units (CAU) in accordance with the International Complement Standard #2 [26].

Soluble P-*selectin and Soluble CD40 Ligand*

The concentrations of soluble P-selectin (sP-selectin; ng/mL) and soluble CD40 ligand (sCD40L; pg/mL) were determined using commercially available quantitative sandwich ELISA kits: the Human P-Selectin/CD62P Quantikine® ELISA [27] and the Human CD40 Ligand/TNFSF5 Quantikine® ELISA [28] (R&D Systems), which were performed according to the manufacturer's instructions. Absorbances were measured at 450 nm with the correction wavelength set at 540 nm (Varian 50 MPR Microplate Reader; Varian Medical Systems). Reported reference range for sP-selectin was 18.3– 57.4 ng/mL. Reported reference range for sCD40L was not detected - 139 pg/mL, with an 18% detection rate among healthy volunteers.

Curve Fits

All ELISA samples were analyzed in duplicates and the concentrations were extrapolated relative to logarithmic standard curves applying four parameter curve fits in GraphPad Prism version 8.0.0 (GraphPad Software, San Diego, CA, USA). The final concentrations were determined as mean of duplicates.

Statistical Analyses

All continuous variables were non-normally distributed and descriptively presented as medians with lower and upper quartiles. Categorical variables were presented with counts and percentages. Statistical differences for binary variables were evaluated using Fisher's exact test. Statistical differences for continuous variables between two independent groups were evaluated using the Mann-Whitney U test. Statistical differences for dependent continuous variables were evaluated using the Friedman test followed by a post hoc Dunn's multiple comparisons test adjusted by the Bonferroni correction. Bland-Altman plots were used to visualize the agreement between the actual and calculated concentration per biomarker after transfusion. A multiple linear regression for all subjects (groups 1–4 and controls) was performed by the log10 transformation of the sP-selectin concentration before transfusion, using the white blood cell count (WBC) at transfusion day and the PLC before transfusion as independent variables. Spearman's rank-order correlation test was used to assess bivariate correlations between variables with non-normally distributed residuals. The p-values < .05 were considered significant. All p-values were two-tailed, and all analyses were performed using IBM SPSS Statistics version 26.0 (IBM, Armonk, NY, USA). Sample size estimations were conducted with respect to the PLC increments reported in the initial study [18]. Consequently, no sample size estimations were determined to detect statistical differences for the biomarkers reported in the present study. For this reason, investigations were primarily assessed for general temporal differences and for differences

between principal categories of groups: groups 1–4 (transfused) *versus* controls (non-transfused) and groups 1–3 (hypo-proliferative; WBC mainly < 0.5 x 10^9/L) *versus* group 4 (non-hypo-proliferative; WBC ≥ 0.5 x 10^9/L).

Results

Forty-three individual subjects were enrolled of which 11 subjects participated twice during the study period. In total, the study encompassed 54 platelet transfusions of which 89% (n = 48/54) were pooled concentrates. The total number of transfusions were administered among the study groups as follows: group 1 (Chemo) = 19, group 2 (Auto-HSCT) = 7, group 3 (Allo-HSCT) = 16 and group 4 (Intervention) = 12. Additionally, 10 non-transfused hematological control subjects were enrolled (Table 1).

Association between Biomarkers and the Corrected Count Increment

To evaluate correlations between CCI and levels of sTCC, sP-selectin and sCD40L, respectively, biomarkers were assessed in the following settings for groups 1–4 combined: 1) *in vitro* in the transfused concentrates (potentially reflecting lost viability during storage), 2) *in vivo* before transfusion (potentially reflecting pre-transfusion activation as an immanent propensity of an insufficient platelet response) and 3) *in vivo* after completed transfusion to determine the 1 h post-transfusion Δconcentration (1 h post-transfusion – pretransfusion; potentially reflecting transfusion-mediated activation). First, the *in vitro* concentration of sTCC, sP-selectin and sCD40L, respectively, did not reveal any significant correlation with the CCI(1 h). Secondly, neither the concentrations of sTCC, sP-selectin or sCD40L before transfusion nor the corresponding 1 h post-transfusion Δconcentrations showed any significant correlations with the CCI(1 h) (Figure 1(a-c)). Thirdly, the corresponding correlations tested for CCI(24 h) were also without significant results. Additionally, subgroup analyses only including cases with actual concentrations at 1 h after transfusion > calculated post-transfusion concentrations (see subsequent section below), for sTCC (n = 35) and sP-selectin (n = 17), did not reveal any significant correlations with the CCI(1 h) or CCI (24 h). Finally, subjects with CCI(1 h) < 5000 (n = 5), that is, by definition transfusion refractory, were compared to subjects with CCI(1 h) ≥ 5000 without significant differences observed for levels of biomarkers in the settings examined above.

