

Ex Vivo Test for Measuring Complement Attack on Endothelial Cells: From Research to Bedside

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Ex vivo test for measuring complement attack on endothelial cells: from research to bedside

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20 Abstract

- As part of the innate immune system, the complement system plays a key role in defense against pathogens and in host cell homeostasis. This enzymatic cascade is rapidly triggered in the presence of
- activating surfaces. Physiologically, it is tightly regulated on host cells to avoid uncontrolled activation
- 24 and self-damage. In cases of abnormal complement dysregulation/overactivation, the endothelium is
- 25 one of the primary targets.
- 26 Complement has gained momentum as a research interest in the last decade because its dysregulation
- has been implicated in the pathophysiology of many human diseases. Thus, it appears to be a promisingcandidate for therapeutic intervention.
- However, detecting abnormal complement activation is challenging. In many pathological conditions, complement activation occurs locally in tissues. Standard routine exploration of the plasma concentration of the complement components shows values in the normal range. The available tests to

32 demonstrate such dysregulation with diagnostic, prognostic, and therapeutic implications are limited. There is a real need to develop tools to demonstrate the implications of complement in diseases and to 33 34 explore the complex interplay between complement activation and regulation on human cells. The 35 analysis of complement deposits on cultured endothelial cells incubated with pathologic human serum holds promise as a reference assay. This ex vivo assay most closely resembles the physiological context. 36 37 It has been used to explore complement activation from sera of patients with atypical hemolytic uremic 38 syndrome, malignant hypertension, elevated liver enzymes low platelet syndrome, sickle cell disease, 39 pre-eclampsia, and others. In some cases, it is used to adjust the therapeutic regimen with a complement-blocking drug. Nevertheless, an international standard is lacking, and the mechanism by 40 41 which complement is activated in this assay is not fully understood. Moreover, primary cell culture remains difficult to perform, which probably explains why no standardized or commercialized assay 42 has been proposed. Here, we review the diseases for which endothelial assays have been applied. We 43 also compare this test with others currently available to explore complement overactivation. Finally, 44 we discuss the unanswered questions and challenges to overcome for validating the assays as a tool in 45 routine clinical practice. 46

47

48 Introduction

49 As part of the complex innate immune surveillance system, the complement system plays a key role in defense against pathogens and in host homeostasis. This enzymatic cascade is rapidly triggered in the 50 51 presence of activating surfaces, such as bacteria or apoptotic necrotic cells. However, the cascade is 52 highly physiologically regulated on host cells to avoid self-aggression. The endothelium is one of the 53 primary targets of complement dysregulation. There is increasing evidence of complement 54 implications in the pathophysiology of many human diseases. Many complement-blocking therapeutics 55 are under development, and some are already available in clinical practice. Nevertheless, detection of 56 abnormal functioning complement is challenging, because in many pathological conditions C3 and C4 57 plasma levels, the two main biomarkers of complement activation, remain within normal ranges. The 58 available tests to demonstrate such overactivation with diagnostic, prognostic, and therapeutic 59 implications are limited. Methods are poorly standardized, and only a few have functional value. 60 Therefore, there is a need to develop a robust and standardized tool for identifying infraclinical 61 complement activation.

- 62 The final objective is to allow better pathophysiologically based therapeutic management of patients. The analysis of complement deposits on cultured endothelial cells (EC) incubated with patient serum 63 64 holds promise as a reference assay. This approach has been used to explore complement activation in the sera of patients with atypical hemolytic uremic syndrome (aHUS), malignant hypertension, 65 hemolysis, elevated liver enzymes, and low platelet (HELLP) syndrome, sickle cell disease (SCD), and 66 67 pre-eclampsia. In some cases, adjusting the complement-blocking drugs has been considered. Nevertheless, the international standard for this test is lacking, and the mechanism by which 68 complement is activated in this assay is not fully understood. 69
- After a brief summary of the complement cascade, we present the mechanisms of complement activation and how they contribute to cell damage in several human diseases. We then provide an overview of the tests currently available to explore complement overactivation in routine practice. Finally, through a comparative analysis of the available endothelial assays for complement exploration, we discuss the unanswered questions and challenges to overcome to validate the study of complement deposition on cultured EC as a tool in routine clinical practice.
- 76

77 The complement system in health and disease

The complement system plays a key role in cell homeostasis, inflammation, and defense against 78 79 pathogens. It is the first line of defense. The system comprises more than 30 soluble and membrane-80 bound proteins. Three different pathways lead to complement activation: the classical (CP), lectin (LP), and alternative (AP) pathways. When activated, these serine protease cascades converge to the 81 82 formation of two enzymes, C3 convertase and C5 convertase, allowing the generation of the main 83 effectors of this system: anaphylatoxins (C3a and C5a), opsonin (C3b/iC3b), and the membrane attack 84 complex (MAC) (C5b-9). CP and LP are initiated by the recognition of pathogen-associated molecular 85 patterns or damage-associated molecular patterns by pattern-recognition molecules (C1q and mannose-86 binding lectin). Conversely, AP is constantly activated at a low level in the fluid phase, generating a 87 small quantity of C3b. In the presence of an activating surface (apopto-necrotic or bacterial), C3b 88 covalently binds to the surface, and thus, initiates cell surface C3 convertase formation (C3bBb) and 89 the AP amplification loop. To avoid self-aggression, AP is highly regulated in the fluid phase and on 90 the host cell surface by soluble (factor H (FH), factor I (FI)) and membrane-bound regulators

(membrane cofactor protein (MCP) or CD46, complement receptor 1 (CR1) or CD35, decay
 accelerating factor or CD55, and CD59). In humans, deficiencies in complement regulatory proteins

are associated with rare diseases, such as aHUS, C3 glomerulopathy (C3G), and paroxysmal nocturnal

hemoglobinuria (PNH). However, complement activation triggered by different pathophysiological
 processes that overwhelm the capacity of regulation has been increasingly described in a wide spectrum

96 of diseases.

