Complement mutations in DGKE-associated atypical hemolytic uremic syndrome.

Running title: Complement mutations in DGKE-associated aHUS.

Subject of manuscript: Inherited and genetics diseases

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ABSTRACT

Background and objectives.

Atypical hemolytic uremic syndrome (aHUS) is characterized by vascular endothelial damage usually due to complement dysregulation. Consistently, complement inhibition therapies are highly effective in most aHUS patients. Recently, it was shown that a significant percentage of early-onset aHUS cases carry mutations in diacylglycerol kinase-ε (DGKE), an intracellular protein with no obvious role in complement. These data support an alternative, complement-independent, mechanism leading to thrombotic microangiopathy that has implications for treatment of early-onset aHUS. To get further insights into this new form of aHUS we analyzed the DGKE gene in our aHUS cohort.

Design, setting, participants and measurements.

Eighty-three patients with early-onset aHUS (<2y), enrolled in the Spanish aHUS registry between 1999 and 2013, were screened for mutations in DGKE. These patients were also fully characterized for mutations in CFH, MCP, CFI, C3, CFB and THBD, CFH-CFH Rs copy number variations and rearrangements, and anti–factor H antibodies.

Results.

We found four patients carrying mutations in DGKE, one p.H536Qfs*16 homozygote and three compound heterozygotes (p.W322*/p.P498R, two patients; p.Q248H p.G484Gfs*10, one patient), who also carry heterozygous mutations in THBD or C3. Extensive plasma infusions controlled aHUS recurrences and prevented renal failure in the two patients with DGKE and THBD mutations. A positive response to plasma infusions and complement inhibition treatment was also observed in the patient with concurrent DGKE and C3 mutations.

Conclusions.

Our data suggest that complement dysregulation influence the onset and disease severity in carriers of DGKE mutations, and that treatments based on plasma infusions and complement inhibition are potentially useful in those patients with combined DGKE
and complement mutations. A comprehensive understanding of the genetic component predisposing to aHUS is therefore critical to guide an effective treatment.

**Keywords:** Complement, DGKE, aHUS, thrombotic microangiopathy.
INTRODUCTION

Hemolytic uremic syndrome (HUS) is a rare, life threatening disease characterized by thrombocytopenia, haemolytic anemia and acute renal failure\(^1\). The most frequent form of HUS follows a diarrheal prodrome and is associated with infections involving shiga-toxin (Stx)-producing \textit{E. coli} strains. Five to ten percent of HUS patients lack an association with this type of infections. This atypical form of HUS (aHUS) has the poorest long-term prognosis; it is characterized by recurrences and presents a mortality rate approaching 30\%\(^1,2\). Genetic analysis in aHUS patients have revealed that most cases associate with mutations and polymorphisms in complement genes and that the disease develops as a consequence of defective protection of cellular surfaces from complement activation\(^3\textendash}^{17}\). The recognition that aHUS is a disorder involving complement-dependent tissue damage provided strong support for the implementation of aHUS therapies based in complement inhibitors\(^18\).

In contrast to this complement-related aHUS, Lemaire et al. (2013)\(^19\) have reported that as many as 27\% of aHUS cases presenting in the first year of life are caused by the deficiency of diacylglycerol kinase-\(\epsilon\) (DGK-\(\epsilon\)) encoded by the \textit{DGKE} gene. It has been proposed that lack of DGK-\(\epsilon\) causes enhanced signaling through arachidonic acid-containing DAGs and results in a pro-thrombotic phenotype\(^19,20\). Because these patients lacked discernable complement alterations it was suggested that \textit{DGKE}-associated aHUS represents an alternative mechanism leading to thrombotic microangiopathy that is independent of complement dysregulation\(^19\).

To get further insights into this new form of aHUS and to estimate the prevalence of carriers of \textit{DGKE} mutations in the aHUS Spanish cohort we performed the analysis of the \textit{DGKE} gene in 83 cases with a disease onset in the first two years of life.
PATIENTS, MATERIALS and METHODS.

Patients.

