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## RESEARCH ARTICLE

# Systemic complement activation influences outcomes after allogeneic hematopoietic cell transplantation: A prospective French multicenter trial

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## Abstract

Complement activation has shown a role in murine models of graft-versus-host disease (GVHD) and in endothelial complications after allogeneic hematopoietic cell transplantation (allo-HSCT). However, its impact on post-transplant outcomes has not been so far fully elucidated. Here, we conducted a prospective multicentric trial (NCT01520623) performing serial measurements of complement proteins, regulators, and CH50 activity for 12 weeks after allo-HSCT in 85 patients receiving a myeloablative conditioning (MAC) regimen for various hematological malignancies. Twenty-six out of 85 patients showed an “activated” complement profile through the classical/lectin pathway, defined as a post-transplant decline of C3/C4 and CH50 activity. Time-dependent Cox regression models demonstrated that complement activation within the first weeks after allo-HSCT was associated with increased non-relapse mortality (hazard ratio [HR]: 3.69, 95% confident interval [CI]: 1.55–8.78,  $p = .003$ ) and poorer overall survival (HR: 2.72, 95% CI: 1.37–5.39,  $p = .004$ ) due to increased

Anne Béatrice Notarantonio, Maud D'aveni-Piney, Olivier Hermine, Marie Agnès Durey-Dragon, and Marie-Thérèse Rubio contributed equally to this study.

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incidence of grade II–IV acute GVHD and in particular gastrointestinal (GI) GVHD (HR: 36.8, 95% CI: 12.4–109.1,  $p < .001$ ), higher incidences of thrombotic microangiopathy (HR: 8.58, 95% CI: 2.16–34.08,  $p = .0022$ ), capillary leak syndrome (HR: 7.36, 95% CI: 2.51–21.66,  $p = .00028$ ), post-engraftment bacterial infections (HR: 2.37, 95% CI: 1.22–4.63,  $p = .0108$ ), and EBV reactivation (HR: 3.33, 95% CI: 1.31–8.45,  $p = .0112$ ). Through specific immune staining, we showed the correlation of deposition of C1q, C3d, C4d, and of C5b9 components on endothelial cells in GI GVHD lesions with the histological grade of GVHD. Altogether these findings define the epidemiology and the clinical impact of complement classical/lectin pathway activation after MAC regimens and provide a rationale for the use of complement inhibitory therapeutics in a post-allo-HSCT setting.

## 1 | INTRODUCTION

Allogeneic hematopoietic cell transplantation (allo-HSCT) remains the only curative strategy in most hematological malignancies. However, its beneficial effect can be hampered by the occurrence of alloreactive complications such as acute and chronic graft versus host disease (a and cGVHD), responsible for a significant rate of morbidity and mortality.<sup>1</sup> The pathophysiology of aGVHD involves cell interaction and activation of donor T cells by recipient and donor antigen presenting cells (APCs), in particular dendritic cells (DCs).<sup>1</sup> The complement system plays a crucial role between the innate and adaptive immunity.<sup>2</sup> It is now well recognized that the C3a and C5a anaphylatoxins produced after the different complement activation pathways can regulate the functions of DCs and T cells expressing the corresponding receptors C3aR and C5aR in mice and humans.<sup>3</sup> Both C3a and C5a activate DCs which in turn enhance alloreactive Th1 and Th17 T cell differentiation and proliferation<sup>4–8</sup> and reduce the generation and stability of regulatory CD4<sup>+</sup> T cells.<sup>9</sup> Additionally, complement inhibitory decay-accelerating factor (DAF or CD55) deficient DCs may enhance C3a and C5a production, increasing T cell activation and reducing Treg induction.<sup>9–11</sup>

Complement activation has been observed in mouse models of aGVHD, mainly due to the activation of the alternative pathway triggered by transplant conditioning regimen-induced damages.<sup>12,13</sup> Complement C3d and C5b9 membrane attack complex deposits have been observed in GVHD target organs.<sup>13,14</sup> Moreover, recipient mice deficient in C3 or C3aR/C5aR were protected from GVHD mortality,<sup>12,15,16</sup> while experiments performed with DAF-null (*Daf1<sup>-/-</sup>*) host BM and *Daf1<sup>-/-</sup>* donor lymphocytes led to exacerbated GVHD.<sup>12</sup> Pharmacological anti-C3aR/C5aR antagonists and the alternative pathway CR2-FH inhibitor can also control experimental GVHD in conventional and xeno-GVHD mouse models.<sup>13,15</sup>

In humans, complement activation has largely been described in thrombotic microangiopathy (TMA) occurring after allo-HSCT and in minor part in other endothelial complications,<sup>17–21</sup> but its potential role in GVHD is far from being completely understood. Monitoring of circulating complement fractions C3, C4, and immune complexes reported increased levels in patients developing chronic GVHD.<sup>22,23</sup> Some studies have suggested an implication of complement activation

in human GVHD since C4d and C3 deposition has been observed in skin and gastrointestinal (GI) GVHD organs during acute and chronic GVHD.<sup>7,24</sup> Moreover, *in vitro* inhibition of C3 by compstatin has been reported to be able to reduce human CD4 and CD8 T cell proliferation and differentiation into Th1 and Th17 T cells.<sup>7</sup>

The presented study was designed to prospectively explore the dynamic of complement activation early after allo-HSCT following a myeloablative conditioning (MAC) regimen. We, therefore, measured complement fractions and regulators once a week after allo-HSCT for 3 months and correlated composite metrics of complement activity with the occurrence of GVHD, endothelial complications, infections, and survival outcomes.

## 2 | METHODS

### 2.1 | Study design and data collection

We performed a prospective multicenter study in three university hospitals in Paris (Necker Hospital, Saint-Antoine Hospital, and Saint Louis Hospital). The study was registered in [clinicaltrials.gov](https://clinicaltrials.gov) under the number NCT01520623. All patients provided written informed consent authorizing the usage of their personal information for research purposes. This research was conducted under the principles and the legacies of Helsinki Declaration.

