Deficiency in mouse hyaluronidase 2: a new mechanism of chronic thrombotic microangiopathy

by Cécile Onclinx, Sophie Dogne, Laurence Jadin, Fabienne Andris, Christian Grandfils, François Jouret, François Mullier, and Bruno Flamion

Haematologica 2015 [Epub ahead of print]

doi:10.3324/haematol.2015.123828

Publisher's Disclaimer.
E-publishing ahead of print is increasingly important for the rapid dissemination of science. Haematologica is, therefore, E-publishing PDF files of an early version of manuscripts that have completed a regular peer review and have been accepted for publication. E-publishing of this PDF file has been approved by the authors. After having E-published Ahead of Print, manuscripts will then undergo technical and English editing, typesetting, proof correction and be presented for the authors' final approval; the final version of the manuscript will then appear in print on a regular issue of the journal. All legal disclaimers that apply to the journal also pertain to this production process.
Deficiency in mouse hyaluronidase 2: a new mechanism of chronic thrombotic microangiopathy

Short title: Microangiopathy due to HYAL2 deficiency

Cécile Onclinx¹, Sophie Dogne¹, Laurence Jadin¹, Fabienne Andris², Christian Grandfils³, François Jouret⁴, François Mullier⁵ and Bruno Flamion¹

¹ Molecular Physiology Research Unit, NARILIS, University of Namur, 61 rue de Bruxelles, 5000 Namur, Belgium.
² Laboratoire d’Immunobiologie, Institut de Biologie et de Médecine Moléculaire, Université Libre de Bruxelles, 12 Rue des Prof. Jeener et Brachet, 6041 Gosselies, Belgium.
³ Interfacultary Research Centre of Biomaterials (CEIB), University of Liège, 3 Allée de la Chimie, 4000 Liège, Belgium.
⁴ Groupe Interdisciplinaire de Génoprotéomique Appliquée (GIGA), Cardiovascular Sciences, University of Liège, Quartier Hôpital, Avenue Hippocrate, 13, B-4000 Liège, Belgium.
⁵ Hematology Laboratory, Namur Thrombosis and Hemostasis Center, CHU Dinant Godinne UCL Namur, 1 Avenue Gaston Therasse, 5530 Yvoir, Belgium.

Corresponding author: Bruno Flamion. Molecular Physiology Research Unit, NARILIS, University of Namur, 61 rue de Bruxelles, 5000 Namur, Belgium. E-mail: bruno.flamion@unamur.be. Phone: +32.81.724332. Fax: +32.81.724329
ABSTRACT

Hyaluronan is a major component of the extracellular matrix and glycocalyx. Its main somatic degrading enzymes are the hyaluronidases 1 and 2, none of which is active in the bloodstream. We generated hyaluronidase 2 deficient mice. They suffer from mild chronic anemia and thrombocytopenia, in parallel with a 10-fold increase in plasma hyaluronan concentration. The current study explores the mechanism of these hematological anomalies. The decreased erythrocyte and platelet counts were assigned to peripheral consumption. The erythrocyte half-life was reduced from 25 to 8 days without signs of premature aging. Hyaluronidase 2 deficient platelets were functional. Major intrinsic defects in erythrocyte membrane or stability, as well as detrimental effects of high hyaluronan levels on erythrocytes, were ruled out in vitro. Normal erythrocytes transfused into hyaluronidase 2 deficient mice were quickly destroyed but neither splenectomy nor anti-C5 administration prevented from chronic hemolysis. Schistocytes were present in hyaluronidase 2 deficient smears at a level of 1 to 6%, while virtually absent in control mice. Hyaluronidase 2 deficient mice had increased markers of endothelial damage and microvascular fibrin deposition, without renal failure, accumulation of ultra-large multimers of von Willebrand factor, deficiency of A Disintegrin And Metalloproteinase with ThromboSpondin type 1 motifs, member 13 (ADAMTS13), or hypertension. There was no sign of structural damage in hepatic or splenic sinusoids, or in any other microvessels. We conclude that hyaluronidase 2 deficiency induces chronic thrombotic microangiopathy with hemolytic anemia in mice. The link between this uncommon condition and hyaluronidase 2 remains to be explored in man.
INTRODUCTION

Hyaluronan (HA), a major constituent of the vertebrate extracellular matrices, belongs to the glycosaminoglycan family. Dependent on its molecular size, it acts as a scaffold and a signaling molecule between the matrix and the cells.\(^1\) It can also be found in the plasma at concentrations of about 10-100 ng/mL in humans\(^2\) and 400-600 ng/mL in C57Bl/6 mice.\(^3\) HA is catabolized by somatic hyaluronidases, principally HYAL1 and HYAL2,\(^4\) none of which is active in the bloodstream at physiological pH. The role of each hyaluronidase in HA turnover is poorly understood. HYAL2 is known to be a ubiquitous glycosylphosphatidylinositol-anchored membrane protein.\(^5\) HYAL2-deficient (Hyal2\(^{-/-}\)) mice were generated in our laboratory through a conditional Cre-lox system.\(^6\) These Hyal2\(^{-/-}\) mice display mild skeletal and hematological abnormalities, which include thrombocytopenia and chronic compensated hemolytic anemia with markedly elevated plasma HA levels. The main purpose of the current study was to characterize the cause of this chronic anemia accompanied by thrombocytopenia and how it relates to HYAL2 deficiency. In particular, we determined if the cause of hemolysis was intra- or extra-corpuscular by performing complete hematological analysis and measuring red blood cell (RBC) intrinsic properties (membrane deformability, signs of senescence) and survival in Hyal2\(^{-/-}\) and control mice.