97

98 **Complement implication in diseases**

99 While AP overactivation is the central mechanism of cell and tissue injury in complementopathies 100 (aHUS, C3G, and PNH), complement is crucial to tissue injury in a wide variety of diseases. These include age-related macular degeneration (AMD), antibody-mediated rejection 101 (ABMR). 102 cryoglobulinemic vasculitis (CV), IgA nephropathy (IgAN), systemic lupus erythematosus (SLE), anti-103 phospholipid syndrome (APS), ANCA-associated vasculitis (AAV), rheumatoid arthritis (RA), 104 HELLP syndrome, pre-eclampsia, myasthenia gravis (MG), neuromyelitis optica spectrum disorder 105 (NMOSD), SCD, and rhabdomyolysis-induced acute kidney injury (RIAKI). To a lesser extent, 106 complement seems to be involved in an increasing spectrum of human pathological conditions, such 107 as inflammatory disorders, ischemia/reperfusion, cancer, degenerative disorders (e.g., Alzheimer's 108 disease, atherosclerosis), and more recently, viral infections that include COVID-19 (1,2) (Figure 1).

109 Complementopathies are characterized by a specific cell target of AP-mediated damage. In aHUS and 110 PNH, AP dysregulation occurs on the cell membrane, EC surface or platelets (3) and erythrocyte 111 surface (4). In C3G, overactivation of C3 and C5 convertases may occur in the fluid phase or locally 112 within the glomeruli, where the targeted surface remains to be determined (suggestions include 113 glomerular EC and mesangial cells). AP dysregulation is a central pathophysiological mechanism in 114 these diseases. It can be related to innate or acquired abnormalities in complement components, mainly

regulators (FH, FI, or MCP) or C3 convertase components (C3 or FB) (5–16).

116 In diseases with major complement contributions, complement activation can be triggered by one or 117 another pathway. In CV (17) and ABMR (18), activation occurs through CP in the presence of immune 118 complexes (IC). In cryoglobulinemia (type II), IC are composed of IgM with rheumatoid factor activity 119 associated with polyclonal IgG. In ABMR, IC are composed of IgG and donor HLA molecules. 120 Conversely, despite the disease being triggered by the presence of IC, AP appears to be essential for 121 disease development in mouse models of RA (19,20) and SLE (21-23). This activation can be 122 enhanced by apoptotic and necrotic cells due to prior damage (24) or by proteins of the extracellular 123 matrix (ECM) from damaged cartilage in RA (25). In IgAN, AP (26), and LP (27) activation is 124 mediated by polymeric IgA. In vitro, a correlation was found between C3 cleavage products (iC3b, 125 C3c, C3dg) and IgA-A-IgG IC levels, suggesting that IC-containing IgA may act as a surface for 126 soluble AP activation (28). In AAV, AP may be activated by neutrophil extracellular traps, thus 127 amplifying complement activation and damage of EC (29). Finally, a disease-specific soluble factor 128 has been implicated in complement activation. Free heme renders EC more sensitive to complement 129 activation in SCD (30), aHUS (31), and RIAKI (32). In vitro, thrombin induces C5 cleavage in C5a in 130 APS (33).

131 Complement activation does not arise from a unique mechanism but can be triggered in several ways 132 according to the disease pathophysiology. Identification of the precise mechanisms of complement

132 according to the disease pathophysiology. Identification of the precise mechanisms of contract activation will help determine different potential therapeutic targets within the cascade.

- 134 Complement activation contributes to cell and tissue injuries in different ways. First, it promotes
- inflammatory cell recruitment mainly in CV (34), ABMR (35), AMD (36), SLE (37) and RA. C5a and
- 136 its receptor C5aR are involved in neutrophil recruitment (38–40) and endothelial activation (41) in 127 AAV Complement activation can appear to a single diagonal Thus MAC can directly affect
- 137 AAV. Complement activation can promote specific disease processes. Thus, MAC can directly affect 138 collagenase production by synovial fibroblasts in RA (42). In IgAN, mesangial cells exposed to
- conagenase production by synovial horoblasts in RA (42). In IgAN, mesangial cells exposed to complement activation and C3 deposition promote phenotypic conversion to a more synthetic and
- proliferative state (43). In AMD, C3a and C5a promote choroidal and C5a induces vascular endothelial
- 141 growth factor secretion by retinal pigment epithelium (36). In pre-eclampsia, it has been suggested that
- 142 the binding of C5a to C5aR expressed on trophoblasts contributes to the acquisition of their anti-
- 143 angiogenic phenotype (44).
- The complement system can also act as an amplifier for other molecules involved in injury. The C5a/C5aR axis participates in neutrophil recruitment and activation, which in turn can induce complement activation in AAV (39). C5a induces tissue factor expression by neutrophils, leading to factor X activation and thrombin generation, which in turn cleaves C5 into C5a in APS (33).
- 148 Ultimately, several triggers of complement activation and effectors may contribute to cell and tissue 149 damage in heterogeneous human diseases. The identification of specific triggers of complement 150 activation and fine pathophysiological mechanisms resulting in cell and tissue complement-mediated 151 injury is needed to determine the best therapeutic target within the cascade. Complement inhibitor anti-152 C5 monoclonal antibody (eculizumab, and more recently its long-acting form, ravulizumab) is the gold 153 standard in two complementopathies, aHUS and PNH, and has obtained Food and Drug Administration 154 (FDA) approval for MG and NMOSD. Avacopan is a C5aR1 antagonist that has also been approved 155 by the FDA for patients with AAV, another disease with a major complement contribution. 156 Understanding the detailed mechanisms of complement activation and complement-mediated damage 157 is necessary to guide the prescription of new complement inhibitors.