Levels of Biomarkers after Transfusion

To evaluate the relation between post-transfusion increments observed for the analyzed biomarkers *in vivo* and the potential contribution of passively infused molecules originating from the platelet concentrates, the following analyses, comprising groups 1–4 combined, were performed. First, assessment of the temporal developments of the *in vivo* concentrations revealed a significant trend for both sTCC ($p < .001$; Figure 2(a)) and sP-selectin ($p < .001$; Figure 2(b)). The trend for sCD40L was not significant (Figure 2(c)). Secondly, the actual concentrations at 1 h after transfusion were compared to the calculated post-transfusion concentrations. Bland-Altman plots for sTCC and sP-selectin revealed good agreement without bias (Figure 2(a-b)). Neither the comparison between the actual and calculated concentration of sTCC (2.4 [1.2, 2.9] *versus* 2.2 [1.3, 3.0] CAU/mL; $p = .65$), nor the corresponding comparison for sP-selectin (15.55 [9.5, 24.7] *versus* 16.8 [14.5, 26.1] ng/mL; $p = .10$) revealed any significant difference. For sCD40L, the Bland-Altman plot revealed an obvious bias for the difference between the actual and calculated concentration as the 95% limit of agreement only contained negative values (−369 to −53 pg/mL; Figure 2(c)). The calculated concentration of sCD40L exhibited significantly higher values compared to the actual concentration at 1 h after transfusion (232 [195, 271] *versus* 42 [10, 79] pg/mL; $p < .001$).

Clinical Characteristics of Study Group Categories

To evaluate clinical differences between categories of study groups (groups 1–4 *versus* controls and groups 1–3 *versus* group 4), the following comparisons were made. First, significant differences for general characteristics (age, sex and spleen status) were neither observed between groups 1–4 and controls nor between groups 1–3 and group 4. Secondly, there were no significant or unexpected differences between the compared categories of groups regarding body temperature or routine laboratory investigations assessed at transfusion day. Thirdly, the prevalence of respective concentrate type (apheresis *versus* pooled) as well as the storage time of concentrates, were not different comparing groups 1–3 to group 4. Finally, no significant difference was observed for the CCI(1 h) or CCI(24 h) comparing groups 1–3 to group 4 (Table 1).

Levels of Biomarkers according to Study Group Categories

To evaluate *in vivo* differences for the analyzed biomarkers between categories of study groups, both before transfusion and at 1 and 8 h after transfusion, respectively, the following comparisons were made. First, the concentration of sTCC before transfusion was not different comparing groups 1–4 to controls, but significantly higher ($p = .04$) in group 4 (2.5 [2.2, 5.7] CAU/mL) compared to groups 1–3 (1.9 [0.9, 2.5] CAU/mL). The 1 h post-transfusion Δconcentration of sTCC was not significantly different comparing groups 1–4 to controls or groups 1–3 to group 4 (Figure 3(a)). The corresponding comparisons of Δconcentration tested at 8 h after transfusion were also without significant differences. Secondly, the concentration of sP-selectin before transfusion was not different comparing groups 1–4 to controls, but significantly higher ($p = .02$) in group 4 (18.4 [6.8, 34.9] ng/mL) compared to groups 1–3 (7.7 [5.2, 13.3] ng/mL). The 1 h post-transfusion Δconcentration of sP-selectin was significantly higher ($p < .001$) in groups 1–4 (5.3 [3.1, 9.6] ng/mL) compared to controls (0.4 [−2.3, 0.6] ng/mL), but not different comparing groups 1–3 to group 4 (Figure 3(b)). Tested at 8 h after transfusion, the Δconcentration of sP-selectin was somewhat higher ($p = .08$) in groups 1–4 (3.6 [3.1, 7.4] ng/mL) compared to controls (0.55 [−4, 4.3] ng/mL) but not different comparing groups 1–3 to group 4. Finally, no significant differences between the compared categories of groups were observed for sCD40L, neither regarding the concentration before transfusion nor the 1 h post-transfusion Δconcentration (Figure 3(c)). The corresponding comparisons of Δconcentration tested at 8 h after transfusion were also without significant differences.