METHODS

**Animals.** Control (Hyal2\(^{+/+}\) or Hyal2\(^{+/-}\); these genotypes had indistinguishable phenotypes) and knockout (Hyal2\(^{-/-}\)) outbred 129P1.CD1 Hyal2\(^{tm1.1BFla}\) mice\(^6\) were used. Males from the same litters were selected except where otherwise noted. For
bone marrow and transfusion experiments, donor and recipient inbred mice, backcrossed on a C57Bl/6 background, were used. Blood was collected via retro-orbital, tail vein, or cardiac puncture. All experiments were approved by the local (University of Namur, Belgium) animal ethics committee.

**Hematologic analyses** were run on ADVIA 120 (Siemens). Eosin-5’-maleimide was assayed using BDIS FACS Canto II® cytometer (BD Biosciences) and immature reticulocyte and platelet fractions, using XE-2100 instrument (Sysmex).^7^

**RBC survival.** Mice received an IV injection of 3 mg sulfo-NHS-LC-biotin (Pierce) and RBCs collected at various time points were incubated with Alexa 488-streptavidin (Life Technologies) and analyzed using flow cytometry (FACS Calibur, BD Biosciences).^8^

**RBC senescence:** Phosphatidylserine (PS),^9^ auto-immunoglobulin G (IgG),^10^ and C3 exposures on RBCs were measured after in vitro incubations with fluorescein isothiocyanate-(FITC)-annexin V (BD Biosciences), FITC-rabbit anti-mouse IgG (Dako), or rat monoclonal anti-mouse C3 antibody (Abcam) followed by Alexa-488-anti-rat antibody (Life Technologies), respectively.

**RBC deformability.** A pre-determined volume of blood (23 to 30 µl for hematocrit values of 33% to 44%, respectively) was added to 5 ml of isotonic polyvinylpyrrolidone solution. One ml of this suspension was submitted to 12 different shear stress values at 37°C using a LORCA ektacytometer (Mechatronics Instruments). A decrease in elongation index indicates a loss of RBC deformability.^11,12^

**Transplantation of bone marrow.** Mice were bi-irradiated (2×6 Gy at 3-h interval).
One hour later, they received an IV injection of either control or Hyal2^- bone marrow (5.10^6 cells). The mice were kept under sterile conditions for 12 weeks to allow bone marrow reconstitution and blood analyses.\textsuperscript{13}

\textbf{Transfused RBC survival.} RBCs of control or Hyal2^- donor mice were labeled by IV injection of sulfo-NHS-LC-biotin, collected 1 h later, washed, and resuspended to achieve 25% hematocrit. Two hundred \(\mu\)l of this suspension were injected into the tail vein of control and Hyal2^- recipient mice. The percentage of biotinylated erythrocytes remaining in the circulation at various time points was calculated using FACS.\textsuperscript{14}

\textbf{RBC incubations.} Buffy coat-free plasma was collected by centrifugation. Complement was inactivated (56°C for 30 min). RBCs were incubated for 24 h in either Hyal2^- plasma, control plasma, or the latter + 7.5 \(\mu\)g/mL high molecular mass (3.9\times10^6 Da) HA, corresponding to plasma HA concentrations in Hyal2^- mice. RBCs were then incubated with FITC-annexin-V and analyzed using FACS.

\textbf{Blood and plasma viscosities} were measured at 38°C under various shear rates using a rotation viscosimeter (LVDV-II+Pro C/P, Brookfield Engineering Laboratories).

\textbf{Blood pressure} was measured using the tail-cuff method (CODA, Kent Scientific, Torrington, CT).\textsuperscript{15,16}

\textbf{Other measurements.} VCAM-1, ICAM-1, and P-selectin were measured using ELISA assays (R&D Systems), and the activity of A Disintegrin And Metalloproteinase with ThromboSpondin type 1 motifs, member 13 (ADAMTS13), using Lifecodes\textsuperscript{®} Activity Assay (Immucor). Von Willebrand factor (vWF) multimers were assessed in the laboratory of Claudine Caron, Lille, France. Fibrin was detected using polyclonal rabbit anti-human fibrinogen antibody (Dako).\textsuperscript{17}
Statistical analyses. Unpaired and Mann-Whitney tests were used for single comparisons; two-way ANOVA and Kruskal-Wallis test, for multiple comparisons. Correlations were assessed using Pearson’s r².

RESULTS

Hyal2−/− mice display chronic macrocytic anemia and thrombocytopenia.