The study included adult patients receiving a first allo-HSCT for hematological malignancies following MAC regimens. Patients and sample recruitment were performed between 2010 and 2012. Patients were followed for at least 2 years or until death. Data collection and final analysis were performed between 2019 and 2022.

### 2.2 | Transplant procedures

Authorized MAC regimens were busulfan cyclophosphamide (BuCy) (intravenous [iv] busulfan 3.2 mg/kg/day  $\times$ 4 days and cyclophosphamide 60 mg/kg iv daily for 2 days), cyclophosphamide/TBI (12 Grays of total body irradiation and cyclophosphamide 60 mg/kg iv daily for

2 days) or fludarabine melphalan (Flu/Mel) (fludarabine 120 mg/m<sup>2</sup> and iv melphalan 140 mg/m<sup>2</sup>). Hematopoietic stem cell source could be peripheral blood or bone marrow stem cells from a sibling, a 10/10 HLA matched or 9/10 HLA mismatched-unrelated donor. Patients who received allo-HSCT from a haplo-identical donor or umbilical cord blood were excluded. GVHD prophylaxis included standard protocols based on cyclosporine A (CSA) and short-term methotrexate or CSA with mycophenolate mofetil. In addition, some patients, in particular recipients of unrelated donor transplants received rabbit anti-thymocyte globulin (Thymoglobulin at 2.5 mg/kg/day for 2 days before transplantation) or anti-lymphocyte globulin (Grafalon at 20 mg/kg/day for 3 days).

Clinical data including gender, age, diagnosis, comorbidities, donor, graft source, human leukocyte antigen (HLA) matching, cytomegalovirus (CMV), and Epstein–Barr virus (EBV) serostatus, conditioning regimen, GVHD prophylaxis, as well as clinical outcomes regarding acute and chronic GVHD, infectious events, relapse, endothelial complications, mortality, causes of death, were prospectively collected in a specific case report clinical form (CRF).

### 2.3 | GVHD grading and endothelial complication definitions

Grading of acute GVHD was performed using the Glucksberg's established criteria.<sup>25</sup> Chronic GVHD was classified during the time of inclusion as limited or extensive according to published criteria<sup>26</sup> and a posteriori reclassified as mild, moderate or severe according to National Institute of Health consensus criteria.<sup>27</sup> Capillary leak syndrome (CLS) was diagnosed in patients presenting with weight gain (>10% increased body weight) with a generalized edematous syndrome resistant to diuretic treatment, within the first 15 days after HSCT in the absence of cardiac and renal dysfunctions.<sup>28</sup> TMA was diagnosed according to the International Working Group (IWG) of the European society for blood and marrow transplantation-EBMT criteria: schisocytosis >8 per high power field on peripheral blood smear, normal coagulation assay, increase in serum lactate dehydrogenase (LDH), thrombocytopenia, anemia, decrease in serum haptoglobin.<sup>29</sup> Diagnosis of sinusoidal obstruction syndrome/veno-occlusive disease (SOS/VOD) was based on the modified Seattle criteria with the presence of at least two of the three following clinical features within 20 days post-HSCT: hyperbilirubinemia (>2 mg/dL), hepatomegaly with hepatalgia, weight gain >2%.<sup>30</sup>

### 2.4 | Complement proteins and regulators measurements

Complement components measurements were performed in a national referral center for complement system investigations (Laboratoire d'Immunologie Biologique, Hôpital Européen Georges Pompidou–HEGP-Paris). Plasma samples were collected from patients at admission for the pre-HSCT timepoint, and subsequent samples

were collected weekly after allo-HSCT until 12 weeks (Figure S1). For flow cytometry study, one EDTA whole blood sample was sent at room temperature to the lab and the analysis was performed less than 48 h after collection. For all plasma protein measurements, after a short transportation in ice to the lab (less than 4 h), centrifugation at +4°C and separation in at least four aliquots of plasma EDTA, the samples were frozen at –80°C and the measurements in the a few days later were performed according to the routine cadence. All results were obtained within 10 days after sample collection. Plasma concentrations of complement factors (C3, C4, factor B) and complement regulator C1-esterase inhibitor were measured by nephelometry (nephelometer BN ProsPec, Siemens), while the alternative pathway regulators, Factor H and Factor I levels were determined by in-house ELISA assays, as previously described.<sup>31,32</sup> Surface expression of complement regulatory molecules CD46 and CD55 (DAF, Decay-Accelerating Factor) were analyzed on leucocytes by flow cytometry using the following monoclonal antibodies: anti-human CD46, (clone Be2eBioscience) and anti-Human CD55, (clone IA10 BD Bioscience).<sup>33</sup> Complement CH50 activity was estimated as previously described.<sup>34</sup> For each patient, the profile of complement activation was carefully assessed in a blinded manner by HEGP investigators who did not have access to clinical data.

### 2.5 | Immunohistochemistry on gastrointestinal biopsies

In selected cases of patients with suspicion of GI acute GVHD, endoscopies GI and gut biopsies were performed for diagnosis and for detection of complement fractions by immunohistochemistry. Gut tissues were placed in 10% formalin, then embedded in paraffin, and sectioned using a microtome (Microm, France) 3.5 µm. After deparaffinization and rehydration step, the samples were colored by hematoxylin–eosin–safran (HES). In addition to HES coloration, immunostaining was performed in several steps. First, we blocked endogenous peroxidases, biotin, and avidin. Second, samples were incubated with the following primary monoclonal antibodies for 1 h (anti-C4d human: Db Biotech [DB107-0.5], 1/100; anti C3d human: Abcam [AB15981], 1/200). Anti-C1q: DakoCytomation (A0136, 1/300; anti-C5b9 human: Hycult Biotech [HM2264]). Third, samples were incubated with secondary antibodies (anti-IgG mice: Abcys, 3A2000, 1/200; anti-IgG rabbit: Abcys, BA-1000, 1/400). GVHD pathological scoring was evaluated using under double-blinded conditions as previously described.<sup>35</sup> Photographs were taken using optic microscope (Grossisment \*40). The number of blood vessels stained by mm<sup>2</sup> was quantified thanks to a NanoZoomer Digital Pathology (Hamamatsu Photonics, Bridgewater, NJ).