Our aHUS cohort includes patients, mainly of Spanish origin, enrolled since 1999. We have performed complement studies that have allowed us to identify mutations, risk haplotypes and auto antibodies in these patients, most of which have already been published. Currently, our cohort includes 289 aHUS patients, 262 from Spain, 9 from other European countries, 4 from the North of Africa (Morocco, Tunisia, Senegal), 6 from USA, and 8 from South America. All these cases were diagnosed at specific centers in the countries mentioned and subsequently submitted to the Spanish aHUS registry for complement functional and genetic analysis. aHUS was diagnosed based on microangiopathic hemolytic anemia and thrombocytopenia defined by an hematocrit (Ht) less than 0.3 (30%), hemoglobin (Hb) level less than 10g/dL, serum lactate dehydrogenase (LDH) level greater than 460U/L, undetectable haptoglobin level, fragmented erythrocytes in the peripheral blood smear, and platelet count less than 150,000/µL, associated with acute renal failure. The studies reported here have Institutional Review Board’s approval. Informed consent was provided to all individuals participating in the study, according to the Declaration of Helsinki.

Complement profile assessment.

Serum concentrations of C3 and C4 were evaluated by nephelometry (Siemens. Marburg. Germany). Factor H (FH) and factor I (FI) serum levels were measured by ELISA. Membrane cofactor protein (MCP; CD46) levels on peripheral blood cells were determined by flow cytometry. The presence of anti FH antibodies and C3 nephritic factor, and the functional analysis of FH using the sheep erythrocytes hemolysis assay were performed as previously described.

Mutation screening and genotyping.

Exons of the CFH, MCP, CFI, C3, CFB and THBD genes were amplified from
genomic DNA using primers derived from the intronic sequences as described\textsuperscript{5, 10, 11, 13}. Exons of \textit{DGKE} were amplified with the primers described in Supplementary Table 1. Automatic sequencing was performed in an ABI3730 sequencer using a dye terminator cycle sequencing kit (Applied Biosystems). Copy number variations and genomic rearrangements were assessed by multiplex ligation-dependent probe amplification (MLPA) and custom-designed high-density 8x15k oligonucleotide CGH arrays spanning the RCA gene cluster (median resolution: 110bp) \textsuperscript{27} (AMADID 040193, Agilent Technologies, Santa Clara, CA). \textit{CFH} and \textit{MCP} genotyping was performed as described previously\textsuperscript{8}.

\textbf{In silico analysis of mutations.}

The probability of a genetic variant to result in structural or functional alterations was calculated using bioinformatics prediction tools that discriminate neutral polymorphisms from amino acid substitutions of likely functional importance. To minimize the possibility of false positive or negative results we have applied four different computational algorithms publicly available, namely, Sorting Intolerant From Tolerant\textsuperscript{28} (SIFT, \url{http://sift.jcvi.org}), Polymorphism Phenotyping\textsuperscript{29} (PolyPhen, \url{http://genetics.bwh.harvard.edu}), Mutation Taster\textsuperscript{30} (\url{http://www.mutationtaster.org}) and Align-GVGD\textsuperscript{31} (\url{http://agvgd.iarc.fr}). The SIFT algorithm predicts whether an amino acid substitution affects protein function based on sequence homology among related genes and domains over evolutionary time, and the physico-chemical properties of the amino acid residues. Polyphen also incorporates the analyses of sequence conservation and the nature of the amino acid residues involved, as well as the location of the substitution within the structure of the protein. By accessing a variety of heterogeneous biological databases and analytical tools, MutationTaster is able to identify the missense mutations most likely to have functional effects, such as changes to the transcriptional level and pre-mRNA splicing. Align-GVGD combines the biophysical characteristics of amino acid
and protein multiple sequence alignments to predict where missense substitutions in
genes of interest fall in a spectrum from enriched deleterious to enriched neutral.
RESULTS

The Spanish aHUS cohort includes 83 patients, enrolled between 1999 and 2013, with a disease onset in the first two years of life; sixty-two of them without a discernable genetic or autoimmune abnormality that helps to explain the development of the disease. We performed the analysis of the DGKE gene and found that one of these patients (HUS299) was homozygote for a frameshift DGKE mutation. We also screened the 21 early-onset aHUS patients with identified genetic factors and, surprisingly, found three additional patients, belonging to two unrelated pedigrees, carrying compound heterozygous mutations in DGKE (Figure 1, Figure 2 and Table 1). We did not find heterozygote carriers of DGKE mutations in our aHUS cohort.