Congenital HYAL2 deficiency in mice is accompanied by thrombocytopenia and mild chronic anemia with high reticulocyte counts and elevated plasma lactate dehydrogenase (LDH) levels, suggesting chronic hemolytic anemia. Erythrocytic and thrombocytic indices were analyzed in Hyal2−/− and control (Hyal2+/+ and Hyal2+/−) outbred young adult mice of 8 weeks of age (Table 1). Whereas the main parameters of Hyal2+/+ and Hyal2+/− mice did not significantly differ, Hyal2−/− mice had a mild (10%) but significant decrease in both circulating hemoglobin levels and hematocrit, as well as a 5-fold increase in reticulocyte count, suggestive of chronic regenerative anemia. In addition, both reticulocyte and thrombocyte immature fractions were elevated in Hyal2−/− mice. This indicates a strong stimulation of erythropoiesis and thrombopoiesis probably due to peripheral consumption. Similar hematological abnormalities were found when comparing control C57Bl/6 inbred vs Hyal2−/− mice (Supplemental Table 1). These observations were thus independent of the mouse genetic background.

HYAL2-deficient mice also displayed macrocytosis (Table 1). The mean corpuscular volume and mean corpuscular hemoglobin content of RBCs were significantly higher in Hyal2−/− mice than in control group, even after in vitro maturation of reticulocytes into RBCs, suggesting macrocytosis was not due to the high
proportion of large reticulocytes. Nor was it due to vitamin B12 deficiency or folic acid deficiency (Table 1). In addition, histograms of RBC volume and hemoglobin concentration revealed anisocytosis. Both macrocytosis and anisocytosis may result from enhanced erythropoiesis.

The absence of a significant difference in mean corpuscular hemoglobin concentration and the results of eosin-5-maleimide binding tests (Table 1) ruled out hereditary spherocytosis.

The half-life of Hyal2−/− RBCs is dramatically reduced without signs of intrinsic membrane anomaly, premature aging, or high C3 deposition.

To confirm peripheral destruction, the lifespan of control and Hyal2−/− RBCs was measured following sulfo-NHS-LC-biotin injections. This system enables simple and efficient labeling of RBC proteins without altering their biological activity. The half-life of biotinylated RBCs was only 8 days for Hyal2−/− RBCs (Figure 1A) compared with 25 days for control RBCs (P<0.001).

The accelerated turnover of Hyal2−/− RBCs suggests premature aging. Therefore, the main markers of RBC aging, i.e. PS exposure and auto-IgG binding, were measured. PS externalization did not differ significantly between Hyal2−/− and control RBCs (1.1±0.1% vs 0.9±0.1%, respectively; N=8; NS). The level of IgG bound to RBCs was significantly lower in Hyal2−/− RBCs than in normal RBCs (1.5±0.2% vs 3.6±0.4%, respectively; N=6; P<0.001).

Besides IgG, C3 deposition on RBC surface may also trigger auto-immune hemolytic anemia. Still, C3 exposure on RBCs was measured using cytometry and found to be
too low (<1% cells were positive for C3) to cause significant hemolysis in either HYAL2-deficient or control RBCs, even though the proportion of C3-positive cells was higher in \textit{Hyal2^-} vs control cells (0.81±0.15% vs 0.03±0.01%, respectively; N=14; P<0.0001).

To further test for intrinsic membrane defects, RBC deformability was measured using ektacytometry (Figure 1B). There was no significant difference between the curves of elongation index measured over a range of shear stresses in both genotypes. In particular, the elongation index at 7.8 Pa (the murine mean physiological shear stress)\textsuperscript{18} was identical in both genotypes (0.41±0.01 vs 0.41±0.01 in control and \textit{Hyal2^-} mice, respectively; N=10; NS), indicating no difference in RBC surface area under physiological conditions.

Concerning the platelets, electron microscopy revealed that \textit{Hyal2^-} platelets did not differ morphologically either before or after ADP- or thrombin-induced aggregation (Supplemental Figure 1A\&F). Furthermore, the amount of platelet microparticles did not differ between genotypes, ruling out premature aging of \textit{Hyal2^-} platelets (Supplemental Figure 1G).

Taken together, these data point to an accelerated clearance of platelets and RBCs without any sign of premature aging or eryptosis.

\textit{Hyal2^-} RBCs include acanthocytes and schistocytes. Close observations of blood smears (Figure 1C-F) revealed abnormalities in \textit{Hyal2^-} RBCs, including global poikilocytosis and shape irregularities (acanthocytosis). Moreover, various amounts of fragmented cells (schistocytes) were visible. A precise
quantification of these schistocytes was performed on blood smears. As shown in Figure 1G, schistocytes was significantly \((P<0.001)\) more frequent in Hyal2\(^{-/-}\) than in control blood smears. Figure 1H shows a strong correlation (Pearson’s \(r^2 = 0.862, P<0.001\)) between the proportion of schistocytes (0 to 6%) and reticulocytes (1 to 10%), pointing to a common process underlying both abnormalities.

The presence of schistocytes points to a mechanical destruction of Hyal2\(^{+/+}\) RBCs. Bone marrow histology did not reveal any abnormality and hemoglobin electrophoresis excluded hemoglobinopathy (data not shown).