158 **Overview of the tests exploring complement activation**

- 159 Quantification of complement components
- 160 Currently available tests mainly consist in quantification of individual complement components or161 activation products.
- For the quantification of individual complement proteins in plasma, various types of immunoassays are used to determine the concentration of individual complement components. The most common is nephelometry. Polyclonal antibodies to component are added in excess of the sample and bind to their target. Quantification is performed by passing a light beam through the sample, which is distorted by the IC that have formed (45).
- Quantification of complement activation products corresponding to cleavage fragments or complement protein complexes (C3a, C3dg, C4a, C4d, Ba, Bb, C5a, C3bBbP, MASP2, and sC5b 9) is possible. Several assays have been described, mostly based on the recognition of a neoepitope
- of the complement component in an enzyme-linked immunosorbent assay (ELISA) format. Thus,
 C4a and C4d reflect CP/LP activation, Ba, Bb, and C3bBbP reflect AP activation, MASP2 is a key
 enzyme in LP activation (46) and increasing soluble C5b-9 reflects TP activation (47). C3a and
 C5a are common to the three activation pathways.
- 173 C5a are common to the three activation pathways.
- Detection of auto-Abs (anti-FH, FB, C3b, C3bBb, and C1q) targeting complement proteins can be
 performed using ELISA (15)

176 **Functional** assays

177 • Quantification of complement function is used to explore the activity of a pathway or the entire 178 cascade.

179 In hemolytic assays, CP activation can be assessed by incubating patient sera with sheep 0 180 erythrocytes coated with rabbit anti-sheep red blood cell antibodies (48). In this assay, termed the 181 CH50 assay, C1q binds to immunoglobulins, initiates the formation of CP C3 convertase, and leads 182 to MAC assembly and erythrocyte lysis. Hemoglobin release is determined to calculate the number 183 of hemolytic sites per cell. Activation through AP can be assessed using rabbit or guinea pig 184 erythrocytes, which are activators of human AP, incubated with patient serum added to ethylene 185 glycol-bis(β -aminoethyl ether) (EGTA), which chelates Ca2+ and inhibits activation via CP and 186 LP (49). This hemolytic assay is termed the AP50 assay.

- 187 Liposomes coated with an activator can be used in a similar manner to CH50 assays (50). The main 0 188 difference is the readout, which consists of the unquenching of a fluorescent dye and not the lysis 189 of erythrocytes.
- 190 0 Assays based on ELISA method can also be used to explore the function of the three pathways. 191 Microtiter plate wells are coated with recognition structures specific to each pathway (IgM for CP, 192 mannan or acetvlated bovine serum albumin for LP, and LPS for AP). Patient serum is added and 193 incubated under conditions in which only one pathway is operative at any given time; the other two 194 pathways are blocked. Finally, activation capacity is detected through the formation of the C5b-9 195 complex by monoclonal antibodies targeting a neo-epitope in complex-bound C9 (51).
- 196
- 197 • Different hemolytic assays have been developed to explore specific steps of the AP.
- 198 Sanchez-Corral et al. (52) developed a hemolytic assay to study FH functional defects in aHUS. 0 199 The assay relies on the knowledge that sheep erythrocytes are highly sialylated and favor FH 200 binding, whereas their membrane complement regulators are incompatible with human 201 complement proteins. Therefore, they are protected from complement lysis due to the binding of human FH to their surface. In the assay, sheep erythrocytes are incubated with human plasma in 202 Mg-EGTA buffer, allowing activation of AP only. Normal plasma does not induce lysis, whereas 203 204 aHUS plasma with FH functional defects (mutations or autoantibodies) induces lysis under these 205 conditions (52).
- 206 Hemolytic assays can also be used to study the stabilization of cell-bound AP convertases (53). 0 207 This assay has been used to detect C3Nef in C3G cells. Sheep erythrocytes bearing C3 convertase 208 C3bBb (generated by exposure of sheep erythrocytes bearing C3b to FB and FD) were incubated 209 with patient IgG. C3Nef activity correlates with residual C3bBb hemolytic sites, and lysis is developed by the addition of rat serum. 210
- 211
- 212 Staining of tissue sections for the deposition of complement activation products can provide • 213 information about local complement activation in tissue. This can be performed by 214 immunohistochemistry or immunofluorescence (54). For example, this technique has been used 215 to study C5b-9 deposition in the skin of patients with aHUS (55).
- 216 These tests allow only the characterization of a specific molecule or step of the complement cascade. 217

To reproduce human pathological conditions and their complexity, several authors have proposed the 218

219 on the EC surface after incubation with human serum. The EC surface is used as the regulating surface.

- 220 The objective is to detect abnormal complement deposition that could result from either complement
- 221 overactivation exceeding the capacity of regulation, or from a defect in complement regulation in fluid
- 222 or on the EC surface. The *ex vivo* endothelial assay is presented in Figure 2.
- 223 We next discuss the advantages and limits of this functional approach.