Post Hoc Analysis of the Soluble P-*selectin Concentration before Transfusion*

To evaluate the effect of the WBC and the PLC before transfusion on the *in vivo* concentration of sP-selectin before transfusion, a multiple linear regression for all subjects was performed. The analysis indicated a positive effect of the PLC ($p = .01$), however, the contribution of the WBC was not significant ($p = .52$).

Table 1. Clinical characteristics.

Variables	Study group (no. of subjects)					
	Chemo (n = 13)	**Auto-HSCT (n = 7)**	**Allo-HSCT (n = 13)**	**Intervention (n = 10)**	**Controls (n = 10)**	***p*-value**
Age, years - median (q1, q3)	64 (35, 69)	49 (36, 54)	45 (26, 55)	72 (39, 76)	53 (36, 59)	.96[a]/.24[b]
Sex, female - no. (%)	5 (38)	2 (29)	2 (15)	2 (20)	2 (20)	.71[a]/.73[b]
Spleen status, enlarged - no. (%)	2 (15)	1 (14)	2 (15)	3 (30)	2 (20)	1.0[a]/.20[b]
Primary diagnosis - no. (%)						
AML	9 (69)	0	5 (38)	5 (50)	1 (10)	N.A.
ALL	1 (8)	0	2 (15)	3 (30)	0	N.A.
MPAL	1 (8)	0	1 (8)	0	0	N.A.
MDS	2 (15)	0	0	0	1 (10)	N.A.
MM	0	4 (57)	0	0	3 (30)	N.A.
Other diagnosis	0	3 (43)	5 (38)	2 (20)	5 (50)	N.A.
Transfusion occasions - no (% of total n)	19 (35)	7 (13)	16 (30)	12 (22)	N.A.	N.A.
Conc. volume, mL - median (q1, q3)	303 (291, 310)	301 (298, 310)	308 (297, 316)	311 (299, 318)	N.A.	.19[b]
PLTs per conc., x 10^9 - median (q1, q3)	242 (199, 274)	214 (164, 246)	252 (211, 269)	267 (229, 297)	N.A.	.10[b]
Conc. storage time, days - median (q1, q3)	6 (4, 7)	6 (6, 7)	6 (4, 7)	5 (3, 7)	N.A.	.18[b]
Conc. type, apheresis[c] - no. (%)	2 (11)	0	3 (19)	1 (8)	N.A.	1.0[b]
Pretransfusion temperature, °C - median (q1, q3)	37.0 (36.7, 37.5)	37.2 (36.6, 37.7)	37.2 (36.8, 37.7)	37.0 (36.6, 37.5)	37.0 (36.7, 37.4)	.52[a]/.61[b]
Pretransfusion lab. tests - median (q1, q3)						
Hb, g/L	96 (87, 104)	102 (94, 104)	93 (89, 96)	86 (79, 95)	91 (87, 106)	.86[a]/**.01**[b]
WBC, x 10^9/L	0.3 (0.1, 0.6)	0.1 (0.1, 0.1)	0.1 (0.1, 0.2)	0.9 (0.6, 4.3)	0.6 (0.2, 1.9)	.08[a]/**<.001**[b]
CRP, mg/L	36 (11, 80)	142 (61, 260)	34 (10, 109)	33 (7, 109)	22 (4, 48)	.11[a]/.47[b]
eGFR, mL/min	90 (75, 102)	107 (91, 113)	110 (98, 118)	82 (55, 92)	93 (87, 103)	.99[a]/**.01**[b]
PLC, x 10^9/L - median (q1, q3)						
Before transfusion	7 (3, 8)	6 (5, 8)	8 (5, 10)	17 (13, 26)	21 (15, 24)	**.001**[a]/**<.001**[b]
1 h post-transfusion	20 (15, 27)	15 (13, 18)	21 (12, 25)	36 (24, 51)	22 (16, 25)	N.A.
24 h post-transfusion	13 (8, 18)	12 (11, 15)	13 (6, 22)	23 (14, 35)	33 (14, 46)	N.A.
ΔPLC, 1 h – pretransfusion	14 (9, 21)	8 (7, 11)	12 (7, 15)	15 (12, 26)	1 (−2, 3)	**<.001**[a]
ΔPLC, 24 h – pretransfusion	7 (5, 11)	5 (3, 8)	5 (1, 12)	5 (1, 13)	11 (−1, 21)	.51[a]
CCI - median (q1, q3)						
1 h post-transfusion	10 200 (7800, 16 000)	8100 (7100, 10 400)	9600 (4900, 11 700)	10 900 (7800, 17 800)	N.A.	.27[b]
24 h post-transfusion	5600 (3100, 10 000)	4400 (2400, 6600)	4000 (500, 8600)	3300 (800, 8200)	N.A.	.37[b]