To clarify the relative contribution of intrinsic defects vs peripheral consumption of RBCs, a more radical experiment, i.e. bone marrow transplantation, was performed. **Bone marrow transplantation does not reproduce all Hyal2\(^{-/-}\) blood abnormalities.**

Fourteen C57Bl/6 normal recipient mice were irradiated. Seven received bone marrow cells from a Hyal2\(^{+/+}\) inbred donor mouse while the others received bone marrow cells from a control inbred mouse. Twelve weeks later (i.e. the time necessary for bone marrow reconstitution), the engraftment of the donor cells was verified by genotyping circulating leukocytes: those of all recipients of Hyal2\(^{+/+}\) cells were indeed homozygous for the null allele (data not shown). Concurrent hematological counts (Table 2) showed significant but small (7 to 8%) differences in hemoglobin concentration between recipients of control and Hyal2\(^{+/+}\) marrows without significant difference in reticulocyte counts. Platelet levels remained 35% lower in recipients of Hyal2\(^{+/+}\) marrows. In summary, these results cannot exclude a minor intrinsic abnormality in Hyal2\(^{-/-}\) RBCs and platelets but they are insufficient to explain
chronic hemolytic anemia in *Hyal2*<sup>−/−</sup> mice. The role of extrinsic factors was therefore further explored using RBC transfusions.

The *Hyal2*<sup>−/−</sup> environment is responsible for a reduced RBC life span.

*In vivo* labeled RBCs from one control and one *Hyal2*<sup>−/−</sup> inbred donor mice were infused into recipient mice of both genotypes and the percentage of labeled RBCs was monitored over a 1-month period (Figure 2). In control recipient mice, the survival of *Hyal2*<sup>−/−</sup> RBCs was completely normal whereas in *Hyal2*<sup>−/−</sup> recipient mice the half-life of both normal and *Hyal2*<sup>−/−</sup> RBCs was dramatically reduced to the level of endogenous outbred *Hyal2*<sup>−/−</sup> RBCs. These observations strongly suggest that the origin of hemolysis in *Hyal2*<sup>−/−</sup> mice is almost exclusively extracorporeal and could be explained by mechanical destruction, a finding in agreement with the presence of schistocytes in *Hyal2*<sup>−/−</sup> blood smears.

*Hyal2*<sup>−/−</sup> mice displayed marked splenomegaly (Supplemental Table 2). It was therefore important to exclude splenomegaly as a cause of RBC and platelet destruction before analyzing various causes of mechanical stress. RBC survival was measured immediately before and 1 month after splenectomy in *Hyal2*<sup>−/−</sup> mice. This removal of the spleen had no influence on RBC half-life (Supplemental Figure 2).

In addition, anti-C5 antibodies were administered for 3 weeks in order to rule out complement-dependent hemolysis. Anti-C5 treatment has been shown to suppress plasma hemolytic activity by at least 60% in C57Bl/6 mice. In our hands, plasma hemolytic activity was reduced to 18.5±3.7% of baseline after 3 weeks of treatment in our outbred mice (N=9; *P*<0.0001). However, after a total of 6 biweekly injections of anti-C5 antibodies, the hematological differences between control and *Hyal2*<sup>−/−</sup> mice
persisted (Supplemental Table 3). We conclude that neither hemolysis nor thrombocytopenia of Hyal2−/− mice is C5-dependent.

**High HA concentrations do not increase PS exposure in vitro or blood viscosity in vivo.**

We hypothesized that the elevated plasma HA levels in Hyal2−/− mice could damage RBCs either through a direct toxicity or through increased blood viscosity and mechanical stress. To test this hypothesis, RBCs were first incubated with HA-rich plasma (either Hyal2−/− plasma or normal plasma supplemented with exogenous high molecular mass HA). These incubations did not induce signs of premature aging, such as increased PS exposure, in any type of RBCs (Figure 3A). Still, Hyal2−/− RBCs were more sensitive than normal RBCs to plasma incubation in general.

Second, whole blood viscosity was measured in blood samples from control and Hyal2−/− mice at different physiological shear rates ranging from 11 to 450 s⁻¹ (Figure 3B). No significant difference was detected between genotypes. Similarly, plasma viscosity did not differ (1.02±0.03 vs 1.03±0.01 Pa.s in control and Hyal2−/− mice, respectively; N=3, NS). These results rule out a toxic effect of HA on RBC, either directly or through blood viscosity.

**Hyal2−/− mice are normotensive.**

Malignant hypertension is another possible cause of RBC mechanical fragmentation. Arterial blood pressure was thus measured using a validated noninvasive method in trained mice. There was no difference in mean blood pressure
between genotypes (76.5 ± 2.0 mmHg vs 72.0 ± 1.5 mmHg in control and *Hyal2*−/− mice, respectively; N=7, NS).

*Hyal2*−/− mice have significant endothelial cell injuries with signs of thrombosis but a normal renal function and no deficiency in ADAMTS13.

