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

3

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13

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20 **Abstract**

21 As part of the innate immune system, the complement system plays a key role in defense against
22 pathogens and in host cell homeostasis. This enzymatic cascade is rapidly triggered in the presence of
23 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
27 has been implicated in the pathophysiology of many human diseases. Thus, it appears to be a promising
28 candidate for therapeutic intervention.

29 However, detecting abnormal complement activation is challenging. In many pathological conditions,
30 complement activation occurs locally in tissues. Standard routine exploration of the plasma
31 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.
33 There is a real need to develop tools to demonstrate the implications of complement in diseases and to
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
36 holds promise as a reference assay. This *ex vivo* assay most closely resembles the physiological context.
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
40 complement-blocking drug. Nevertheless, an international standard is lacking, and the mechanism by
41 which complement is activated in this assay is not fully understood. Moreover, primary cell culture
42 remains difficult to perform, which probably explains why no standardized or commercialized assay
43 has been proposed. Here, we review the diseases for which endothelial assays have been applied. We
44 also compare this test with others currently available to explore complement overactivation. Finally,
45 we discuss the unanswered questions and challenges to overcome for validating the assays as a tool in
46 routine clinical practice.

47

48 **Introduction**

49 As part of the complex innate immune surveillance system, the complement system plays a key role in
50 defense against pathogens and in host homeostasis. This enzymatic cascade is rapidly triggered in the
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.
63 The analysis of complement deposits on cultured endothelial cells (EC) incubated with patient serum
64 holds promise as a reference assay. This approach has been used to explore complement activation in
65 the sera of patients with atypical hemolytic uremic syndrome (aHUS), malignant hypertension,
66 hemolysis, elevated liver enzymes, and low platelet (HELLP) syndrome, sickle cell disease (SCD), and
67 pre-eclampsia. In some cases, adjusting the complement-blocking drugs has been considered.
68 Nevertheless, the international standard for this test is lacking, and the mechanism by which
69 complement is activated in this assay is not fully understood.

70 After a brief summary of the complement cascade, we present the mechanisms of complement
71 activation and how they contribute to cell damage in several human diseases. We then provide an
72 overview of the tests currently available to explore complement overactivation in routine practice.
73 Finally, through a comparative analysis of the available endothelial assays for complement exploration,
74 we discuss the unanswered questions and challenges to overcome to validate the study of complement
75 deposition on cultured EC as a tool in routine clinical practice.

76

77 **The complement system in health and disease**

78 The complement system plays a key role in cell homeostasis, inflammation, and defense against
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),
81 and alternative (AP) pathways. When activated, these serine protease cascades converge to the
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

91 (membrane cofactor protein (MCP) or CD46, complement receptor 1 (CR1) or CD35, decay
92 accelerating factor or CD55, and CD59). In humans, deficiencies in complement regulatory proteins
93 are associated with rare diseases, such as aHUS, C3 glomerulopathy (C3G), and paroxysmal nocturnal
94 hemoglobinuria (PNH). However, complement activation triggered by different pathophysiological
95 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
101 include age-related macular degeneration (AMD), antibody-mediated rejection (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
115 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
133 activation will help determine different potential therapeutic targets within the cascade.

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134 Complement activation contributes to cell and tissue injuries in different ways. First, it promotes
135 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
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
139 complement activation and C3 deposition promote phenotypic conversion to a more synthetic and
140 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).

144 The complement system can also act as an amplifier for other molecules involved in injury. The
145 C5a/C5aR axis participates in neutrophil recruitment and activation, which in turn can induce
146 complement activation in AAV (39). C5a induces tissue factor expression by neutrophils, leading to
147 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 or
161 activation products.

- 162 ○ For the quantification of individual complement proteins in plasma, various types of immunoassays
163 are used to determine the concentration of individual complement components. The most common
164 is nephelometry. Polyclonal antibodies to component are added in excess of the sample and bind
165 to their target. Quantification is performed by passing a light beam through the sample, which is
166 distorted by the IC that have formed (45).
- 167 ○ Quantification of complement activation products corresponding to cleavage fragments or
168 complement protein complexes (C3a, C3dg, C4a, C4d, Ba, Bb, C5a, C3bBbP, MASP2, and sC5b-
169 9) is possible. Several assays have been described, mostly based on the recognition of a neoepitope
170 of the complement component in an enzyme-linked immunosorbent assay (ELISA) format. Thus,
171 C4a and C4d reflect CP/LP activation, Ba, Bb, and C3bBbP reflect AP activation, MASP2 is a key
172 enzyme in LP activation (46) and increasing soluble C5b-9 reflects TP activation (47). C3a and
173 C5a are common to the three activation pathways.
- 174 ○ Detection of auto-Abs (anti-FH, FB, C3b, C3bBb, and C1q) targeting complement proteins can be
175 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
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
188 difference is the readout, which consists of the unquenching of a fluorescent dye and not the lysis
189 of erythrocytes.
- 190 ○ 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 acetylated 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.
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
202 human FH to their surface. In the assay, sheep erythrocytes are incubated with human plasma in
203 Mg-EGTA buffer, allowing activation of AP only. Normal plasma does not induce lysis, whereas
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).
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
210 developed by the addition of rat serum.