224 Study of complement deposition on cultured EC

225 Heterogeneity of endothelial cells populations and their complement regulation

226 EC line blood vessels and constitute an active regulatory organ that has been implicated in vascular 227 homeostasis, permeability regulation, vasomotor tone, angiogenesis, and diapedesis of immune cells (56). As first barrier between the blood and interstitium it is in constant equilibrium with the 228 229 environment. Thus, heterogeneity in the structure and function of EC is a core property of the endothelium, allowing diverse vascular functions and regional specificity (57,58). This diversity can 230 be partially explained by a distinct transcriptional profile (59) in relation to neighboring cells (60). 231 232 Hence, EC from different blood vessels have distinct and dynamic expression profiles of complement 233 components and regulators, which may explain the different susceptibility and specific organ tropism 234 observed in some complement-mediated diseases (61).

235 At a steady state, EC can produce most complement components and express high levels of 236 complement regulators on their membranes (Table S1). Under inflammatory conditions, complement 237 component production and regulatory protein expression are modified (Table 1). In addition to the steady state, the modulation of complement protein expression under inflammatory conditions differs 238 239 according to the EC type and probably contributes to a specific damage mediated by AP and the 240 different organ tropisms observed in complement-mediated diseases. Sartain et al. demonstrated that 241 resting or tumor necrosis factor (TNF)-stimulated brain microvascular EC expressed higher levels of regulatory molecules (FH, FI, CD46, CD55, and THBD), generated lower levels of C3a and C4a, and 242 243 enhanced lower degree AP activation (measured by lower Ba generation) than human renal glomerular 244 EC (HRGEC) (62). The authors also demonstrated a slight increase in CD46 expression, decrease in thrombomoduline (TM), and increase in C3 and FB transcription in HRGEC exposed to TNF (63). 245 246 These results agree with the prior demonstrations of an increase in C3 and FB production by human 247 umbilical vein EC (HUVEC) exposed to TNF (64), increased FH transcription and production by HUVEC exposed to interferon (INF) gamma (65), increased C2, FH, FB, and C1inh transcription, and 248 249 decreased C3 production by HUVEC exposed to INF gamma (66). May et al. compared the properties 250 of four EC types (HRGEC, glomerular EC (GEnC), human microvascular EC (HMEC), and HUVEC) in the resting state and after overnight exposure to heme (67). While there was no difference in 251 252 expression of regulatory factors (MCP, CD55, TM) at resting state, after overnight heme exposure, C3 253 deposits on glomerular EC were greater than on other EC. This was associated with, and possibly 254 explained by, weaker FH binding and TM upregulation and lower upregulation of heme-oxygenase 1 255 (cytoprotective heme-degrading enzyme) compared to HUVEC. Moreover, HUVEC, but not EC, of 256 glomerular origin were protected from complement deposition after re-challenge with heme (Table 257 **S2**).

EC used for *ex vivo* experiments comprise two types: conditionally immortalized EC (CI-EC) and
 primary EC (**Table 2**). Primary EC can be difficult to isolate and maintain in culture, and have
 a limited lifespan. Moreover, differences in the genetic background of individual donors can
 lead to interexperimental variability. In particular, inter-individual heterogeneity in

262 complement regulator expression at the EC surface cannot be excluded. CI-EC has been developed to overcome these difficulties. HMEC-1 and CI-GEnC are HMEC and GEnCs, 263 264 respectively, that have been transfected with SV40 large T antigen (68,69). EA.hy926 cells 265 were obtained by fusing HUVEC with A549 cells obtained from human lung carcinoma (70). The EA.hy926 cells were used to generate glycosylphosphatidylinositol-anchored complement 266 regulatory protein-deficient cells when treated with phosphatidylinositol-specific 267 268 phospholipase C. These cells have been used along with the PIGA-mutant TF-1 to study complement deposits by confocal microscopy and flow cytometry after incubation with serum 269 270 from patients with thrombotic microangiopathy (TMA), this test was called the modified Ham 271 test (71). After incubation with serum from aHUS patients, cell surface C5b-9 deposits were reportedly higher than after incubation with thrombotic thrombocytopenic purpura (TTP) 272 273 serum. Therefore, this test has been considered a tool to distinguish aHUS from TTP. It is 274 important to note that sC5b-9, reflecting terminal pathway activation and regulation, is elevated 275 under both aHUS and TTP plasma conditions (72,73). One possibility is that both conditions are associated with complement activation. However, in aHUS, complement overactivation 276 277 exceeds alternative and terminal pathway regulation, leading to C5b-9 deposits. In contrast, in 278 TTP, complement activation is counterbalanced by complement regulation, leading to sC5b-9 279 release, but not C5b-9 deposits in the modified Ham test.

Micro- or macrovascular origin of the EC tissue lineages also needs to be considered. Complementmediated EC injury demonstrates specific cell tropism according to pathophysiological processes. In HUS and TTP, microvascular EC of dermal, renal, and cerebral origin are more sensitive to apoptosis, whereas microvascular EC of pulmonary and hepatic origin and macrovascular EC are resistant (74). Distinct sensitivity of EC to complement attack has also been explored in aHUS and heme exposure. The demonstration of a distinct EC response in terms of complement regulator expression after a trigger

286 (here heme) was proposed to partially explain the kidney tropism in this disease (67).

HUVEC are primary macrovascular EC isolated from human umbilical cords. These are the most frequently used cells for *ex vivo* assays (75). If tissue specificity is required, HRGEC (76) or GEnCs (77), which are both isolated from human glomeruli, can be used. More recently, the use of blood outgrowth EC obtained from the differentiation of circulating marrow-derived endothelial progenitor cells isolated from peripheral blood has been proposed (78).