### 2.6 | Statistical analysis

Quantitative variables were presented as median and interquartile range (Q1–Q3). Qualitative variables were presented as number and

percentage. Association between quantitative variables and groups of interest was compared using the non-parametric Wilcoxon rank sum test and between qualitative variables and groups of interest using the Fisher exact or Chi-square tests, as appropriate. Overall survival (OS) was measured from the date of HSCT to the date of death from any cause or of last follow-up. Non-relapse mortality (NRM) was defined as death occurring in continuous complete remission (CR). Cumulative incidence function (CIF) of complement activation was assessed in competing risk setting with death before day 100, without complement activation, considered as competing event. Proportional hazard models for the competing risk subdistribution were used to assess the impact of baseline variables on complement activation.<sup>36</sup> In this analysis, the incidence of complement activation was censored at 12 weeks after allo-HSCT.

Impact of the complement activation on outcomes was evaluated as a time-dependent variable using univariable and multivariable Cox models.<sup>37</sup> For graphical representation of the impact of complement activation on outcomes Simon-Makuch plots were used.<sup>38</sup>

This method takes into account changes in an individual's covariate status over time, with individuals at risk within each level not fixed at time 0.<sup>39</sup>

For multivariable models, stepwise regression consisting of alternating forward and backward elimination steps was used to find the most parsimonious set of significant covariates.

All statistical tests were two-sided, and a  $p$ -value  $< .05$  was considered statistically significant.

Two-sided tests were used in all analyses. Statistical analyses were performed with R-Project 4.2 (R Foundation for Statistical Computing, Vienna, Austria) software packages.

## 3 | RESULTS

### 3.1 | Landscape of complement activation after allo-HSCT

In total 85 patients were included in this study (Figure S1, Table 1). None of the patients received complement inhibitors during the study.

We first asked which was the unsupervised pattern of complement activation in post-transplant plasma, in comparison with pre-transplant time-points. At baseline, before transplantation, all the patients showed normal to increased levels of complement proteins C3, C4, and of CH50 activity (Figure 1A–C). After allo-HSCT, overall, increased levels of C3, C4 fractions, CH50 activity, as well as C1 esterase inhibitor, were detected in the early post-transplantation period (Figure 1A–C, Figure S2). Nonetheless, in post-transplant patients, we saw significant interindividual variations of plasma C3 and C4 levels and of CH50 activity (Figure S3). Starting from this variability, one of the biggest challenges was to define the criteria of complement activation in the post-transplant phase. Analyzing the density of distribution of each studied complement component, we observed that each patient presented a unique post-transplant profile strictly

dependent on pre-transplant complement values. In particular, two patterns of complement activity were evident: patients with sustained or increased levels after HSCT in comparison with pre-transplant levels (with all of the points  $> 80\%$  of pre-transplant levels, Figure 1D,E) and others showing a decline, below normal values, of C3, C4, and CH50 during the three first months post-HSCT (with at least two consecutive points below 100% of pre-transplant levels, Figure 1F,G). Based on these profiles we defined as criteria for complement activation a decrease of C3 and C4 proteins and of CH50 activity below normal values and, at least at one point during the follow-up, a drop of 50% of C3 and C4 and of 70% of CH50 activity in comparison with pre-HSCT levels. We then categorized all patients as complement “activated” and “non-activated.” Among the 85 patients, 26 met the criteria of complement activation and were considered in the “complement activated” group, and 59 were “non-activated” (Table 1). Of note, one patient died at day +95 without complement activation and all the other “non-activated” patients were alive at day +100 post-transplant. Complement activation occurred during the first weeks after HSCT in most patients, with the lowest C3 or C4 levels between day 10 and day 70 post-HSCT in the activated group (median at 21 days). Means of C3, C4 levels and of CH50 activity of the complement-activated group patients were significantly reduced as compared with the complement non-activated group patients between weeks (W) two and seven post-HSCT (W2 to seven for C3, W2 to six for C4, W2 to four for CH50) (Figure 1I–K). Patients in the complement-activated group had slightly lower levels of B factor, but the differences were only significant at Weeks 3, 5, and 11 post-HSCT (Figure S4B), possibly due to the amplification loop of the classical/lectin cascade more than a sustained, independent activation of the alternative pathway. Levels of C1-esterase-inhibitor, factor H and factor I, negative complement regulators, were similar in both complement-activated and non-activated groups indicating that complement activation was not related to impaired control mechanisms (Figure S4A,C). DAF (CD55) expression on leukocytes was globally comparable between the two complement activation groups (Figure S4D). A slight increase of DAF was observed on Week 9 post-transplantation in the activated group, possibly due to the establishment of a negative retro-control after complement activation. Although there was a slight decrease of CD46 expression on leukocytes in the complement-activated group, this was only observed between 9 and 12 weeks post-HSCT, a long time after the occurrence of complement activation (Figure S4E).

### 3.2 | Activation of classical/lectin complement pathway correlates with post-transplant outcomes

We then asked whether patients with complement activation could display a different clinical pattern in terms of baseline characteristics and post-transplant outcomes. Baselines characteristics of patients with a complement-activated profile were mostly similar to those of patients without complement activation (with no differences in terms of stem cell source, type of MAC regimen, GVHD prophylaxis, use of T-depletion),