Patients with isolated DGKE mutations.

HUS299 is a 4y-old male from Marroco who presented with aHUS at 13m-old, coincident with a diarrhea episode. He required peritoneal dialysis during 22 days, but evolved well, without recurrences. Three years after remission, the patient remains asymptomatic with only a residual microalbuminuria (Table 2; Supplementary Data). HUS299 is homozygous for a frameshift mutation in exon 12 (c.1608_1609del; p.H536Qfs*16) of DGKE that results in a truncated DGK-ε protein lacking the last 31 amino acids (Figures 1 and 2 and Table 1), which is very likely deleterious. This is a novel DGKE genetic variation that has not been found in 80 control chromosomes from North African populations and it is not described in the available databases. Additional genetic analysis in this patient failed to reveal other genetic abnormalities. He was also negative for anti FH autoantibodies and his plasma complement protein levels, including C3 were normal (Table 3).

Patients with concurrent mutations in DGKE and THBD.

HUS40 and HUS39 are siblings from an Argentinean family of European ancestry. HUS40 presented with HUS at 7mo of age. Five months later she had a recurrence that
required hemodialysis and continuous ambulatory peritoneal dialysis for few months. She evolved satisfactorily, partially recovering renal function without dialysis requirement. A third recurrence occurred at 2y old that responded well to a protocol of daily plasma infusions, which were suspended one year later. She had not suffered any more recurrences. She is now 17y-old and is clinically well, with normal blood parameters, serum creatinine of 1.5mg/dl and Ccr of 41ml/min/1.73m² (Table 2; Supplementary Data). Her brother, HUS39, presented with aHUS at 3mo-old. He was anuric, requiring acute peritoneal dialysis. Based on the experience with his sister (HUS40), he was immediately treated with daily plasma infusions. After 10 days, his renal function improved and he was discharged maintaining the daily plasma infusions for two months (10ml/kg/day). Interestingly, when plasma infusions were spaced biweekly he had an aHUS recurrence that responded well when plasma infusions were returned to daily basis. Based on his clinical improvement, plasma infusions were again spaced progressively and finally suspended five years later. He is now 11y-old and is clinically well, with normal blood parameters, serum creatinine of 0.8mg/dl and Ccr of 57ml/min/1.73m² (Table 2; Supplementary Data).

HUS40 and HUS39 are compound heterozygotes for a non-sense mutation (c.966G>A; p.W322*) in exon 6 and a missense mutation (c.1493C>G; p.P498R) in exon 11 of the DGKE gene (Figures 1 and 2 and Table 1). The p.W322* mutation has been described previously as a pathogenic mutation associated with a rare European haplotype19. Interestingly, the p.W322* mutation in our patients is also associated with the same DGKE haplotype (Supplementary Table 2). The second mutation p.P498R is a novel genetic variation that results in an amino acid substitution in exon 11 of the DGKE gene; a region that encodes the kinase accessory domain (Figure 2). p.P498R was not found in 140 normal control chromosomes and has not been reported previously in the available DGKE mutation databases. We lack assays to test the functionality of the DGKE mutations and have no biopsy material from the patients to test DGK-ε expression. However, a very strong argument supporting that p.P498R is a DGKE
deleterious mutations is that the patients carrying this mutation present it together with the deleterious p.W322* mutation (patients are compound heterozygotes), which is in agreement with the recessive model of inheritance described for pathogenic DGKE mutations. It would be extremely unlikely that this pairing of mutations would have occurred by chance if such infrequent genetic variants were not pathogenic. Furthermore, we have used bioinformatics prediction tools that discriminate neutral polymorphisms from amino acid substitutions of likely functional importance to support that the identified DGKE changes represent true disease mutations (see Materials and Methods). These in silico analyses indicate that p.P498R is a damaging mutation (Table 4).