292 <u>Comparative analysis of the available endothelial assays</u>

These tests consist of the quantification of complement activation products (C3 activation fragments and C5b-9) deposits on EC by immunofluorescence (IF) measured by confocal microscopy or flow cytometry (fluorescence-activated cell sorting, FACS) after incubation with a serum sample of interest. Different protocols have been proposed to study complement activation on the EC surface in several pathological conditions, including aHUS (31,79–89), TMA of other etiologies (90,91), HELLP syndrome and pre-eclampsia (92), C3G (15,93), lupus nephritis (LN) (94,95), APS (96,97), SCD (30), hemolytic anemia (98) and hyperhemolytic transfusion reaction without hemoglobinopathy (99).

- 300 The general procedure of the *ex vivo* assay and the different protocols are presented in **Figure 3**.
- 301 *To pre-activate or not pre-activate EC?*
- Resting EC or EC pre-activated by cytokines, ADP, or heme can be used (**Table S3**) to provide additional information.

304 When resting HUVEC were incubated with aHUS FB mutants added to FB-depleted normal human 305 serum (NHS), enhanced C3b/iC3b-fragment deposition as measured by an anti-C3c-reacting antibody 306 was observed (79). The same result was obtained for some cases when aHUS patient serum was 307 incubated with resting HUVEC (31,80). Nevertheless, incubation with NHS depleted in FB and 308 reconstituted with other aHUS FB variants (81) or incubation with aHUS serum from patients carrying 309 some C3 or FH variants (31,80) may be insufficient to induce C3 or C5b-9 deposits. When quiescent 310 HMEC-1 were incubated with aHUS serum from patients carrying mutations in FH, FI, C3, or FH/CFHR1 hybrid, enhanced C3c or C5b-9 deposition was reported only if serum was collected during 311 312 the acute phases of the disease and not after reaching remission (83,90). Furthermore, deposits on 313 quiescent HMEC-1 are better correlated with relapse risk during the tapering or discontinuation of 314 eculizumab (86).

- 315 To increase test sensitivity, the authors proposed pre-activating EC. Modifications in surface-bound protein expression enable complement activation. This was achieved in the case of P-selectin 316 317 expression on HMEC-1 pre-activated with ADP, LPS, or thrombin (83) or P-selectin expression on 318 HUVEC or GEnC pre-activated with heme (31,81,100), which could allow C3b binding and C3 319 convertase formation. Enhanced formation of C3 fragments by TNF/IFN pre-activation and C5b-9 320 deposition by ADP pre-activation on HUVEC or HMEC-1 cells was described after incubation of these 321 cells with serum from asymptomatic carriers of mutations in AP regulatory proteins or C3 (80,83). The 322 normal range was established when pre-activated EC were incubated with sera from healthy donors. 323 In addition, serum from healthy family members without the mutation was within the normal range in
- 324 this assay (80).

325 What kind of blood samples might be incubated with EC?

326 Serum has been used as the source of complement proteins in the vast majority of the tests described 327 above. One limitation of these tests, particularly when deposits are detected by IF, is the variation in 328 the results, reportedly from 30% to 52% when activated HMEC-1 were incubated with serum collected 329 at the acute phase of aHUS (92). To reduce this variation, Palomo et al. proposed the use of activated 330 plasma, which refers to citrated plasma mixed 1:1 with a control serum pool. Using this approach, the 331 authors derived a coefficient of variation of 9% to 18% (92). C3 consumption by the patient or loss of 332 C3 activity during the pre-analytical phase are also potential factors responsible for this variation (101). 333 Finally, for all complement assays and to avoid in vitro complement activation, proper blood collection and processing must be achieved (102). Processing of plasma or serum sample must be performed 334 335 within a few hours of collection, with storage at -80°C and defrosting immediately before use to avoid 336 repeated freezing and thawing.

337 To explore the functional consequences of autoantibodies against C3 and properdin in SLE, Vasilev et 338 al. and Radanova et al. incubated HUVEC with NHS supplemented with purified IgG from patients 339 positive for such autoantibodies (94,95). Using this strategy, complement deposition on EC can be 340 directly ascribed to the addition of autoantibodies to NHS. The same approach was applied for anti-341 C3b/FB autoantibodies in patients with C3G (15). To understand the mechanism behind complement 342 deposits on EC from patients with SCD, microvesicles from normal or patient-derived erythrocytes 343 were added to normal serum to model the disease condition. Enhanced binding of the C3 activation 344 products was demonstrated (30,103).

345 Which controls are relevant?

346 Most often, NHS is used as a negative control (15,30,31,79-84) (Table S4). An important aspect to 347 consider is the inter-individual variability in deposits induced by normal sera. FACS analysis has 348 revealed that this variability was relatively low when sera from 50 healthy donors were tested (80). 349 However, this is a concern, particularly when deposits are detected by IF. This has not been directly 350 reported, but has been suggested by the use of pooled sera in more recent papers (86,92) and our own experience. Aiello et al. reported that C3 and C5b-9 deposits obtained after a single healthy subject 351 352 serum (N=12) incubation range from 0.5 to 1.5 fold increase of stained surface area compared to pooled 353 serum (from 10 healthy donors) run in parallel (88).

Several authors did not use any positive controls for their experiments (83,84,86,91,92). The comparison was only made with the deposits obtained with negative controls. It might be interesting to position the results on a scale. Positive controls with published data are FH or FI depleted NHS (15,80,82) or normal serum supplemented with blocking anti-FH antibodies targeting the N-terminus or C-terminus (31,81) or with FH19-20, corresponding to the two last domains of FH, able to compete with the full FH protein for cell surface binding (98).