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 use of an *ex vivo* endothelial assay. The assay detects and quantifies complement component deposition

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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
228 (56). As first barrier between the blood and interstitium it is in constant equilibrium with the
229 environment. Thus, heterogeneity in the structure and function of EC is a core property of the
230 endothelium, allowing diverse vascular functions and regional specificity (57,58). This diversity can
231 be partially explained by a distinct transcriptional profile (59) in relation to neighboring cells (60).
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
238 steady state, the modulation of complement protein expression under inflammatory conditions differs
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
242 regulatory molecules (FH, FI, CD46, CD55, and THBD), generated lower levels of C3a and C4a, and
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
245 thrombomodulin (TM), and increase in C3 and FB transcription in HRGEC exposed to TNF (63).
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
248 HUVEC exposed to interferon (INF) gamma (65), increased C2, FH, FB, and C1inh transcription, and
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)
251 in the resting state and after overnight exposure to heme (67). While there was no difference in
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**).

258 EC used for *ex vivo* experiments comprise two types: conditionally immortalized EC (CI-EC) and
259 primary EC (**Table 2**). Primary EC can be difficult to isolate and maintain in culture, and have
260 a limited lifespan. Moreover, differences in the genetic background of individual donors can
261 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
263 developed to overcome these difficulties. HMEC-1 and CI-GENC are HMEC and GENCs,
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).
266 The EA.hy926 cells were used to generate glycosylphosphatidylinositol-anchored complement
267 regulatory protein-deficient cells when treated with phosphatidylinositol-specific
268 phospholipase C. These cells have been used along with the PIGA-mutant TF-1 to study
269 complement deposits by confocal microscopy and flow cytometry after incubation with serum
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
272 reportedly higher than after incubation with thrombotic thrombocytopenic purpura (TTP)
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
276 are associated with complement activation. However, in aHUS, complement overactivation
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.

280 Micro- or macrovascular origin of the EC tissue lineages also needs to be considered. Complement-
281 mediated EC injury demonstrates specific cell tropism according to pathophysiological processes. In
282 HUS and TTP, microvascular EC of dermal, renal, and cerebral origin are more sensitive to apoptosis,
283 whereas microvascular EC of pulmonary and hepatic origin and macrovascular EC are resistant (74).
284 Distinct sensitivity of EC to complement attack has also been explored in aHUS and heme exposure.
285 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).

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

292 Comparative analysis of the available endothelial assays

293 These tests consist of the quantification of complement activation products (C3 activation fragments
294 and C5b-9) deposits on EC by immunofluorescence (IF) measured by confocal microscopy or flow
295 cytometry (fluorescence-activated cell sorting, FACS) after incubation with a serum sample of interest.
296 Different protocols have been proposed to study complement activation on the EC surface in several
297 pathological conditions, including aHUS (31,79–89), TMA of other etiologies (90,91), HELLP
298 syndrome and pre-eclampsia (92), C3G (15,93), lupus nephritis (LN) (94,95), APS (96,97), SCD (30),
299 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?*

302 Resting EC or EC pre-activated by cytokines, ADP, or heme can be used (**Table S3**) to provide
303 additional information.

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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
311 FH/CFHR1 hybrid, enhanced C3c or C5b-9 deposition was reported only if serum was collected during
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
316 protein expression enable complement activation. This was achieved in the case of P-selectin
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
334 and processing must be achieved (102). Processing of plasma or serum sample must be performed
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
351 experience. Aiello et al. reported that C3 and C5b-9 deposits obtained after a single healthy subject
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).

354 Several authors did not use any positive controls for their experiments (83,84,86,91,92). The
355 comparison was only made with the deposits obtained with negative controls. It might be interesting
356 to position the results on a scale. Positive controls with published data are FH or FI depleted NHS
357 (15,80,82) or normal serum supplemented with blocking anti-FH antibodies targeting the N-terminus
358 or C-terminus (31,81) or with FH19-20, corresponding to the two last domains of FH, able to compete
359 with the full FH protein for cell surface binding (98).