Genetic analysis in these patients revealed that both patients are also heterozygotes for a mutation in THBD (c.1456G>T; p.D486Y) previously found associated with aHUS and demonstrated to be pathogenic16 (Figure 1). CFH and MCP genotyping showed that HUS39 and HUS40 are heterozygotes for the MCP and CFH aHUS risk haplotypes, respectively. The search for anti FH autoantibodies was negative and the complement plasma levels were normal, although C3 levels in these individuals, particularly HUS40, were in the lower part of the normal range (Table 3).

Patients with concurrent mutations in DGKE and C3.

HUS272 is a 4y-old female from a German-Spanish family who presented with aHUS at 8mo of age, coincident with an upper respiratory tract infection. She had periorbital edema, low plasma total protein levels, hematuria and nephrotic proteinuria (Table 2). After 48h she developed generalized edema, hypertension, thrombocytopenia and schistocytes were observed at the peripheral blood smear. A biopsy supported the diagnosis of aHUS. She evolved favorably with a progressive decrease of proteinuria levels. Eight months later, coincident with a vaccination, she suffered an episode of oliguria and edema, associated with increased proteinuria, which resolved raising the Enalapril dosis. However, three months later, after an acute upper tract infection, she
had an aHUS recurrence and her clinical situation deteriorated. C3 was slightly below normal range. She initiated biweekly plasma infusions, which led to stabilization of the clinical symptoms and near normalization of all blood parameters (Figure 3); however proteinuria and gross hematuria persisted. One year later, C3 levels dropped to 0.77g/l, the edema returned and her clinical situation worsened again. It was decided to switch her from the biweekly plasma infusions to Eculizumab treatment and thereafter her clinical situation improved significantly. However, a urine sample revealed ongoing proteinuria. She is currently treated biweekly with Eculizumab and the clinical situation had much improved. Previously, she always had peripheral edema when she had infections, under Eculizumab treatment, however, she remained without obvious edema (Table 2; Supplementary Data).

Genetic analysis showed that HUS272 is a compound heterozygote for a non-sense mutation (c.1452del; p.G484Gfs*10) in exon 11 of DGKE that truncates the last 69 amino acids of the protein (most likely deleterious) and a missense mutation (c.744G>C; p.Q248H) in exon 4 of the DGKE gene, encoding the kinase catalytic domain of DGK-ε (Figures 1 and 2 and Table 1). p.G484Gfs*10 and p.Q248H are novel mutations that were not found in 140 normal control chromosomes and have not been reported previously in the available DGKE mutation databases. Similarly to the case of the p.P498R mutation found in HUS40 and HUS39, the concurrence of p.G484Gfs*10 and p.Q248H in HUS272 and the results of their analysis in silico strongly support that these DGKE mutations are pathogenic (Table 4).

Critically, the analysis of all known aHUS genetic risk factors showed that HUS272 carries in addition to the DGKE mutations a novel mutation in the C3 gene (c.784G>T; p.G262W) (Figure 1) that in silico analysis also clearly identified as a damaging mutation (Table 4). p.G262W was not found in 140 normal control chromosomes and is not included in genetic variation databases. Interestingly, this patient carries the C3 mutation in combination with a MCP risk haplotype in homozygosity (Table 3), which is characteristic of aHUS patients carrying gain-of-function mutations in C3 and CFB13, 32.
Complement plasma levels were normal, although C3 levels in this patient were always below or in the lower part of the normal range (Table 3). Notably, her mother who is also a carrier of the p.G262W C3 mutation also presents decreased plasma C3 levels (Figure 3A).
DISCUSSION

Previous studies have identified two groups of patients with mutations in the \textit{DGKE} gene. Ozaltin et al.\textsuperscript{20} reported 9 patients from three consanguineous pedigrees presenting with MPGN with histological signs of both glomerular microangiopathy and endothelial distress at ages ranging from 0.8 to 17y-old. No complement data were reported for these patients\textsuperscript{20}. A second study by Lemaire et al.\textsuperscript{19}, reported 13 patients from 9 unrelated pedigrees also carrying mutations in \textit{DGKE}. In contrast, all these patients presented within the first year of life with aHUS. Notably, these patients belong to a group of pediatric-onset aHUS that was selected to include only patients in whom mutation in known aHUS-associated genes or anti FH auto-antibodies have not been identified\textsuperscript{19}. Here we have analyzed the \textit{DGKE} gene in our aHUS cohort and found that 5% (4/83) of our early-onset patients carry \textit{DGKE} mutations, including both carriers and non-carriers of mutations in other aHUS-associated genes.