In total, 54 platelet transfusions were administered to 43 individual subjects. Statistically significant differences for routine laboratory tests were either expected or clinically negligible. *p*-values were calculated using Fisher's exact test or the Mann-Whitney U test.
(1) Chemo: subjects diagnosed with AML, ALL, MPAL or MDS who had developed hypo-proliferative thrombocytopenia due to induction or consolidation chemotherapy during the current admission. **(2) Auto-HSCT**: subjects diagnosed with MM, CNS lymphoma or MS who had developed hypo-proliferative thrombocytopenia due to autologous HSCT during the current admission. **(3) Allo-HSCT**: subjects diagnosed with AML, ALL, MPAL, myelofibrosis or sideroblastic anemia who had developed hypo-proliferative thrombocytopenia due to allogeneic HSCT) during the current admission. **(4) Intervention**: subjects diagnosed with AML, ALL or other hematologic conditions who were scheduled for insertion of a central venous catheter and who had a pre-intervention PLC < 50 x 10^9/L. **Controls**: non-transfused patients diagnosed with AML, MDS, MM or other hematological conditions, with a morning PLC within the approximate range of 10– 20 x 10^9/L, who were sampled in accordance with the study protocol to account for non-transfusion-related temporal variations in the levels of investigated biomarkers.
[a] Groups 1–4 (transfused) *versus* controls (non-transfused).
[b] Groups 1–3 (hypo-proliferative) *versus* group 4 (non-hypo-proliferative).
[c] Apheresis *versus* pooled platelet concentrate.
ALL: acute lymphoblastic leukemia; AML: acute myeloid leukemia; CCI: corrected count increment; CNS: central nervous system; Conc.: concentrate; CRP: C-reactive protein; eGFR: estimated glomerular filtration rate; Hb: hemoglobin; HSCT: hematopoietic stem-cell transplantation; MDS: myelodysplastic syndrome; MM: multiple myeloma; MPAL: mixed phenotype acute leukemia; MS: multiple sclerosis; N.A.: not applicable; PLC: platelet count; PLT: platelet; sCD40L: soluble CD40 ligand; sTCC: soluble terminal complement complex; WBC: white blood cells.

Discussion

In this prospective cohort study, it was investigated whether elevated biomarkers reflecting complement and platelet activation, respectively, were associated with the post-transfusion response in patients with hematological conditions. Previous studies have mainly relied on *in vitro* investigations of platelet concentrates. To the best of our knowledge, potential *in vivo* effects of complement and platelet activation, respectively,