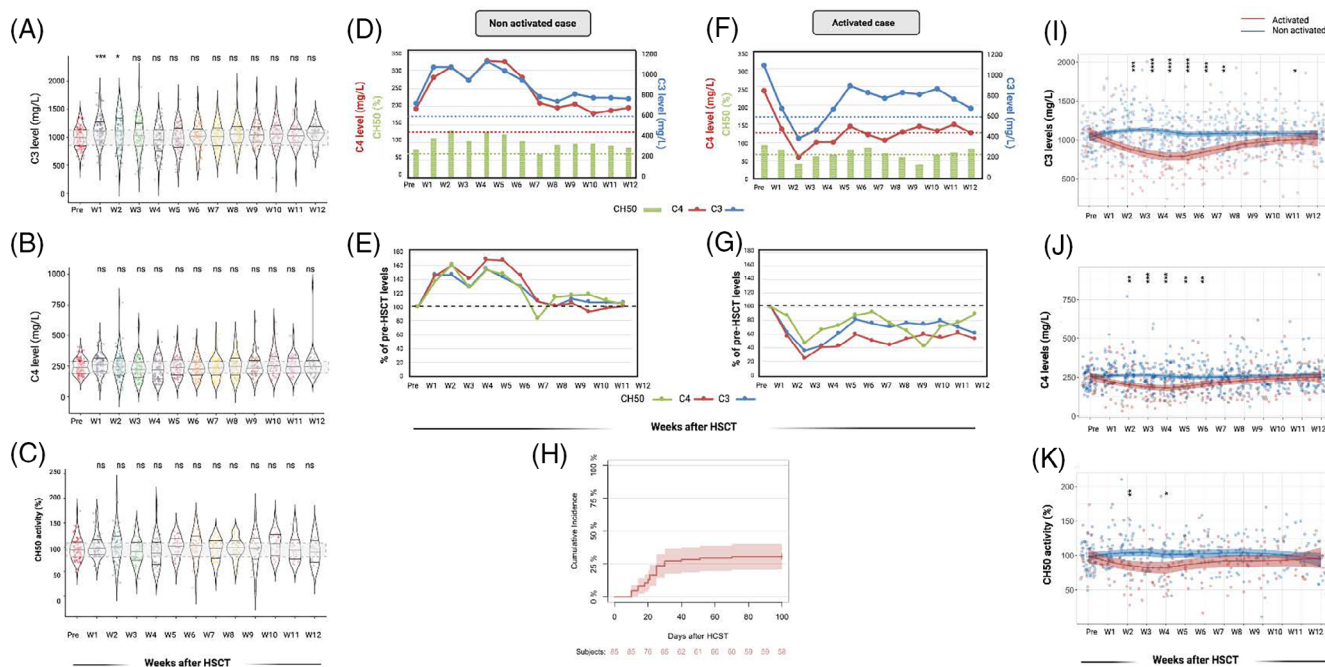
TABLE 1 Patients' characteristics.

	All N or median (%/IQR)	Activated	Non activated	p-Value
	85	26	59	
Age at transplant (years)	39.28 (29.3–49.4)	38.6 (26.6–51.6)	40.2 (31.1–49)	.7566
Age at transplant (years) cut-off				
≤45 years	53 (62%)	15 (58%)	38 (64%)	.7004
>45 years	32 (38%)	11 (42%)	21 (35%)	
Recipient gender				
Male	53 (62%)	13 (50%)	40 (68%)	.1186
Female	32 (38%)	13 (50%)	19 (32%)	
Hematological malignancies, n (%)				
AML, MDS	37 (43%)	8 (31%)	29 (49%)	.5531
ALL	23 (27%)	9 (35%)	14 (24%)	
MPD	10 (12%)	3 (11%)	7 (12%)	
Lymphoma	9 (7%)	4 (15%)	5 (8%)	
Myeloma	6 (11%)	2 (8%)	4 (7%)	
Disease status, n (%)				
CR	56 (66%)	12 (46%)	44 (75%)	.0373
PR	20 (23%)	10 (39%)	10 (17%)	
Active	9 (11%)	4 (15%)	5 (8%)	
Conditioning regimens, n (%)				
TBI-Cy	38 (45%)	14 (54%)	24 (41%)	.0797
Bu-Cy	31 (36%)	5 (19%)	26 (44%)	
Flu-Mel	16 (19%)	7 (27%)	9 (15%)	
Type of donor, n (%)				
Identical sibling	40 (47%)	10 (38%)	30 (51%)	.1415
Matched unrelated (10/10)	38 (45%)	13 (50%)	25 (42%)	
Mismatched unrelated (9/10)	7 (8%)	3 (12%)	4 (7%)	
Stem cell source, n (%)				
Bone marrow	38 (45%)	13 (50%)	25 (42%)	.4247
Peripheral blood	47 (55%)	13 (50%)	34 (58%)	
CMV risk, n (%)				
Low-risk (donor negative to recipient negative)	24 (29%)	10 (38%)	14 (24%)	.1001
Intermediate-risk (others)	41 (48%)	8 (31%)	33 (56%)	
High-risk (donor negative to recipient positive)	20 (23%)	8 (31%)	12 (20%)	
In vivo T-cell depletion, n (%)				
No ATG	13 (15%)	4 (15%)	9 (15%)	.9987
ATG	72 (85%)	22 (85%)	50 (85%)	
GVHD prophylaxis				
Ciclosporin A or tacrolimus + MTX	72 (85%)	21 (81%)	51 (86%)	.5032
Ciclosporin A + MMF	13 (15%)	5 (19%)	8 (14%)	

Note: For linear variables Wilcoxon signed-rank test was used, for categorical variables Chi-square test was deployed.

Abbreviations: ALL, acute lymphoid leukemia; AML, acute myeloid leukemia; ATG, anti-thymocyte globulin; Bu, busulfan; CMV, cytomegalovirus; CR, complete response; CsA, cyclosporin A; Ct, complement; Cy, cyclophosphamide; Flu, fludarabine; GVHD, graft versus host disease; IQR, interquartile range; MDS, myelodysplastic syndrome; Mel, melphalan; MMF, mycophenolate mofetil; MPD, myeloproliferative disease; MTX, methotrexate; PR, partial response; TBI, total body irradiation.

\*p-Value of comparison of activated versus non activated groups.