Disease presentation and evolution in our four patients was comparable to that of patients carrying \textit{DGKE} mutations described by Lemaire et al.\textsuperscript{19} These patients met the clinical criteria for aHUS at presentation, with disease recurrences within the early childhood. Later on, one of them clearly progressed to the chronic glomerulopathy with hematuria, proteinuria, hypertension and renal failure, which is distinctive of \textit{DGKE} mutation carriers from other aHUS patients (Table 2).

Interestingly, we found that three of our patients also carry mutations in other known aHUS-associated genes. Clinical course and severity of the disease in these patients was different from those of the patient with isolated \textit{DGKE} mutations. HUS39 and HUS40, who also carry a pathogenic heterozygous mutation in \textit{THBD}, had various aHUS recurrences shortly after the disease onset that responded well to daily plasma infusions. Notably, the course of the disease was more benign in the affected brother (HUS39) who was intensively treated with plasma immediately after the onset of the disease (Table 2). These data point to a potential beneficial effect of plasma infusions in our patients and argue previous remarks, based on data from patients carrying
exclusively loss-of-function DGKE mutations, not supporting the use of plasma in affected carriers of DGKE mutations\textsuperscript{19}. Notably, it has been reported that most (88\%) aHUS patients associated with THBD mutations responded well to plasma therapy\textsuperscript{33}. Therefore, we like to suggest that the association of a THBD mutation with the DGKE mutations in HUS39 and HUS40 results in a potentially more severe phenotype due to an increased frequency of aHUS recurrences caused by the THBD mutation. Consistently with other aHUS patients carrying THBD mutations, our patients responded well to plasma therapy.

HUS272, who in addition to the DGKE mutations also carries a likely pathogenic heterozygous mutation in C3 and the MCP risk haplotype in homozygosity, also presented several aHUS recurrences, shortly after onset. Biweekly plasma infusions were effective in normalizing blood parameters, and subsequent Eculizumab treatment resolved the infection-associated edemas that were typical in this patient (Figure 3). In comparison with the rest of patients in this case series with DGKE mutations, the association of a C3 gene mutation in this particular patient possibly contributed to a more severe disease with chronic activation of thrombotic microangiopathy despite plasma treatment and, more importantly, to a positive response to complement C5 blockage with Eculizumab, which conducted to remission.

Finally, HUS299, carrying exclusively DGKE mutations, recovered renal function without plasma infusions or Eculizumab treatment and has remained stable without recurrences since the aHUS onset, three years ago (Table 2).

These findings support a potential role of mutations in complement genes in patients carrying DGKE loss-of-function mutations. The novelty of our approach has been searching for DGKE mutations in aHUS patients with and without mutations in known aHUS associated complement genes. It is remarkable that after screening 83 aHUS patients with a very early onset (<2y), without excluding those with previously identified mutations in known aHUS-associated genes (21 patients), we found three patients carrying recessive DGKE mutations who also carry mutations in other known aHUS-
associated genes and just one patient with isolated DGKE mutations. These data, and the evident correlation of disease recurrences with infection in our patients (Supplementary Data), suggest that the onset and severity of the chronic renal pathology associated with DGKE mutations may be influenced by additional complement-related aHUS risk factors. If these data are replicated in other aHUS cohorts and a role for complement dysregulation is finally established in DGKE-related aHUS, the suggestion that current treatment based on plasma therapy and complement inhibition are not beneficial in individuals with DGKE mutations should, perhaps, be reconsidered; at least for patients in whom there is coexistence of DGKE and complement gene mutations. Preventing complement-related thrombotic microangiopathy episodes may result in a more benign course of the renal disease in carriers of DGKE mutations.