360 The main issues with this type of assay are the lack of validated international standards as well as
361 standardized positive and negative controls. The variability of the results in samples from healthy
362 donors needs to be studied extensively to determine the appropriate cutoff. In addition, the impact of
363 C3 or other complement protein consumption in the patient and the influence of the pre-analytical
364 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
368 the deposition of complement component products resulting from activation or regulation. C3c (a
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
385 settings. The test can be performed under different conditions to avoid CP and LP contributions, which
386 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,

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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
393 complement components or protein complexes when activated components bind their target (i.e., C3a,
394 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
403 systematically digitized along the surface is quantified. The quantified results expressed as the mean
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
416 of complement on endothelial injury. Analysis of transcriptomic modifications in EC exposed to
417 complement deposits under several conditions could also be of interest.

418

419 Clinical and therapeutic relevance of the obtained results

420 The *ex vivo* EC assay, consisting in the quantification of complement activation products (C3 activation
421 fragments or C5b-9) deposits on EC (by IF measured on confocal microscopy or FACS), after
422 incubation with a serum sample of interest, was first used for specific characterization of complement
423 component abnormalities (79–82,84) or exploration of mechanisms implicated in EC injury (31) in the
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
435 contrast, only one patient without relapse showed increased deposits (86). In clinical practice, CH50 is
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
438 monitoring of eculizumab dosage tapering or discontinuation, as it is not well correlated with relapse
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
441 of interest to a better personalized complement-blocking therapy, but first needs to be more
442 standardized.

443 This test can also be used to better classify and assess the prognosis of specific diseases. This is the
444 case for hypertensive TMA, as Timmermans et al. demonstrated in a cohort of hypertension
445 emergencies associated with TMA(108). The authors demonstrated a statistical association between
446 increased C5b-9 deposition in the EC *ex vivo* test and kidney survival. Moreover, they reported an
447 improvement in renal function for those with increased deposits treated with eculizumab. The authors
448 proposed a classification of TMA-hypertensive emergency based on the EC *ex vivo* test (108).

449 Finally, many new anti-complement drugs targeting specific steps of the cascade have been under
450 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**

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

459 The development of tests with these capabilities could allow for a deeper exploration of the
460 mechanisms of complement activation in several diseases. This information could inform the
461 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.

466 However, there are still unanswered questions hindering broad used. The first is the variability in the
467 results and the inter-individual variability in deposits induced by normal sera. Comprehension of the
468 precise mechanism responsible for complement deposition in this assay would improve its better use.
469 The second issue is to standardize the main steps of the procedure to improve the interexperimental
470 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**

476 MSM, SC, and LR conceptualized and conceived the manuscript. MSM drafted the manuscript,
477 including the literature search, reading, and writing. SC, VFB, AD, and LR edited and critically
478 evaluated the manuscript. All authors have contributed to the manuscript and approved the submitted
479 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

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	C3	↑	↑	→*	
	C4	→*	↑	→*	
	C5	→*		→*	
	FB	↑		→*	
	FD	→*		→*	
	Properdin	↓*		→*	
	FH	→		→*	
	FI			→*	
	TM	↓		↑	
	CD46	↑		→	↓
	CD55	→		→	↓
	CD59	→		→	
	E-selectine	↑	→		
	C3aR	↑			
	C5aR	0			
BMVEC	C3	↑			
	C4	→*			
	C5	→*			
	FB	↑			
	FD	→*			
	Properdin	↓*			
	FH	→			
	CD46	↑			
	CD55	→			
	C3aR	↑			
	C5aR	0			
HMEC	E-selectine	↑	→		
	C3		→		
	C4		↑		
	CD46				↓
	CD55				→
HUVEC	C2		↑		
	C3	↑	→/↓	↑	
	C4			→*	
	C5			→*	
	FB	↑	↑	↑*	
	FD			→*	
	Properdin			→*	
	FH		↑	↓	
	FI			→*	
	TM	↓		→	
	CD46	↑		→	↓
	CD55	↑		↑	↓
	CD59	→		→	→
	E-selectin	↑	→		
	P-selectin				↑
	C1-inh		↑		

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863 The data presented here are mainly concerned with protein expression. * denotes transcriptomic data. For details, please
864 refer to **Table S2**. Abbreviations: 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	CI-GEEnC HMEC-1	BMVEC HRGEC BOEC

869 Abbreviations: BMVEC, brain microvascular endothelial cells; BOEC, blood outgrowth endothelial cells; CI-GEEnC,
870 conditionally immortalized human glomerular endothelial cells; HMEC, human microvascular endothelial cells; HRGEC,
871 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
878 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 immunoglobulins, 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.

902 **Figure 3: Comparative analysis of different protocols used for the *ex vivo* complement**
903 **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
906 umbilical vein endothelial cells (HUVEC), blood outgrowth endothelial cells (BOEC), and glomerular
907 endothelial cells (GEnC). 2: Cultured EC are then used at their resting state or after an activation by
908 either ADP, heme, LPS, TNF/INF gamma, or apoptonecrotic cells. 3: EC are incubated with sample of
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

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

917 **Figure 4: Current and future application fields of the *ex vivo* complement activation test on** 918 **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