Hemolytic anemia of extrinsic origin can also be a sign of microangiopathy. Therefore, markers of endothelial injury, i.e. VCAM-1, ICAM-1, and P-selectin, were measured. As shown in Figure 4A-C, all three markers were significantly higher in *Hyal2*−/− than in control mice. This endothelial impairment was not accompanied by changes in serum creatinine or urine albumin/creatinine ratio at 6 and 11 months of age (Supplemental Table 4). In other words *Hyal2*−/− mice showed no sign of renal dysfunction, even at an advanced age. Moreover, careful screening of microvessel histology in various tissues, particularly in liver and spleen, revealed no obvious signs of endothelium damage (data not shown) or glycocalyx scarcity (Supplemental Figure 3). On the contrary, endothelial glycocalyx tended to be thicker in *Hyal2*−/− than in control mice, while vascular fibrin deposition, a sign of thrombotic events, was found in numerous microvessels of *Hyal2*−/− mice but not in control mice (Figure 4D-G).

In humans, thrombotic microangiopathy is often associated with a deficiency in the vWF-cleaving protease, ADAMTS13. However, the activity of ADAMTS13 was not decreased in *Hyal2*−/− mice and was even higher than in control mice (Figure 4H). In
addition, no ultra-large multimers of vWF were detected in either genotype (Figure 4I), further supporting a normal activity of ADAMTS13.

**DISCUSSION**

*Hyal2*-* mice have been developed in our laboratory to study the exact function of HYAL2 in HA catabolism. Surprisingly, *Hyal2*-* mice were found to suffer from mild thrombocytopenia and compensated hemolytic anemia with increased plasma LDH levels. Working out these hematological defects, the current study revealed chronic thrombotic microangiopathic hemolytic anemia (MAHA) with fragmented RBCs (i.e. schistocytes) in *Hyal2*-* mice. HYAL2 deficiency seems to be a unique cause of chronic MAHA.

A thorough analysis of RBC parameters using an ADVIA 120 equipment showed that the absence of HYAL2 leads to macrocytosis (without folate or vitamin B12 deficiency) and anisocytosis without spherocytosis (the latter was also ruled out by normal eosine-5-maleimide binding data). Blood smears confirmed poikilocytosis and showed numerous damaged RBCs with a few schistocytes. RBC deformability was normal. Thus, poikilocytosis and macrocytosis likely result from accelerated erythropoiesis secondary to increased peripheral consumption. Bone marrow transplantation of either control or knockout stem cells into irradiated wild-type mice confirmed that the causes of erythrocyte and thrombocyte abnormalities must be mostly environmental since erythrocyte and thrombocyte production arising from *Hyal2*-* precursors was only minimally affected and there was no sign of hemolysis. Similarly, and perhaps even more convincingly, wild-type RBCs transfused into *Hyal2*-* mice disappeared as quickly as *Hyal2*-* RBCs.
RBCs turn over very quickly in \textit{Hyal2}⁻/⁻ mice; their half-life is a mere 8 days instead of 25 days in normal mice. Several experiments were performed to demonstrate that their rapid removal is not related to an overactive reticulo-endothelial system or spleen. Indeed, despite splenomegaly (probably a sign of extramedullary erythropoiesis in \textit{Hyal2}⁻/⁻ mice), splenectomy had no effect on the fast RBC turnover. In addition, no obvious platelet dysfunction or markers of platelet and RBC senescence were found.

Several elements including the presence of schistocytes point to a mostly intravascular mechanical destruction of RBCs and platelets in \textit{Hyal2}⁻/⁻ mice. The cause of this destruction could be the elevated plasma levels of HA. For instance, HA has recently been found to interact with the RBC surface leading to a reduction in erythrocyte deformability and aggregability.\textsuperscript{22} However, potential changes in erythrocyte rheological properties in the presence of HA do not necessarily imply hemolysis. In addition, we found that \textit{in vitro} incubation of RBCs with \textit{Hyal2}⁻/⁻ plasma or with high concentrations of HA itself was not toxic to the RBCs. High concentrations of HA could also increase blood viscosity. For this reason, we measured plasma and blood viscosity at different shear rates, including those present in murine microvessels under physiological conditions\textsuperscript{18} but there was no significant difference in viscosity between control and \textit{Hyal2}⁻/⁻ mice. Thus, HA does not seem to be directly involved in RBC destruction.

A typical or even atypical hemolytic uremic syndrome can also be excluded because (i) the hemolytic process in \textit{Hyal2}⁻/⁻ mice is chronic, (ii) renal function remains normal even at an advanced age, and (iii), more importantly, while activated C5 is required for complement-dependent destruction of RBCs, repeated administrations of anti-C5 antibody (BB5.1, the murine equivalent of eculizumab), though effectively repressing
plasma hemolytic activity, did not prevent erythrocyte elimination. Eculizumab is very efficient for the treatment of paroxysmal nocturnal hemoglobinuria and atypical hemolytic uremic syndrome. BB5.1 is similarly effective in several complement-dependent murine disease models such as collagen-induced arthritis or cardiac allograft rejection. Therefore we can reliably exclude C5 activation as a key step in HYAL2 deficiency-induced hemolysis. A few forms of chronic atypical hemolytic uremic syndrome, such as defects in membrane cofactor protein, may present with preserved renal function.