In conclusion, we found that 5% of the patients in our aHUS cohort who had a very early onset of the disease (<2y) carry recessive mutations in DGKE. Although this is a small percentage compared to the 27% reported for the French cohort, it is still a significant number. Our patients carry a total of 5 different mutations in the DGKE gene. Four of them are novel mutations and one (p.W322*) seems to be a relatively prevalent mutation of European origin. The disease presentation and evolution in our patients was comparable to that of patients carrying DGKE mutations described by Lemaire and coworkers in the French aHUS cohort. However, in contrast with that previous study, we found that three of our patients carry additional mutations in the known aHUS candidate genes C3 and THBD. Although further analysis in other aHUS cohorts are needed to replicate our findings, these data suggest that complement-mediated aHUS episodes have a role modulating the onset and severity of renal disease in carriers of DGKE mutations. Finally, this work further illustrates that a comprehensive understanding of the genetic component predisposing to the pathology is critical to guide appropriate diagnostics and effective treatment in aHUS.
ACKNOWLEDGEMENTS

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DISCLOSURES

SRdeC, BH, MA and LL have received honoraria from Alexion Pharmaceuticals for giving lectures and participating in advisory boards. None of these activities has had any influence on the results or interpretation in this article. Other authors declare no conflicts of interest.
REFERENCES


FIGURE LEGENDS

Figure 1. Pedigrees of the aHUS patients carrying DGKE mutations.
For each pedigree the index cases are indicated with arrows and the chromatograms for the corresponding mutations are shown. Segregation of the mutations is indicated with colored symbols. ND, indicates that genetic analysis have not been performed yet.

Figure 2. Location of the DGKE mutations found in Spanish aHUS patients.
A diagram of the DGK-ε protein depicting the different protein domains (HD, hydrophobic domain; C1 domain; kinase catalytic domain and kinase accessory domain) is shown. Arrows identify the position of the five mutations found in the screening of the early-onset Spanish aHUS patients.

Figure 3. Evolution of blood parameters in HUS272.
The evolution of the C3 (A), platelets (B) and lactate dehydrogenase (LDH) (C) serum levels in patient HUS272 from the disease onset are shown. Arrows indicate the start of the plasma therapy (FFP, fresh frozen plasma) and of the Eculizumab (Ecu) treatment, respectively. C3 levels in the mother (Mo) and father (Fa) are also indicated.
Table 1. *DGKE* and complement gene mutations found in early onset aHUS patients

<table>
<thead>
<tr>
<th>Patient ID</th>
<th><em>DGKE</em></th>
<th>Other mutations</th>
</tr>
</thead>
<tbody>
<tr>
<td>HUS40</td>
<td>Exon 6; c.966G&gt;A; p.W322*</td>
<td>Exon 11; c.1493C&gt;G; p.P498R</td>
</tr>
<tr>
<td>HUS272</td>
<td>Exon 4; c.744G&gt;C; p.Q248H</td>
<td>Exon 11; c.1452del; p.G484Gfs*10</td>
</tr>
<tr>
<td>HUS299</td>
<td>Exon 12; c.1608_1609del; p.H536Qfs*16</td>
<td>Exon 12; c.1608_1609del; p.H536Qfs*16</td>
</tr>
</tbody>
</table>
Table 2. Clinical characteristics of the aHUS patients carrying DGKE mutations.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Gender</th>
<th>Age (years)</th>
<th>Age at onset (months)</th>
<th>Proteinuria onset</th>
<th>Thrombocytopenia onset</th>
<th>LDH(U/L) / Schisto.</th>
<th>sCr onset (mg/dL)</th>
<th>Dialysis onset</th>
<th>Histology</th>
<th>aHUS recurrences</th>
<th>Plasma infusions</th>
<th>Ecu therapy</th>
<th>Last sCr (mg/dL)</th>
<th>Last Hematuria</th>
<th>Last Proteinuria Alb(g)/Cr(g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HUS299</td>
<td>M</td>
<td>4</td>
<td>13</td>
<td>Yes</td>
<td>Yes</td>
<td>2138/Yes</td>
<td>7.7</td>
<td>Yes</td>
<td>ND</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>0.4</td>
<td>None</td>
<td>0.05</td>
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<td>11</td>
<td>3</td>
<td>&gt;3</td>
<td>Yes</td>
<td>2727/Yes</td>
<td>2.8</td>
<td>Yes</td>
<td>ND</td>
<td>Yes (1)</td>
<td>Yes</td>
<td>No</td>
<td>0.8</td>
<td>None</td>
<td>None</td>
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<tr>
<td>HUS40</td>
<td>F</td>
<td>17</td>
<td>7</td>
<td>&gt;3</td>
<td>Yes</td>
<td>4560/Yes</td>
<td>4</td>
<td>Yes</td>
<td>TMA</td>
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<tr>
<td>HUS272</td>
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<td>4</td>
<td>8</td>
<td>12</td>
<td>Yes</td>
<td>1032/Yes</td>
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<td>TMA</td>
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<td>Yes</td>
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<td>+++</td>
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Abbreviations: Alb, albumin; Cr, creatinine; LDH, lactate dehydrogenase; Schisto, schistocytes; sCr, serum creatinine; Ecu, eculizumab; M, male; F, female; TMA, thrombotic microangiopathy.
Table 3. Complement profiles in aHUS patients carrying mutations in DGKE.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Polymorphisms</th>
<th>Complement assessment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CFHR3-CFHR1</td>
<td>CFH Risk haplotype</td>
</tr>
<tr>
<td>HUS299</td>
<td>Het</td>
<td>Het</td>
</tr>
<tr>
<td>HUS39</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>HUS40</td>
<td>No</td>
<td>Het</td>
</tr>
<tr>
<td>HUS272</td>
<td>Het</td>
<td>No</td>
</tr>
</tbody>
</table>