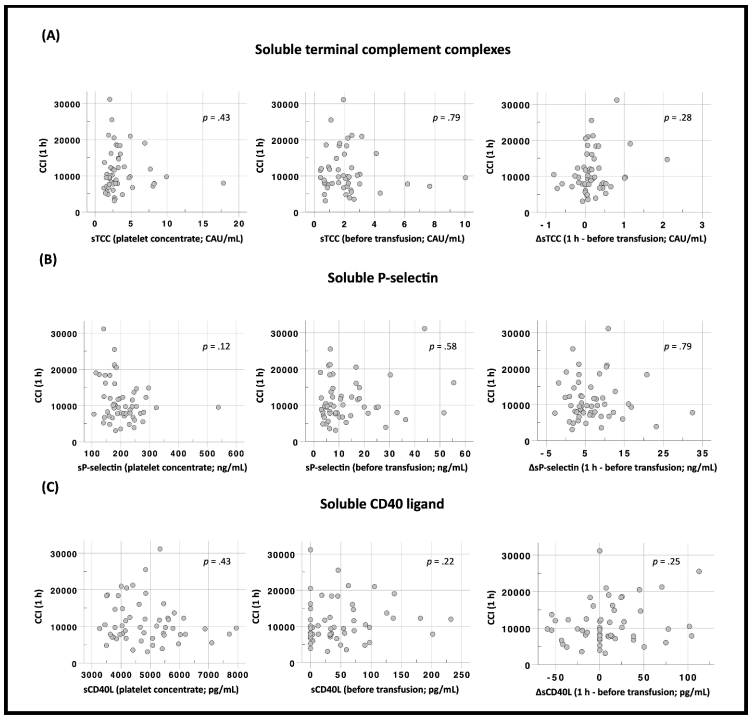

Figure 1. Data encompassed all 54 transfusion occasions. Scatter plots to the left represent correlations between the concentrations in the platelet concentrates (*in vitro*) and the corrected count increment (CCI) at 1 h after transfusion. Scatter plots in the center represent correlations between the *in vivo* concentrations before transfusion and the CCI(1 h). Scatter plots to the right represent correlations between the *in vivo* Δconcentrations at 1 h after transfusion (1 h post-transfusion – pretransfusion) and the CCI(1 h). Spearman's rank-order correlation test was used to assess statistical correlations.

investigated for sequential time points after transfusion, have never been studied before.

In the present study, complement activation was determined by levels of sTCC, the non-membrane-bound fraction of formed TCC. The TCC, also referred to as the membrane attack complex or C5b-9, is the final product of the terminal complement pathway, thus reflecting overall complement activity regardless of the initiating pathway involved [29]. Platelet activation was determined by levels of sP-selectin and sCD40L. Soluble P-selectin is the proteolytically cleaved byproduct of membrane-bound P-selectin, a thrombo-inflammatory glycoprotein stored in a preformed state in granules of both platelets and endothelial cells. Upon cell activation, P-selectin translocates to the cell membrane where it eventually is shed due to cell interactions [30]. Soluble CD40 ligand is the byproduct of membrane-bound CD40L, a glycoprotein primarily expressed in T-cells and platelets, which similarly is released upon cell activation. Platelet-derived sCD40L has shown thrombo-inflammatory capabilities and has previously been associated with acute transfusion reactions [31].

We aimed to determine whether the post-transfusion response was correlated with any of three scenarios possibly associated with complement and/or platelet activation: 1) lost viability of platelet concentrates due to activation *in vitro*, 2) ongoing *in vivo* activation before transfusion and 3) transfusion-mediated *in vivo* activation. Neither scenario was significantly correlated with the CCI at 1 or 24 h post-transfusion. Hence, the hypothesis that complement and/or platelet activation would be correlated with the post-transfusion response could not be confirmed.

Previous studies have suggested a loss of *in vivo* viability for activated platelets in stored concentrates [13]. Herein, we report no significant correlations between the CCI at 1 or 24 h after transfusion and levels of sP-selectin and sCD40L in the platelet concentrates, respectively. Thus, indications of presumably pre-activated platelets did not appear to correlate with the CCI. Other previous studies have indeed reported that degranulated platelets, losing their membrane-bound P-selectin, continue to circulate and function normally [14]. Additionally, the interaction of activated platelets with leukocytes via P-selectin has been reported not to result in increased clearance [32]. Thus, our results support these findings.

Significant post-transfusion trends were observed for the levels of sTCC and sP-selectin. However, comparisons between actual and calculated concentrations after transfusion were not significant, suggesting that the trends, rather than being caused by transfusion-mediated activation, primarily were a result of the passive infusion of plasma residuals. Additionally, the Δconcentration of sTCC was not different in transfused compared to non-transfused subjects, implying that the trend observed with sTCC was clinically negligible. Interestingly, no significant trend