The remaining possibility is a complement-independent form of MAHA. In man, these forms, essentially characterized by thrombocytopenia and the presence of schistocytes in addition to anemia, are described as thrombotic microangiopathies (TM), thrombotic microangiopathic anemias (TMA), or simply MAHA. They represent a spectrum of disorders that usually present as hemolytic uremic syndrome or thrombotic thrombocytopenic purpura. They are mostly acute conditions linked to bacterial toxin exposure, pregnancy, organ transplant, malignancy, malignant hypertension, or vasculitis. Most TMAs are linked to ADAMTS13 deficiency or endothelial damage leading to partial occlusion of small vessels, mechanical trauma to RBCs, and platelet consumption. In Hyal2−/− mice, plasma levels of three typical endothelial cell injury markers, i.e. VCAM-1, ICAM-1, and P-selectin, were all significantly elevated but, unlike most human forms of MAHA, HYAL2-deficient MAHA was not accompanied by abnormalities in regulatory elements of the complement cascade, deficiency in vWF-cleaving protease, or any degree of organ dysfunction. RBC fragmentation (1 to 6%) and thrombocytopenia (to one third of normal levels) were mild. There was no visible purpura. Thrombotic events were identified through fibrin deposition in microvessels without visible tissue damage.
Since we did not find evidence of any of the spontaneous causes of TMA, such as autoimmune disorders, chronic vasculitis, hypertension, or the Kasabach-Merritt phenomenon, i.e., the presence of hemangiosarcoma, hemangioendothelioma or tufted angioma, we assume that HYAL2 deficiency represents a novel form of chronic MAHA. The link between HYAL2 deficiency, high plasma levels of HA, and endothelial damage remains to be elucidated. Although the final mechanism for intravascular hemolysis or TMA in Hyal2−/− mice has not been uncovered so far, we have demonstrated that a yet undetermined environmental factor present in Hyal2−/− mice (and perhaps in so far unexplained human cases of TMA) induces fibrin deposition, peripheral platelet consumption and RBC fragmentation (schistocytes). This observation represents the general mechanism of TMA in humans. Other causes of intravascular hemolysis or TMA such as lack of ADAMTS13 were clearly excluded. Our next objective will be to identify the specific Hyal2−/− environmental particularity at the origin of this novel form of TMA. Thereby, we will hopefully bring a better comprehension of the mechanism creating intravascular hemolysis in these mice and possibly in some forms of similar diseases in humans.

ACKNOWLEDGEMENTS
We thank Alexion Pharmaceuticals, Cheshire, CT, USA, for the gift of anti-murine C5 antibodies; Halozyme Therapeutics Inc., San Diego, CA, USA, for breeding Hyal2−/− mice; Katia Donadello, MD, Université Libre de Bruxelles, Belgium, for the ektacytometry experiments; Chantal Sevrin, CEIB, Université de Liège (ULg), Belgium, for the blood viscosity experiments; André Van Escote, MD, from Gamma-Médic clinical laboratory, Gembloux, Belgium, for blood parameters measurements; Jean-François Gaussin, UNamur, for help with histological sections; Laurence Poma (ULg) for help with blood pressure measurements; Corry Charlier for help with
transmission electron microscopy acquisition; Jennifer Defoux and Geoffroy Parmentier for help with platelet phenotype; and Professors Christian and Bernard Chatelain, both from CHU Dinant Godinne UCL Namur, for their support and advice.

FJ is an MD Postdoctoral Fellow of the Fonds National de la Recherche Scientifique (FNRS), and receives support from the FNRS (Research Credit 3309), the University of Liège (Fonds Spéciaux à la Recherche) and from the Fonds Léon Fredericq.

AUTHORSHIP CONTRIBUTION AND DISCLOSURE OF CONFLICTS OF INTEREST

The study was supported by institutional funds. CO, SD and FA performed experiments. CO and BF conceived the study and wrote the manuscript. LJ, FM, CG and FJ provided advice and materials. The authors declare no conflict of interest.

REFERENCES


24. Loirat C, Frémeaux-Bacchi V. Atypical hemolytic uremic syndrome. Orphanet J Rare Dis. 2011;6-60


### TABLES

Table 1. Corpuscular indices in outbred mice.