(*) Complement studies were performed at different times after onset as follows: HUS39, 7 months; HUS40, 6 years; HUS272, 10 weeks; and HUS299, 3 weeks.

Abbreviations: Het, heterozygote; Hom, homozygote; CFH, gene encoding factor H; MCP, gene encoding membrane cofactor protein; FH, factor H; C3, complement C3; C4, complement C4; FI, factor I; MCP, Membrane cofactor protein; PBLs, peripheral blood lymphocytes; Anti-FH, auto antibodies against factor H; Neg, negative.
Table 4. *In silico* analyses of missense mutations.

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Polyphen (0-1)</th>
<th>SIFT (1-0)</th>
<th>Align-GVGD (C0 – C65)</th>
<th>MutationTaster (0-1)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>DGKE</em>; c.744G&gt;C; p.Q248H</td>
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<td>0</td>
<td>C15 Low risk</td>
<td>0.999 Disease causing</td>
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<td>0.05</td>
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HUS299

HUS299 is a 4y-old male from Marroco who presented with aHUS at 13m-old, coincident with a 7d-long vomiting and diarrhea episode. The analytic parameters at admission were: hemoglobin 7.7g/dL; hematocrit 21.8%; platelets 202,000/μL; LDH 2138U/L; haptoglobin <0.15mg/dL; creatinine 7.7mg/dL and urea 245mg/dL. Schistocytes were present in the peripheral blood smear. He was oliguric and required peritoneal dialysis during 22 days. During that period, anemia progressed and thrombocytopenia dropped to 17,000 platelets/μL, requiring transfusions. Severe hypertension was controlled with Enalapril and Amlodipine. He evolved well and recovered renal function, with only a residual proteinuria of 0.28g/L, discrete anemia (Hg 10g/dL) and normal platelets and serum LDH. Two years after remission, the patient remains asymptomatic and without recurrences. By August 2013 he had normal blood pressure and proteinuria further decreased, remaining only a residual microalbuminuria (MAU/Cr 53.4mg/g). A renal ultrasound at that time was normal.