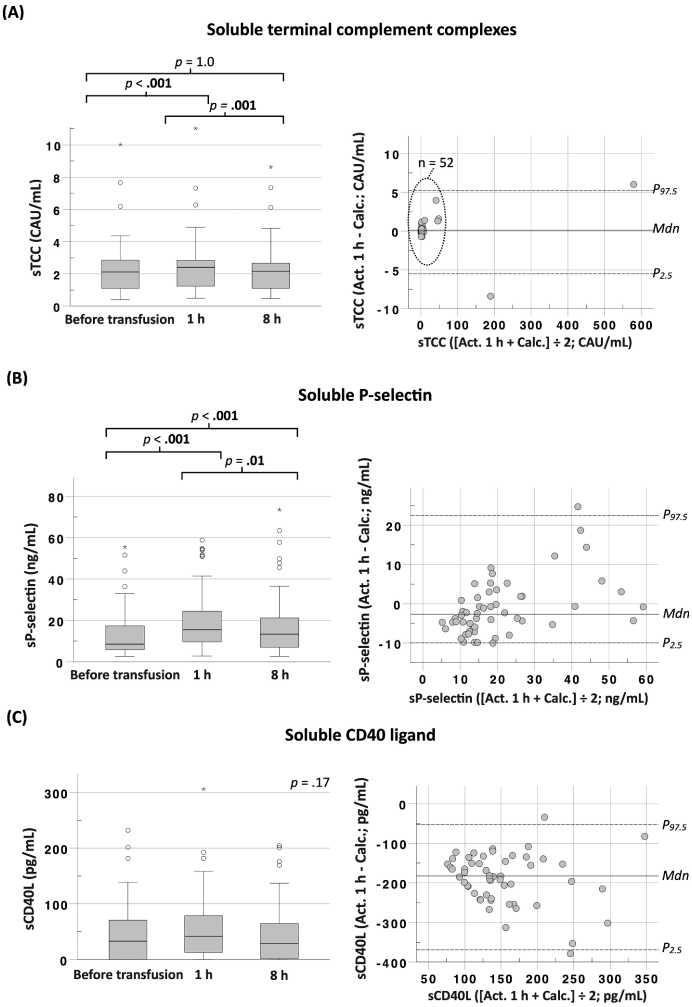

Figure 2. Data encompassed all 54 transfusion occasions. Box plots represent the temporal developments of the *in vivo* concentrations measured before transfusion and at 1 and 8 h after transfusion, respectively. The Friedman test followed by Dunn's multiple comparisons test was used to assess statistical differences. Bland-Altman plots were used to visualize the 95% limit of agreement (LoA) for the difference between actual concentrations measured at 1 h after transfusion and the calculated post-transfusion concentrations. **(A) Soluble terminal complement complexes**. A significant difference ($p < .001$) for the trend of the temporal concentrations was observed between the mean ranks of at least one pair of time points. A significant increment was revealed between before transfusion (2.1 [1.1, 2.9] CAU/mL) and 1 h after transfusion (2.4 [1.2, 2.9] CAU/mL) and a significant decrement was revealed between 1 and 8 h (2.2 [1.1, 2.8] CAU/mL) after transfusion, respectively. The Bland-Altman plot indicated a median difference of 0.10 (−0.04, 0.29) CAU/mL between the actual and calculated concentration. The 95% LoA ranged from −5.5 to 5.2 CAU/mL. **(B) Soluble *P*-selectin**. A significant difference ($p < .001$) for the trend of the temporal concentrations was observed between the mean ranks of at least one pair of time points. A significant increment was revealed between before transfusion (8.6 [5.8, 17.6] ng/mL) and 1 h after transfusion (15.6 [9.5, 24.7] ng/mL) and between before transfusion and 8 h after transfusion (13.4 [6.9, 21.8] ng/mL). A significant decrement was revealed between 1 and 8 h after transfusion, respectively. The Bland-Altman plot indicated a median difference of −2.7 (−6.0, 2.2) ng/mL between the actual and calculated concentration. The 95% LoA ranged from −10.0 to 22.5 ng/mL. **(C) Soluble CD40 ligand**. A non-significant difference was observed for the trend of

was observed with sCD40L. Additionally, the Bland-Altman plot revealed a bias toward significantly higher calculated than actual levels of sCD40L after transfusion. This may reflect the skewed condition that the comparison was made between calculated levels theoretically achieved after perfect plasma distribution and the actual levels measured at 1 hour post-transfusion. The finding implies that sCD40L was cleared from plasma at a faster rate than sP-selectin, potentially due to interactions with the endothelium [33] or more efficient *in vivo* metabolization. Indeed, a previous study by Larsen et al. [34] also reported a high discrepancy between calculated and actual levels of sCD40L after transfusion. Whether infused sCD40L interacts with the endothelial lining and