<table>
<thead>
<tr>
<th></th>
<th>Hyal2^{+/+} (N=15)</th>
<th>Hyal2^{+/-} (N=24)</th>
<th>Hyal2^{-/-} (N=49)</th>
<th>( P^1 )</th>
<th>( P^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBC (10^6 cells/µl)</td>
<td>9.79 ± 0.20</td>
<td>9.66 ± 0.10</td>
<td>7.75 ± 0.13</td>
<td>NS</td>
<td>***</td>
</tr>
<tr>
<td>Hb (g/dl)</td>
<td>14.14 ± 0.59</td>
<td>13.67 ± 0.16</td>
<td>12.51 ± 0.23</td>
<td>NS</td>
<td>**</td>
</tr>
<tr>
<td>HCT (%)</td>
<td>46.03 ± 1.30</td>
<td>43.17 ± 0.47</td>
<td>39.47 ± 0.68</td>
<td>NS</td>
<td>**</td>
</tr>
<tr>
<td>Ret (10^9 cells/L)</td>
<td>327 ± 24</td>
<td>240 ± 14</td>
<td>922 ± 46</td>
<td>NS</td>
<td>***</td>
</tr>
<tr>
<td>Ret (%)</td>
<td>3.60 ± 0.24</td>
<td>2.39 ± 0.11</td>
<td>12.12 ± 0.67</td>
<td>NS</td>
<td>***</td>
</tr>
<tr>
<td>IRF (%) (N=9)</td>
<td>44.97 ± 1.60</td>
<td>56.86 ± 2.08</td>
<td></td>
<td>***</td>
<td></td>
</tr>
<tr>
<td>MCV (fL)</td>
<td>45.87 ± 0.56</td>
<td>44.50 ± 0.34</td>
<td>51.16 ± 0.43</td>
<td>NS</td>
<td>***</td>
</tr>
<tr>
<td>MCH (pg)</td>
<td>15.42 ± 0.13</td>
<td>14.14 ± 0.15</td>
<td>16.17 ± 0.12</td>
<td>**</td>
<td>***</td>
</tr>
<tr>
<td>MCHC (g/dl)</td>
<td>32.69 ± 0.44</td>
<td>31.71 ± 0.20</td>
<td>31.66 ± 0.20</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>CHCM (g/dl)</td>
<td>32.04 ± 0.71</td>
<td>30.90 ± 0.11</td>
<td>30.97 ± 0.21</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>CH (pg)</td>
<td>14.94 ± 0.25</td>
<td>13.77 ± 0.11</td>
<td>15.78 ± 0.12</td>
<td>*</td>
<td>***</td>
</tr>
<tr>
<td>RDW (%)</td>
<td>13.61 ± 0.31</td>
<td>12.65 ± 0.27</td>
<td>14.72 ± 0.32</td>
<td>NS</td>
<td>***</td>
</tr>
<tr>
<td>HDW (g/dl)</td>
<td>2.25 ± 0.07</td>
<td>1.95 ± 0.03</td>
<td>2.73 ± 0.05</td>
<td>NS</td>
<td>***</td>
</tr>
<tr>
<td>PLT (10^3 cells/µl)</td>
<td>1140 ± 79</td>
<td>1252 ± 45</td>
<td>402 ± 26</td>
<td>NS</td>
<td>***</td>
</tr>
<tr>
<td>IPF (%) (N=9)</td>
<td>0.58 ± 0.04</td>
<td>6.33 ± 0.61</td>
<td></td>
<td>NS</td>
<td>***</td>
</tr>
<tr>
<td>MPV (fL)</td>
<td>7.06 ± 0.13</td>
<td>6.06 ± 0.18</td>
<td>7.71 ± 0.15</td>
<td>*</td>
<td>***</td>
</tr>
</tbody>
</table>

Vitamin 12 (nmol/L) 23.0 ± 3.7 33.1 ± 2.3 *

Serum folates (nmol/L) 52.7 ± 5.5 98.0 ± 11.9 **

*After in vitro reticulocyte maturation:*

<table>
<thead>
<tr>
<th></th>
<th>(N=9)</th>
<th>(N=9)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>MCV (fL)</td>
<td>40.70 ± 0.44</td>
<td>47.70 ± 0.63</td>
<td>***</td>
<td></td>
</tr>
<tr>
<td>RDW (%)</td>
<td>14.88 ± 0.29</td>
<td>15.07 ± 0.66</td>
<td>NS</td>
<td></td>
</tr>
</tbody>
</table>
RBC, red blood cell count; Hb, hemoglobin; HCT, hematocrit; Ret, reticulocytes; IRF, immature reticulocyte fraction; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration (calculated); CHCM, corpuscular hemoglobin concentration mean; CH, corpuscular hemoglobin content; RDW, red cell volume distribution width; HDW, hemoglobin concentration distribution width; PLT, platelet count; IPF, immature platelet fraction; MPV, mean platelet volume. \( P \) for ANOVA 1 (using Kruskal-Wallis test) was <0.001 for all parameters except for MCHC (\( P=\text{NS} \)) and CHCM (\( P=\text{NS} \)). Values of \( P^1 \) for Hyal2\(^{+/+} \) vs Hyal2\(^{+/-} \) and \( P^2 \) for Hyal2\(^{+/-} \) vs Hyal2\(^{-/-} \), calculated based on Dunn's Multiple Comparison test, are indicated. Mann Whitney test was used for IRF and IPF comparisons as well as for MCV and RDW after reticulocyte maturation. NS, non significant; *, \( P<0.05 \); **, \( P<0.01 \); ***, \( P<0.001 \).

Table 2. Effect of bone marrow reconstitution on blood parameters of irradiated Hyal2\(^{+/-} \) mice.