HUS40 and HUS39

HUS40 and HUS39 are siblings from an Argentinean family of European ancestry. HUS40 is a 17y-old female who at 7mo of age presented an episode of thrombotic microangiopathy. Since STEC-HUS is endemic in that country, it was taken as a typical case evolving satisfactorily. Five months later, however, she had a recurrence that required hemodialysis and continuous ambulatory peritoneal dialysis for few months. aHUS was then suspected and a renal biopsy performed at that time revealed glomerular thrombotic microangiopathy. She evolved satisfactorily from this recurrence, partially recovering renal function without dialysis requirement. A third recurrence occurred at 2y old, following an upper respiratory tract infection. She responded well to a protocol of daily plasma infusions that were spaced progressively and suspended one year later. During the last 14 years she had not suffered any more recurrences.
the last control, on December 2013, she was clinically well, with normal blood parameters, serum creatinine of 1.5mg/dL and Ccr of 41mL/min/1.73m². Her hypertension and residual proteinuria is currently treated with Enalapril and Losartan. HUS39 is a 11y-old male, who in December 2002 presented with aHUS at the early age of 3mo-old. He had severe thrombocytopenia and petechial rash and was anuric, requiring acute peritoneal dialysis. Based on the experience with his sister (HUS40), he was immediately treated with daily plasma infusions. After 10 days, his renal function improved and he was discharged maintaining the daily plasma infusions for two months (10mL/kg/day). Interestingly, when plasma infusions were spaced biweekly he had an aHUS recurrence that responded well when plasma infusions were returned to daily basis. Based on his clinical improvement, plasma infusions were again spaced progressively and finally suspended on December 2007. At the last control, on December 2013, he was clinically well, with normal blood parameters, serum creatinine of 0.8mg/dL and Ccr of 57mL/min/1.73m². Like his sister, he is under Enalapril and Losartan.

**HUS272**

HUS272 is a 4y-old female from a German-Spanish family who in October 2010, at 8mo of age, presented with aHUS coincident with an upper respiratory tract infection. She had periorbital edema, low plasma total protein levels (35g/dL), sCr 0.6mg/dL, Hb 13.4g/dL, 362,000 platelets/μL, hematuria and nephrotic proteinuria (597mg/m²/h). After 48h she developed generalized edema, hypertension and thrombocytopenia (129,000 platelets/μL) and 6-16% schistocytes were observed at the peripheral blood smear. A biopsy performed on January 2011 revealed the presence of glomerular thrombotic microangiopathy, supporting the diagnosis of aHUS. ADAMST-13 activity was 98.8%. She evolved favorably under Furosemide and Enalapril treatment with a progressive decrease of proteinuria levels. In June 2011, coincident with a vaccination
she suffered an episode of oliguria and edema, associated with increased proteinuria, which resolved raising the Enalapril dosis. In November 2011, again after an acute upper tract infection, she had an aHUS recurrence and her clinical situation deteriorated. Her blood parameters at that time were: total plasma protein 52g/L, platelets 94,000/µL, Hemoglobin 11.2g/dL, LDH 635U/L, uric acid 7.7mg/dL. Her serum creatinine was 0.4mg/dL, but her cystatin C levels were slightly elevated (1.2mg/L). A kidney ultrasound revealed enlarged, hyperechoic kidneys. C3 was slightly below normal range. She initiated biweekly plasma infusions, which after four months led to stabilization of the clinical symptoms and near normalization of all blood parameters (LDH 346U/L, Hb 11.7g/dL, Platelets 350,000/µL, C3 1.06g/L); however proteinuria and gross hematuria persisted.

In November 2012, C3 levels dropped to 0.77g/L, the edema returned and her clinical situation worsened again. It was decided to switch her from the biweekly plasma infusions to Eculizumab treatment and thereafter her clinical situation improved significantly. Her plasma total protein increased to 64g/L, her serum albumin to 42.5 g/L, serum creatinine was 0.18mg/dL, serum cystatin C 0.56mg/dL, LDH 366U/L and C3 0.99g/L. Platelets and LDH were normal (no alteration of these parameters were ever seen again after November 2011, neither under plasma infusions nor Eculizumab treatment). However, a urine sample in May 2013 revealed ongoing proteinuria. She is currently treated biweekly with Eculizumab and the clinical situation had much improved. Previously, she always had had peripheral edema when she had infections, under Eculizumab treatment, however, she remained without obvious edema. Her current values are: serum creatinine 0.3mg/dL, LDH 315U/L, total protein 48g/L, albumin 23g/L, C3 1.32 g/L, 465,000 platelets/µL, Hb 10.9g/dL and Alb(g)/Cr(g) of 4.4.
Supplementary Table 1. PCR primers for *DGKE* exon amplification and sequencing.

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<th>Primer Name</th>
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<td>DGKE-Exon 2R</td>
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Supplementary Table 2. DGKE haplotype associated with the W322* mutation.

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