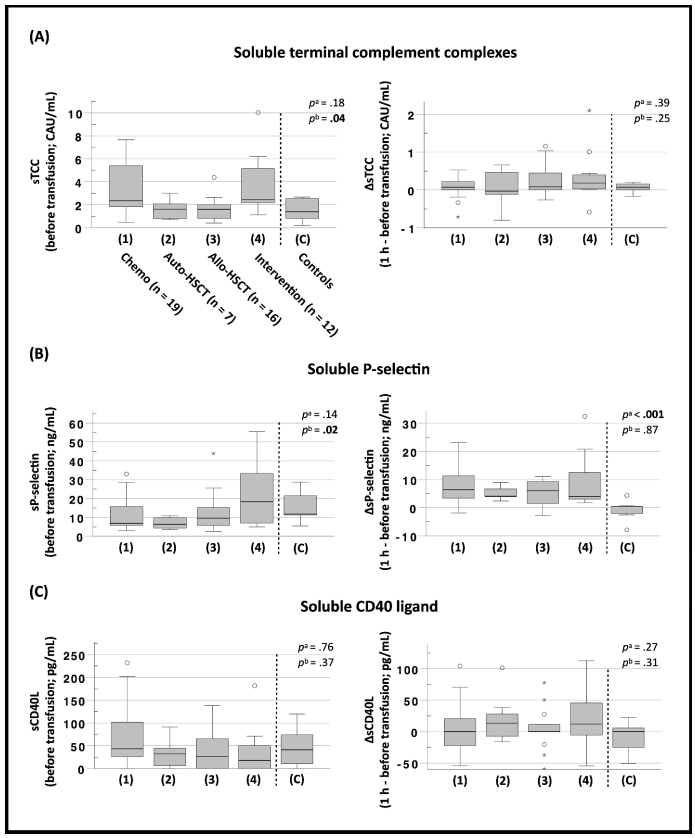

Figure 3. Data encompassed all 54 transfusion occasions and are presented according to study groups. Box plots to the left represent comparisons of the *in vivo* concentrations before transfusion. Boxplots to the right represent comparisons of the *in vivo* Δconcentrations at 1 h after transfusion (1 h post-transfusion – pretransfusion). The dashed lines separate the control subjects (C) from the remaining cohort. Controls (n = 10) were non-transfused hematological patients with a morning platelet count of 10– 20 x 10^9/L, who were sampled in accordance with the study protocol to account for non-transfusion-related temporal variations in the levels of investigated biomarkers. The Mann-Whitney U test was used to assess statistical differences between categories of groups. p^a: groups 1–4 (transfused) *versus* controls (non-transfused).p^b: groups 1–3 (hypo-proliferative) *versus* group 4 (non-hypo-proliferative).

(1) Chemo: hypo-proliferative thrombocytopenia due to induction or consolidation chemotherapy. (2) Auto-HSCT: hypo-proliferative thrombocytopenia due to autologous hematopoietic stem-cell transplantation (HSCT). (3) Allo-HSCT: hypo-proliferative thrombocytopenia due to allogeneic HSCT. (4) Intervention: scheduled insertion of a central venous catheter and a platelet count < 50 x 10^9/L.

what potential implications it may have, remains to be investigated.