The main issues with this type of assay are the lack of validated international standards as well as standardized positive and negative controls. The variability of the results in samples from healthy donors needs to be studied extensively to determine the appropriate cutoff. In addition, the impact of C3 or other complement protein consumption in the patient and the influence of the pre-analytical phase must be determined to avoid false positive and false negative results.

365 Which deposits should be measured?

366 The objective of these tests is to demonstrate and explore complement overactivation or dysregulation 367 on the EC surface after incubation with blood samples of interest. This is enabled by quantification of the deposition of complement component products resulting from activation or regulation. C3c (a 368 369 common epitope to C3, C3(H2O), C3b, and iC3b) (which reflects C3 convertase activity and the early 370 phase of the complement cascade) can be detected by polyclonal anti-C3c antibody. Antibody targeting 371 C5b-9 reveals the final step of the cascade. When a signal is detected on the cell membrane, it can be 372 assumed that the detected fragment is C3b or iC3b covalently attached to the surface. Nevertheless, 373 heme-activated EC and likely ADP-activated EC (104,105) express P-selectin, which recruits C3b, 374 C3(H2O), and a C3(H2O)-like form of C3 generated after contact with heme (31,100). Properdin also 375 binds to heme-exposed or stressed EC, promoting complement activation in a similar manner without 376 covalent C3b binding (98). This is an additional mechanism for amplification of complement activation 377 on the EC surface. C5b-9 deposits may be more relevant in identifying dysregulation at any step. 378 Nevertheless, early dysregulation can induce C3 activation fragment deposits without C5b-9 formation 379 because of TP regulation. C5b-9 is readily detectable by IF but is much more difficult to detect by 380 FACS because of the weak shifts of the peaks. To test for CP participation, the presence of C4d-positive 381 deposits was also investigated (83,90). Staining can also be performed under the same conditions for 382 von Willebrand Factor, C5aR1, P-selectin, and others (88).

383 Evaluation of activated pathways

384 The test can be modified to assess which complement pathway is activated in given pathological

- settings. The test can be performed under different conditions to avoid CP and LP contributions, which
- include C2 (31) or C1q (106) depleted NHS, addition of SCR1 (88) or Mg-EGTA buffer (31,79,94,95).
- 387 EGTA chelates Ca2+, which is crucial for CP and LP activation, whereas AP depends on Mg2+. If AP
- 388 has to be inhibited, FB-depleted NHS can be used. These reagents are applicable for test conditions,

389 where the activating factor is added externally to the serum (i.e., IgG, heme, microvesicles, etc.). When

390 patient samples are used directly, the same effect can be achieved by inhibiting C1q, C4, FB, or 391 properdin with blocking antibodies, protein constructs, or small molecules, if available (98).

- 392 Quantification of complement activation products (split fragments generated by cleavage of
- complement components or protein complexes when activated components bind their target (i.e., C3a,
- C4a, Ba, Bb, C5a, and sC5b-9) in the supernatant might be an additional element to study complement
- 395 cascade activation.

396 Which techniques are used for detection and quantification?

397 The two main detection techniques commonly used are FACS and IF. HUVEC pre-activated with heme 398 and then incubated with NHS or aHUS serum showed results similar by FACS or IF detection(31). IF 399 directly analyzes deposits on EC grown on slides. FACS requires a cell detachment step before 400 staining, with the potential risk of losing a part of the deposit signal. In contrast, as mentioned by 401 Gavriilaki et al., obtaining quantitative data by IF requires confocal microscopy and further analysis 402 using specialized software (71). When IF is used, the area occupied by fluorescent staining in fields systematically digitized along the surface is quantified. The quantified results expressed as the mean 403 404 of the square number of pixels per field are compared with the negative control (83,84,86,90,92). 405 Considering the number of EC on which fluorescence has been measured and the staining intensity 406 might appear relevant. In contrast, FACS allows the rapid and objective quantification of deposits.

407 What are the functional consequences of such deposits?

408 If enhancement of complement fragment deposits on EC is interpreted as pathogenic, the functional 409 consequences of such deposits must be questioned. Lactate dehydrogenase release from EC reflects 410 cell damage. This release can be measured in the cell culture supernatant (106). Analysis of 411 complement deposits can also be associated with a cell viability assay, corresponding to a colorimetric 412 assay based on cleavage of the WST-1 tetrazolium salt by mitochondrial dehydrogenases in viable cells 413 (71). Cellular integrity can be verified by May-Grunwald Giemsa staining (89). Direct cell death rarely 414 occurs under these experimental conditions. Experiments testing cell activation status by complement 415 overactivation have not been reported in the literature and are needed to further understand the impact of complement on endothelial injury. Analysis of transcriptomic modifications in EC exposed to 416 417 complement deposits under several conditions could also be of interest.

418

419 <u>Clinical and therapeutic relevance of the obtained results</u>

420 The exvivo EC assay, consisting in the quantification of complement activation products (C3 activation fragments or C5b-9) deposits on EC (by IF measured on confocal microscopy or FACS), after 421 incubation with a serum sample of interest, was first used for specific characterization of complement 422 component abnormalities (79-82,84) or exploration of mechanisms implicated in EC injury (31) in the 423 424 main complementopathy, aHUS. The assay was then used to demonstrate and explore complement 425 activation and participation in the pathophysiology of several diseases, including C3G (15,93), HELLP 426 syndrome and pre-eclampsia (92,107), TMA associated with severe hypertension (90,108), drug-427 induced TMA (109), SCD (30), hemolytic anemia (98,99), SLE (94,95), and APS (97). Demonstration 428 of increasing complement deposits on EC incubated with pathological sera is not sufficient to 429 determine what is responsible for complement activation at the EC surface. Modulation of the test

- 430 conditions can help in detailing complement activation. This was the case when complement activation
- 431 was inhibited by the addition of hemopexin to the sera of patients with SCD (30).