**FIGURE 1** Distribution and evolution of complement components in transplant recipients across time. Violin plots depicting the distribution of C3 (A), C4 (B) complement components and CH50 activity (C) levels across the time points in the study. Each dot represents one plasma sample, the gray area denotes the range of pre-transplant specimens. Pairwise comparisons were performed with non-parametric Wilcoxon signed rank test using as referent group the pre-hematopoietic stem cell transplantation (HSCT) distribution. Ns, non significant; \* $p < .05$  but  $> .01$ ; \*\* $p < .001$ ; \*\*\* $p < .0001$ ; \*\*\*\* $p < .00001$ . Kinetics of C3, C4, and CH50 in absolute value and in the percentage of pre-HSCT levels in a representative case of no-complement activation patient (D, E) and in case of complement activation (F, G). The dashed lines represent the lower values of C3 (blue), C4 (red), and CH50 (red). (H) Cumulative incidence of complement activation within the first 100 days after allo-HSCT. (I–K) Comparison of the kinetics of absolute level values of C3 (I), C4 (J), and CH50 (K) between patients with complement activation (red) and those without complement activation (blue) \* $p < .05$ , \*\* $p < .01$ , \*\*\* $p < .001$ . [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

except for a significant enrichment in advanced/progressive diseases in the activated group ( $p = .03$ ) (Table 1). Cumulative incidence of complement activation was estimated at 27.1% (95% confident intervals [CI]: 18.1–36.8) at day +30, 29.4% (95% CI: 20.1–39.3) at day +60, and 30.6% (95% CI: 21.1–40.6) at day +100 (Figure 1H). Univariable analysis confirmed disease activity as a factor associated with higher cumulative incidence of complement activation (Table S1).

Median time to neutrophil engraftment was similar in both groups, while median time to platelet engraftment was longer in the activated (23 days, range 10–80) compared with the non-activated group (16 days, range 7–89,  $p = .029$ ). Of note, no differences in terms of incidence of neutrophil and platelet engraftment were seen between the two groups (Figure S5).

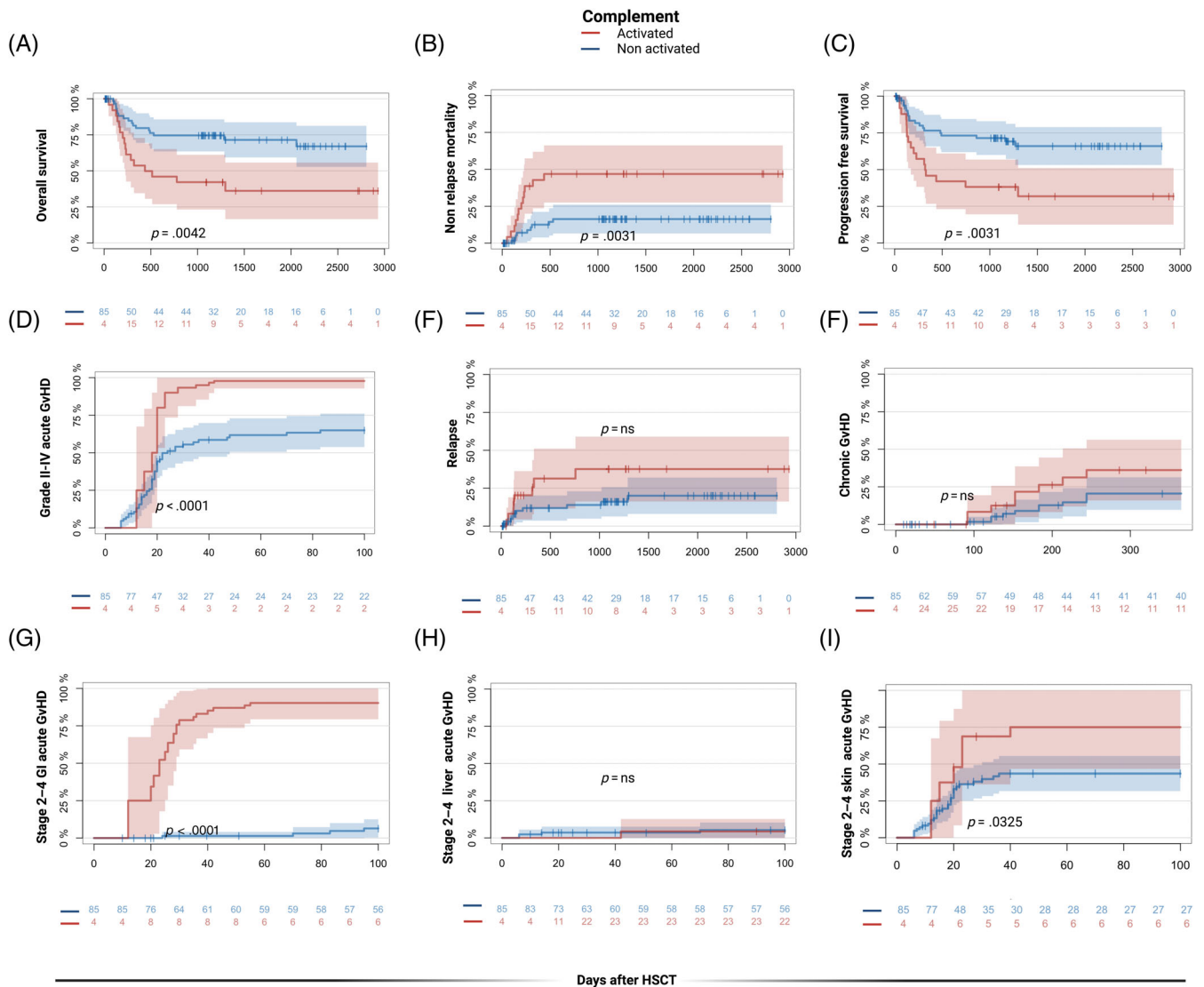
Univariable time-dependent analysis showed for patients with complement activation worse clinical outcomes in terms of OS (hazard ratio [HR]: 2.72, 95% CI: 1.37–5.39,  $p = .00427$ ), NRM (HR: 3.69, 95% CI: 1.55–8.78,  $p = .00317$ ), progression-free survival (PFS) (HR: 2.69, 95% CI: 1.39–5.20,  $p = .00318$ ) and grade II–IV acute GVHD (HR: 4.24, 95% CI: 2.12–8.49,  $p = .00005$ ) (Table S2, Figure 2A–D). No significant impact of complement activation was observed on the incidence of relapse and chronic GVHD (Table S2, Figure 2E,F). These results were confirmed through the application of time-dependent multivariable Cox regression models, adjusted for donor, recipient

age, and disease status. More specifically, the negative impact of complement activation was independently shown on OS, PFS, and acute GVHD. In these models, a mismatched unrelated donor, was an additional negative predictor for OS, PFS, and acute GVHD, a matched unrelated donor also impacted acute GVHD, while refractory disease status impacted alone on relapse and in addition to complement activation on NRM and PFS (Figure 3, Table S3).