Prior to transfusion, the concentration of sP-selectin was significantly higher in group 4 compared to groups 1–3. The corresponding Δconcentrations were, however, not significantly different. Two characteristics distinguished group 4 from groups 1–3. First, group 4 was not sampled through a central venous catheter before transfusion, but blood was collected via peripheral venous cannulation. Small-bore cannulas induce higher shear stress [35], potentially resulting in platelet activation and therefore increased shedding *in vitro*. As opposed to this suggestion, however, Milburn et al. reported no difference in the concentration of sP-selectin between blood samples that were simultaneously collected from vascular accesses and peripheral veins in hemodialysis patients [36]. Secondly, group 4 had a significantly higher WBC and PLC before transfusion compared to groups 1–3. Previous studies have reported that mobilized P-selectin molecules support platelet-leukocyte interactions via binding of P-selectin glycoprotein ligand-1 (PSGL-1), a glycoprotein predominantly present on the surface of neutrophils, monocytes and eosinophils [37][38]. The interaction with PSGL-1 secondarily leads to the proteolytic shedding of the P-selectin ectodomain, thus resulting in increased levels of sP-selectin [38]. The effect of the WBC on the concentration of sP-selectin in leukopenic patients is unknown. Therefore, we performed a post hoc multiple linear regression, showing that levels of sP-selectin were unrelated to the WBC, however, significantly associated with the number of platelets. This finding explains why the concentration of sP-selectin was higher among subjects in group 4, who exhibited a significantly higher PLC compared to groups 1–3 before

transfusion. Additionally, it explains why the concentration of sP-selectin among the participating subjects in general, was below the lower normal limit of healthy controls.

The present study was limited by several factors. First, the presence of HLA- and HPA-specific antibodies was not consistently investigated among the participating subjects. Although repeated platelet transfusion refractoriness likely served as a clinical indication for further investigation, we cannot exclude that unconfirmed alloimmunized subjects were included in the study. However, we argue that the presence of such cases potentially would have strengthened any correlation between the post-transfusion response and complement and platelet activation, respectively. Previous studies have reported increased activation of both platelets [39] and complement [17] in association with alloimmunization. The development of platelet transfusion refractoriness in alloimmunized patients, however, requires transfusions with platelets expressing the relevant antigens. Although only a fraction of alloimmunized patients actually develops transfusion refractoriness [39], these patients would likely manifest with a poor CCI while simultaneously exhibiting increased levels of respective type of biomarker. Therefore, this limitation would have been more troubling in the presence of significant results. Secondly, the benefits of prophylactic platelet transfusions are not solely reflected by the observed numerical CCI. A previous study reported reduced risk of bleeds in prophylactically platelet-transfused patients despite having a post-transfusion PLC similar to the platelet-poor plasma-transfused control arm [4]. This suggests the contribution of undefined transfusion-mediated effects on cytokine reactions, cell-mediated interactions between platelets and endothelial cells or intravascular cascade systems, potentially also involving crosstalk with the complement cascade [40]. Our study was not designed to investigate such correlations as active bleeds served as an exclusion criterion. Thirdly, concentrate properties were heterogenous in terms of storage times and the type of concentrate administered (apheresis *versus* pooled). However, no significant difference regarding the median storage time or the ratio of respective type of concentrate was observed between the two categories of transfused groups (hypo-proliferative *versus* non-hypo-proliferative). Neither was it reflected by significantly different CCI(1 h) or CCI(24 h). However, the study suffered from the lack of power calculations. Consequently, it was potentially underpowered to discover true differences between the categories of study groups. Similarly, and more importantly, the non-significant general correlations (group 1–4 combined) observed between the investigated biomarkers and the CCI at 1 and 24 h post-transfusion, respectively, potentially suffered from type II errors. However, upon visual examination of the scatter plots, tendencies of true negative correlations cannot be proposed.

To conclude, in this study we showed that prophylactic platelet transfusions among hematological patients were associated with the passive infusion of negligible quantities of sTCC. No significant transfusion-mediated complement activation was observed. The transfusions were associated with a significant increase in the concentration of sP-selectin, primarily reflecting the passive infusion of sP-selectin-containing plasma residuals. However, the increase in levels of sP-selectin was not significantly correlated with the post-transfusion response as indicated by the CCI. Future studies should include larger cohorts and investigate additional intravascular pathways and cell-mediated interactions, potentially contributing to a poor post-transfusion response, in order to better understand the likely heterogenous causes of platelet transfusion refractoriness.

Declaration of interest statement

The authors declare no conflicts of interest.

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Contributions

T.K., E.Z., A.Å. and U.S. participated in the study design. A.Å. and M. L. carried out the laboratory work. A.Å. compiled the data, performed the statistics and wrote the draft. A.B. established the used complement assay, contributed essential laboratory reagents and offered valuable input. M.M. and J.K. offered valuable input. All authors reviewed and contributed to the paper.

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