432 Noris et al. and Galbusera et al. also proposed the use of this ex vivo EC test to monitor eculizumab 433 therapy in patients with aHUS (83,86). During eculizumab tapering or discontinuation, disease relapse 434 preceded or was associated with an increase in C5b-9 deposits on resting HMEC-1 in all patients. In contrast, only one patient without relapse showed increased deposits (86). In clinical practice, CH50 is 435 436 the only routine test used to monitor eculizumab therapy. CH50 is reportedly strongly suppressed in 437 patients receiving eculizumab according to the standard protocol. However, CH50 does not allow monitoring of eculizumab dosage tapering or discontinuation, as it is not well correlated with relapse 438 439 risk (83,86). Eculizumab therapy monitoring using the Wieslab® complement system screen (110) or 440 the modified Ham test (111) has also been proposed. Thus, the ex vivo EC assay could represent a test of interest to a better personalized complement-blocking therapy, but first needs to be more 441 442 standardized.

- This test can also be used to better classify and assess the prognosis of specific diseases. This is the case for hypertensive TMA, as Timmermans et al. demonstrated in a cohort of hypertension emergencies associated with TMA(108). The authors demonstrated a statistical association between increased C5b-9 deposition in the EC *ex vivo* test and kidney survival. Moreover, they reported an improvement in renal function for those with increased deposits treated with eculizumab. The authors proposed a classification of TMA-hypertensive emergency based on the EC *ex vivo* test (108).
- Finally, many new anti-complement drugs targeting specific steps of the cascade have been under development in recent years (112). A standardized and validated assay to study complement activation
- 451 could be a useful tool in their development.
- 452

453 Discussion and Conclusion

The increasing demonstration of complement involvement in the pathophysiology of many human diseases has mandated the development of tools to finely explore complement activation. Complement is a complex enzymatic cascade that is highly regulated in constant interplay with its environment. The current arsenal for complement exploration does not provide functional characterization and does not report on the complex interplay between complement and its environment, particularly the cell surface.

The development of tests with these capabilities could allow for a deeper exploration of the mechanisms of complement activation in several diseases. This information could inform the development of a complement blocking therapeutic strategy based on pathophysiological mechanisms.

462 *Ex vivo* complement activation on EC represents a promising tool for demonstrating and exploring 463 complement activation. It not only recapitulates complex complement cascade regulation *in vivo*, but 464 also allows modification of several steps of the experimental procedure to characterize complement 465 activation mechanisms.

However, there are still unanswered questions hindering broad used. The first is the variability in the
results and the inter-individual variability in deposits induced by normal sera. Comprehension of the
precise mechanism responsible for complement deposition in this assay would improve its better use.
The second issue is to standardize the main steps of the procedure to improve the interexperimental
comparison.

471 The use of such a test could be multiple, including molecular functional characterization, disease

472 pathophysiology exploration, prognosis classification, complement targeting drug development, and

- 473 complement therapeutic monitoring. The use of standardized conditions will expand the field of this
- 474 promising tool.

475 Authors Contributions

MSM, SC, and LR conceptualized and conceived the manuscript. MSM drafted the manuscript,
including the literature search, reading, and writing. SC, VFB, AD, and LR edited and critically
evaluated the manuscript. All authors have contributed to the manuscript and approved the submitted
version.

480 **Conflict of Interest**

481 The authors declare that the review was conducted in the absence of any commercial or financial 482 relationships that could be construed as potential conflicts of interest.

483

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859



860 Tables

861 **Table 1: Production of distinct complement components and expression of regulators according**

862 to endothelial cell type after stimulation

		TNF	INF gamma	IL1 beta	Heme
HRGEC	С3	\uparrow	\uparrow	\rightarrow^*	
	C4	\rightarrow^*	\uparrow	\rightarrow^*	
	C5	\rightarrow^*		\rightarrow^*	
	FB	\uparrow		\rightarrow^*	
	FD	→*		\rightarrow^*	
	Properdin	↓*		\rightarrow^*	
	FH	\rightarrow		→*	
	FI			→*	
	тм	¥		<u></u>	
	CD46	\uparrow		\rightarrow	, k
	CD55	\rightarrow		\rightarrow	↓ ↓
	CD59	\rightarrow		\rightarrow	•
	E-selectine	\uparrow	\rightarrow	,	
	C3aB	1 个	,		
	C5aB	0			
BMVEC	C3	*			
Dirivee	C4	→*			
	C5	→*			
	EP	7			
		*			
	Dropordin				
	Fioperulli	¥ 、			
	FH	→ ◆			
	CD46	T			
	CD55	7			
	C3aR	<u>ተ</u>			
	C5aR	0			
HIVIEC	E-selectine	T	\rightarrow		
	03		\rightarrow		
	C4		个		
	CD46				↓
	CD55				\rightarrow
HUVEC	C2		个 ·		
	C3	\uparrow	\rightarrow/\downarrow	Ϋ́	
	C4			→*	
	C5			→*	
	FB	\uparrow	\uparrow	个*	
	FD			→*	
	Properdin			→*	
	FH		\uparrow	↓	
	FI			\rightarrow^*	
	ТМ	\downarrow		\rightarrow	
	CD46	\uparrow		\rightarrow	\downarrow
	CD55	\uparrow		\uparrow	\checkmark
	CD59	\rightarrow		\rightarrow	\rightarrow
	E-selectin	\uparrow	\rightarrow		
	P-selectin				\uparrow
	C1-inh		\uparrow		