The main causes of NRM in the complement activation group were GVHD ( $n = 9$ ), infections ( $n = 7$ ), and vascular complications ( $n = 1$ ); while deaths in the non-activated group were due to sepsis ( $n = 6$ ), GVHD ( $n = 4$ ). One patient developed colorectal cancer ( $n = 1$ ) while in hematological remission.

### 3.3 | Association between the complement activation and the risk of acute gastrointestinal GVHD

When dissecting the increased risk of developing acute GVHD, we observed no association between complement activation and hepatic GVHD, while only a mild correlation was found with skin stage 2–4 acute GVHD (Table S2, Figure 2H,I). In contrast, patients with complement activation were characterized by an exponentially higher risk of acute GI GVHD (HR: 36.8, 95% CI: 12.4–109.1,  $p < .00001$ ) (Table S2,



**FIGURE 2** Impact of complement activation profile on post-transplant outcomes. Simon-Makuch plots representing the time-dependent impact of complement activation on overall survival (A), non-relapse mortality (B), progression-free survival (C), acute grade II-IV graft-versus-host disease (GVHD) (D), relapse (E), chronic GVHD (F). [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

Figure 2G), with 20/26 (77%) patients in the complement-activated group versus 2/59 (3%) in the non-activated subcohort showing this event. Moreover, complement activation mainly occurred before the onset of clinical signs of GI GVHD with a median time between systemic complement activation and the appearance of GI GVHD symptoms of 3.5 days (interquartile range 2–6.5).

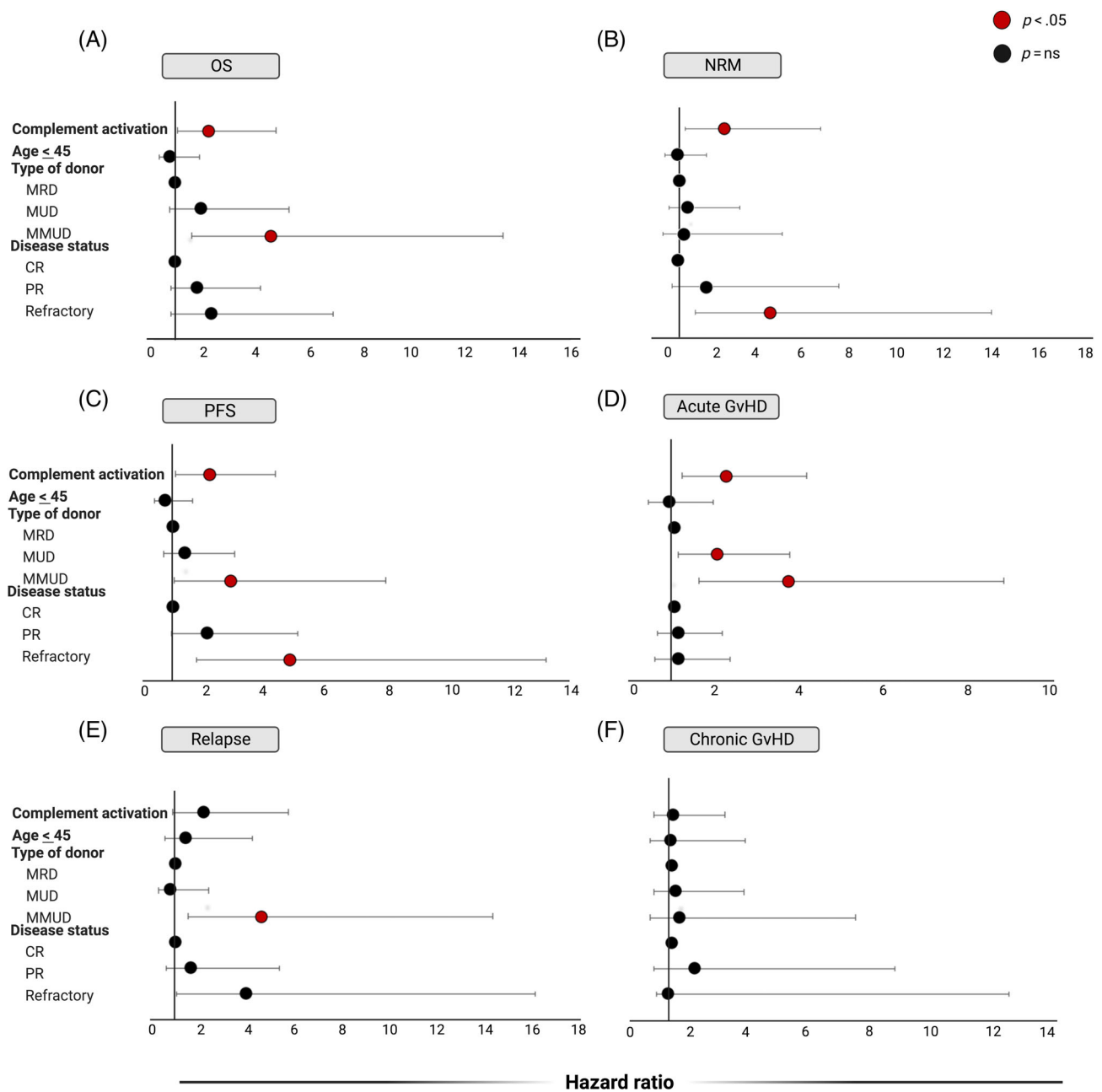
We then asked whether the activation of the classical pathway translated onto histologically confirmed lesions and we analyzed complement protein deposition by immunohistochemistry on gut biopsies of eight patients with grade II-IV GI GVHD using control patients without GVHD available in the pathology laboratory as comparators. In line with a possible pathophysiological contribution of classical complement activation, we observed a relevant deposition of complement components C1q, C3d, C4d, and C5b9 on endothelial cells in GVHD lesions (Figure S6A). Furthermore, the intensity of C1q and C5b9 deposits on blood vessel

sections of GI GVHD cases was proportional to the histological severity of GVHD (Figure S6B). Of note, seven out of the eight patients with proven complement deposits in GI GVHD biopsies showed a complement-activated profile. Interestingly while five of them developed TMA concomitantly or 1 month after the onset of GI GVHD, the remaining three showed C5b9 deposition on gut endothelial cells without the occurrence of TMA. At the time of the study, no histological signs of GI TMA were reported on GI biopsies.