863 The data presented here are mainly concerned with protein expression. * denotes transcriptomic data. For details, please

- 864 refer to **Table S2**. <u>Abbreviations:</u> BMVEC, brain microvascular endothelial cells; HMEC, human microvascular endothelial
- 865 cells; HRGEC, human renal glomerular endothelial cells; HUVEC, human umbilical vein endothelial cells
- 866

867

868 Table 2: Endothelial cells used for *ex vivo* experiments

	Conditionally immortalized	Primary
Macrovascular		HUVEC
Microvascular	Cl-GEnC	BMVEC
	HMEC-1	HRGEC
		BOEC

869 <u>Abbreviations:</u> BMVEC, brain microvascular endothelial cells; BOEC, blood outgrowth endothelial cells; CI-GEnC,

870 conditionally immortalized human glomerular endothelial cells; HMEC, human microvascular endothelial cells; HRGEC,

human renal glomerular endothelial cells; HUVEC, human umbilical vein endothelial cells

872



873 Figures Legends

874 Figure 1: Complement implication in human diseases

875 Complement dysregulation has been implicated in the pathophysiology of several human diseases.

876 Complementopathies in which alternative pathway dysregulation is the central mechanism of cell and 877 tissue injury are represented in red. Conditions in which the complement system has been demonstrated

to contribute significantly to tissue injury are represented in pink. Other diseases in which complement

879 plays an accessory role are represented in gray.

880 Figure 2: Concept of *ex vivo* complement deposition on endothelial cells

881 An *ex vivo* endothelial assay was developed to reproduce human pathological conditions and their 882 complexity. The assay consists of the detection and quantification of complement component 883 deposition on the cultured endothelial cells (EC) surface after incubation with human serum. The EC 884 surface was used as the regulatory surface. (A): In serum from healthy individuals, the alternative 885 pathway is active at low levels but tightly regulated in the fluid phase by regulators, resulting in a very 886 low level of complement activation product deposition on the EC surface. The detection of an increased 887 complement deposition when incubation is performed with pathological serum (B) could result in 888 either i) complement overactivation that overwhelms EC capacity of regulation (orange) or ii) defect 889 in complement regulation in fluid or solid phase. Both are induced by tested human serum incubated 890 with EC. Orange arrows represent some mechanisms involved in complement overactivation in serum: 891 (1) the participation of a coactivation of classical/alternative pathway due to pathological 892 immunoglobins, immune complexes, or lectin pathway activation by polymeric IgA in IgA 893 nephropathy, (2) an increase in the formation of fluid phase C3 convertases in the presence of heme or 894 fluid phase activating surface, and the stabilization of C3 (3) or C5 (4) convertases by pathological 895 immunoglobulins, such as C3 and C5 nephritic factors. Red crosses represent potential defects in 896 alternative complement pathway regulation in the fluid phase (1) and on the cell surface (2, 3). These 897 defects in complement regulation could be the consequence of inhibition of the main alternative 898 pathway regulator FH due to anti-factor H antibodies (such as in aHUS), a lack of function, or a 899 quantitative deficiency of FH and FI due to pathological genetic variants. *CR1: weak expression of 900 CR1 on endothelial cells Abbreviations: CR1: complement receptor 1 (CD35), FB: factor B, FD: factor 901 D, FH: factor H, FI: factor I, FP: properdin, MCP: membrane cofactor protein.

Figure 3: Comparative analysis of different protocols used for the *ex vivo* complement activation test with endothelial cells

904 1: The *ex vivo* test for measuring complement attack on endothelial cells can be performed on different 905 endothelial cells, including human dermal microvascular endothelial cells (HMEC-1), human umbilical vein endothelial cells (HUVEC), blood outgrowth endothelial cells (BOEC), and glomerular 906 907 endothelial cells (GEnC). 2: Cultured EC are then used at their resting state or after an activation by either ADP, heme, LPS, TNF/INF gamma, or apoptonecrotic cells. 3: EC are incubated with sample of 908 909 interest. Either serum or activated plasma (consisting of patient citrated plasma mixed 1:1 with control 910 serum pool) or normal human serum with addition of the protein of interest (e.g., IgG). Complement 911 activation can be modulated in by addition of sCR1, anti-C5 antibody, anti-FH antibody, anti-properdin 912 antibody, or EGTA-Mg buffer. 4: Complement activation products are then revealed by fluorescent 913 tagged antibody. Antibody directed again C3c or C5b9 can be used. According to the context, staining 914 for other molecules have been proposed and include IgG, P-selectin, vWF, and CD31. 5: Quantification

- 915 is then performed using immunofluorescence scanning, flow cytometry, or ELISA. 6: Controls are
- 916 required and vary according to the protocols.

Figure 4: Current and future application fields of the *ex vivo* complement activation test on endothelial cells

- 919 There is a wide range of potential applications of *ex vivo* complement activation tests in endothelial
- 920 cells. Currently used to decipher *in vitro* complement pathophysiology in research, a standardized test
- 921 would represent a promising tool in clinical and therapeutic fields, paving the way for tailored medicine
- 922 in complementopathies.

923