### 3.4 | Endothelial complications, infections, and complement activation

Next, we examined the impact of complement activation on endothelial complications observed in our cohort. Overall, in our study, we





**FIGURE 3** Multivariable Cox regression models of complement activation profile on post-transplant outcomes. Forest plots representing the time-dependent multivariable Cox regression analysis of the impact of complement activation on: overall survival (OS) (A), non-relapse mortality (NRM) (B), progression-free survival (PFS) (C), acute grade II–IV graft-versus-host disease (GVHD) (D), relapse (E), chronic GVHD (F). CR, complete remission; MMUD, mismatched unrelated donor; MRD, minimal residual disease; MUD, matched unrelated donor; PR, partial response. [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

observed a 100-day cumulative incidence of 24.9% (95% CI: 16.2–34.6) for CLS, of 11.8% (95% CI: 6–19.6) for TMA and of 3.5% (95% CI: 0.9–9.1) of VOD/SOS. Time-dependent analysis of complement activation showed a significant association with the occurrence of CLS (HR: 7.36, 95% CI: 2.5–21.6,  $p = .0002$ ) and of TMA (HR: 8.58, 95% CI: 2.16–34,  $p = .0022$ ), while no significant association was seen with VOD/SOS, also in consideration of the low incidence of this complication (Table S2, Figure S7). In total, TMA was diagnosed in 10 patients, seven in the complement-activated group and three in the non-activated group. Among the 26 patients of the complement

activation group, one developed TMA without GI GVHD while six developed TMA after GI GVHD and five of them developed concomitant CLS. TMA occurring in the three non-activated patients was not associated with GI GVHD or CLS.

Looking at infections, we observed a significant association between complement activation and the occurrence of bacterial infections after engraftment (HR: 2.37, 95% CI: 1.22–4.63,  $p = .0108$ ), and EBV infections (HR: 3.33, 95% CI: 1.31–8.45,  $p = .0112$ ) but no correlation was found with CMV reactivation (Table S2, Figure S8).

## 4 | DISCUSSION

We report on the largest prospective study sequentially exploring complement system proteins and regulators in 85 patients during the first 12 weeks after receiving an allo-HSCT from three French centers. We provide the first evidence that systemic complement activation can occur in about 30% of patients transplanted following a MAC regimen representing an independent predictive factor of poor transplant outcome, due to an increase of severe GI acute GVHD, endothelial dysfunctions and infections, definitely impacting on survival, PFS and NRM. On a general note, in our outcome analysis, complement activation is always considered as a time-dependent variable, with incidences computed taking into account events censored after the moment of complement activation. Although clear characterization of the causal link could be odd in certain situations (i.e., complement activation and endothelial syndromes or bacterial infections, well known to trigger themselves complement activity) this analysis allows to consider only the events generated after complement activation, underlying how the development of a certain event could be a consequence more than a cause.

The pattern of complement activation observed in this study included concomitant decline of C3 and C4 levels together with drop in CH50 activity. This significant reduction of C3 and C4 proteins was particularly notable when we considered the inflammatory context observed in the first weeks following the allograft. Indeed, most patients in the non-activated group have an increase of C3 and C4 during the first month post-HSCT due to inflammation, while those in the activated group have a drop of C3/C4 despite high CRP levels. Similarly to the profile observed in systemic lupus erythematosus, the commonly observed pattern of concomitant decrease of C3 and C4 fractions with mild reduction of Factor B, argues for an activation of the classical +/- lectin pathways, rather than an activation of the alternative pathway. This observation needs however to be confirmed with measurement of split fragments such as C4b and Bb/Ba, which were not available at the time of this study.

Although, activation of the classical/lectin pathway has recently been observed in few patients developing TMA after autologous or allo-HSCT,<sup>40,41</sup> activation of the alternative pathway has been demonstrated in mouse models of HSCT<sup>13</sup> and in human studies in post-transplant associated TMA.<sup>21</sup> Indeed, even if the pathophysiology of TMA is not totally elucidated, most human studies reported a biological pattern of complement activation with isolated increase of C5b9 complex in serum, normal C3/C4 serum levels, and implicated the causative role of genetic mutation of factors involved in the alternative pathway.<sup>17,21</sup> The common association between complement activation, independent of the initial activated pathway, and increased TMA after allo-HSCT is supposedly due to common mechanisms of endothelial injury by the terminal components of the complement activation cascade. Complement terminal C5b9 deposition on skin and bone marrow vascular cells recently described in TMA are in line with this statement.<sup>42</sup> In our study, concomitant occurrence of GI GVHD, TMA, and CLS, observed in five patients of the activated group, argues for the possible association of severe endothelial

toxicity with complement activation, corroborated by the observation of C5b9 deposit on endothelial cells of GI GVHD, and the induction of GI GVHD, in agreement with higher incidence TMA in GI GVHD patients.<sup>43</sup> Although not documented, it is possible that at least part of those patients associating TMA, severe GI-GVHD and complement activation and deposition in the gut had also intestinal TMA. However, most of the patients in the activated group developed GI GVHD without TMA nor CLS, suggesting distinct biological patterns of complement activation after allo-HSCT. Our results also suggest that, by contrast with the study describing C5b9 and MASP2 deposition on vascular cells in the skin and bone marrow specifically in TMA patients,<sup>42</sup> complement deposition can be observed on endothelial cells of gut biopsies after allo-HSCT in the absence of TMA. Histological analyses of complement deposits were not primary endpoints of this prospective study but were used as an ancillary proof of concept to determine whether complement activation could feature GI GVHD phenotypes. GVHD features were observed in all the examined biopsies underlying TMA-independent processes. However, we acknowledge that the link between alloreactivity and endothelial activation is very tight and these pathophysiological pictures can overlap in many cases, not allowing proper distinction.<sup>21</sup>

Complement activation occurred in a median delay of 21 days after transplantation and preceded by a few days the appearance of GI acute GVHD symptoms. These results suggest that complement activation can occur during the alloreactive response after HSCT, concomitantly with cytokine release and tissue damage. They are in line with consistent demonstration that the cascade initiation, and in particular the consequent release of the anaphylatoxins C3a and C5a, acts as costimulatory signals enhancing the activation of alloreactive human Th1 and Th17 T cells expressing C3aR/C5aR.<sup>5-7</sup> Our data are in line with the observation that C3a/C5a generated by DCs are essential for fully donor T-cell activation after HSCT in a xeno-GVHD model.<sup>15</sup> Therefore, monitoring plasma levels of complement fraction C3, C4, and of CH50 activity, widely available biological parameters, might be useful to predict the intensity of alloreactivity and the risk of developing severe GI GVHD in patients after allogeneic HSCT.

The reason for the occurrence of complement activation in only 30% of the patients (at day +100), remains unclear. One explanation could be a genetic susceptibility for the classical pathway activation. However, we did not observe reduced levels of the C1-inhibitor, the main regulator of the classical pathway. We observed a secondary increase of CD55 (DAF) as a negative control after the activation, demonstrating its functionality. It would be of interest to measure plasmatic soluble C5b9 and C4d, new biomarkers that reflect the classical and final pathway activation also to investigate genetic alterations of complement proteins and regulators in this context.<sup>44</sup>

Intriguingly, there was no correlation between complement activation and the development of liver acute GVHD and a weaker association with skin GVHD, suggesting the presence of tissue-specific susceptibility factors, arguing for a role of the intensity of the conditioning regimen in the induction of complement activation. Factors that might activate the classical and lectin pathways after allogeneic HSCT following high-intensity chemotherapy or total body

irradiation-based conditioning regimen include apoptotic bodies, lipopolysaccharide, viral RNA, all of which are released from damage tissues, but also CRP, some bacteria species or anti-bacteria immune complexes or leukemic cells.<sup>45</sup> Interestingly, we observed a higher proportion of patients transplanted with an active disease in the activation group, however the lack of minimal residual disease (MRD) detection data in our series precludes a formal analysis of the potential impact of disease burden in the complement activation.

Gut microbiota might be another link between GI acute GVHD and complement activation. In line with this hypothesis, we observed a higher incidence of bacterial infections in the complement-activated group, suggesting a higher use of antibiotics in this group that might have impacted the microbiota. Several studies have reported microbiota dysbiosis in patients undergoing HSCT characterized by a higher proportion of *Enterococcus*, *Enterobacteriaceae*, *Clostridial* bacteria, *proteobacteria*, and a decrease of *Blautia* Genus.<sup>46–49</sup> Patients with an important loss of microbiota diversity have a higher risk of NRM due to a high incidence of GI GVHD.<sup>47</sup> Interestingly, bacteria-like *Enterococcus* can lead to the activation of classical complement pathway,<sup>50</sup> whereas other species, such as *Enterobacteria*, produce LPS and favors the complement lectin pathway activation. Therefore, it would be of interest to analyze gut microbiota diversity and dysbiosis in transplanted patients and correlate the results to complement activation.

Mirroring similar strategies adopted for TMA, pharmacological inhibition of complement activation could be an option for controlling the development of GI GVHD and endothelial dysfunctions in patients developing complement activation. In mouse model of GVHD, despite the distinct complement activation pathways described, it has been shown that targeting recipient C3aR/C5aR by specific antagonists may attenuate GVHD while preserving the GVL effect.<sup>13</sup> More recently, CR2-FH, an inhibitor of C3d and of the complement amplification loop, demonstrated its capability to control xeno-GVHD.<sup>13</sup> In addition, C3 inhibitors are available in clinics and proximal anti-complement inhibitors are under investigation in other disease settings. Our data provide rationale to explore the potential interest of complement inhibitors targeting both the release of anaphylatoxins but also inhibiting the terminal cascade of complement activation.

To conclude, these findings suggest that low levels of complement C3 and C4 are highly associated with poorer outcomes after allogeneic HSCT due to severe acute GVHD and to some extent to vascular complications. Our results suggest weekly measurement of C3, C4, and CH50 during the 3 months following allo-HSCT might be used to predict the occurrence of severe GI GVHD and poor post-transplantation outcomes. Genetic mutation profiles, microbiota sequencing, and deeper immune explorations could improve the understanding of the mechanisms involved in aberrant complement activation after allo-HSCT. These data provide a rationale for evaluating early complement inhibitors such as C3 or C3a/C5a inhibitors in patients showing complement activation profiles after allo-HSCT to improve their outcomes.

#### AUTHOR CONTRIBUTIONS

Marie-Thérèse Rubio, Olivier Hermine, and Marie Agnès Durey-Dragon designed the study, managed all the phases of the trial from

the approval from authorities to patient recruitment and sample centralization; supervised data collection and data analysis and interpreted the results. Their contribution in this work is therefore considered as equal; Marie Agnès Durey-Dragon and Véronique Frémeaux-Bacchi performed the complement factors' measurements; Yayha Ashraf, Tchao Meatchi, and Patrick Bruneval performed the histology and immunohistochemistry analyses; Saik Urien, Naim Bouazza, and Simona Pagliuca performed the statistical analyses and data visualization, Jacques-Emanuel Galimard gave intellectual inputs on study design and statistical analysis; Anne Béatrice Notarantonio, Maud D'aveni-Piney, Simona Pagliuca, and Marie-Thérèse Rubio wrote the manuscript; Marie-Thérèse Rubio, Aliénor Xhaard, Ambroise Marçais, Felipe Suarez, Eolia Brissot, Régis Peffault De Latour included patients in the study. All authors reviewed and approved the final version of the manuscript.

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#### CONFLICT OF INTEREST STATEMENT

This research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

#### DATA AVAILABILITY STATEMENT

The data that support the findings of this study will be openly available in a public repository once the manuscript is accepted for publication.

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#### SUPPORTING INFORMATION

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

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