<table>
<thead>
<tr>
<th></th>
<th>Control marrow (( N=7 ))</th>
<th>Hyal2(^{+/-} ) marrow (( N=6 ))</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC (10(^3) cells/( \mu l ))</td>
<td>14.19 ± 0.62</td>
<td>13.86 ± 0.60</td>
<td>NS</td>
</tr>
<tr>
<td>RBC (10(^6) cells/( \mu l ))</td>
<td>9.87 ± 0.19</td>
<td>9.13 ± 0.19</td>
<td>*</td>
</tr>
<tr>
<td>Hb (g/dl)</td>
<td>13.42 ± 0.19</td>
<td>12.49 ± 0.24</td>
<td>**</td>
</tr>
<tr>
<td>HCT (%)</td>
<td>45.13 ± 0.61</td>
<td>42.55 ± 0.99</td>
<td>NS</td>
</tr>
<tr>
<td>Ret (%)</td>
<td>3.21 ± 0.10</td>
<td>3.49 ± 0.09</td>
<td>NS</td>
</tr>
</tbody>
</table>
Bone marrow engraftment was confirmed using whole blood PCR. Blood analysis was performed 12 weeks after irradiation when blood parameters were stable. WBC, white blood cell count; RBC, red blood cell count; Hb, hemoglobin; HCT, hematocrit; Ret, reticulocytes; PLT, platelet count. One mouse receiving Hyal2−/− bone marrow was excluded and euthanized due to rectal prolapse with visible bleeding. Each value represents the mean ± SEM. P values for the differences between control and Hyal2−/− marrows were calculated using Mann Whitney test. NS, non significant; * P<0.05, ** P<0.01.
FIGURE LEGENDS

Figure 1. Erythrocyte phenotype. (A) Endogenous RBC clearance. Control (closed circle, ●) and Hyal2⁻/⁻ (open circles, ○) outbred mice were injected with sulfo-NHS-LC-biotin. Then, 5 µl of blood were taken at the indicated time points. The percentage of biotinylated erythrocytes (biotin+ RBCs) was calculated as the ratio of positive cells to all RBCs. Each value represents the mean ± SEM of 6 mice in each group. Hyal2⁻/⁻ mice differed significantly from control mice (two-way ANOVA, \( P<0.001 \)). Half-lives of control and Hyal2⁻/⁻ RBCs were estimated to be 25 days and 8 days, respectively. (B) Deformability curve (ektacytometry). Elongation index values vs shear stress (Pa) are shown for RBCs from wild-type (closed circle, ●) and Hyal2⁻/⁻ (open circles, ○) inbred mice. Each value represents the mean ± SEM of 10 measurements on 5 mice in each group. Two-way ANOVA showed no significant difference between genotypes (\( P=0.18 \)). Similar results were found for outbred mice. (C-F) Typical blood smears of control (C) and Hyal2⁻/⁻ (D-F) outbred mice. The bar indicates 20 µm. Arrows point to schistocytes. (G) Percentage of schistocytes counted on blood smears in each genotype based on at least 500 RBCs. Individual values of control (closed circle, ●, \( N=7 \)) and Hyal2⁻/⁻ (open circles, ○, \( N=9 \)) mice, and means ± SEM are shown. ***, \( P<0.001 \) (Mann-Whitney test). (H) Correlation (Pearson’s \( r^2 \)) between the proportions of schistocytes (%) and reticulocytes (%) counted on blood smears of 7 control (closed circle, ●) and 9 Hyal2⁻/⁻ (open circles, ○) mice. \( P<0.001 \).

Figure 2. RBC transfusion. In vivo survival of sulfo-NHS-LC-biotin labeled RBCs transfused into wild-type and Hyal2⁻/⁻ recipient inbred mice. The donors were 1 female control inbred mouse (control RBCs represented by plain symbols, ● and ▲) and 1 female Hyal2⁻/⁻ inbred mouse (Hyal2⁻/⁻ RBCs represented by empty symbols, ○...
and △). The recipients were 3 female inbred wild-type (circles, ● and ○) and 3 female inbred Hyal2⁻/⁻ (triangles, ▲ and △) mice. Transfusion was performed via the tail vein. The percentage of circulating donor biotinylated erythrocytes (donor biotin+ RBCs) was calculated as the ratio of labeled RBCs to all RBCs. Approximately 7% of the RBCs were labeled immediately after the transfusion. Each value represents the mean ± SEM of 3 recipient mice in each group. Hyal2⁻/⁻ recipient mice differed significantly from control recipient mice (two-way ANOVA, P<0.001) but there was no difference in survival between control and Hyal2⁻/⁻ donor RBCs infused into mice of the same genotype.

**Figure 3. In vitro incubations with HA and blood viscosity.** (A) PS exposure (annexin V labeling) on RBCs was measured using FACS after 24-h incubation of control (grey bars, ) and Hyal2⁻/⁻ (white bars, ) RBCs. 1: No incubation. 2: Incubation in control HA-poor plasma. 3: Incubation in Hyal2⁻/⁻ HA-rich plasma. 4: Incubation in control plasma + 7.5 μg/mL high molecular mass (3.9×10⁶ Da) HA. The differences between genotypes were statistically significant for all samples except those which underwent no incubation (two-way repeated measure ANOVA [**, P<0.01] followed by Bonferoni tests [P<0.01-0.001] for each condition). (B) Blood viscosity was measured in 5 control (grey bars, ) and 6 Hyal2⁻/⁻ (white bars, ) mice at 4 predefined shear rates ranging from 11 s⁻¹ to 450 s⁻¹ using a rotation viscosimeter. The differences between genotypes were not statistically significant (two-way ANOVA, P=0.06).

**Figure 4. Endothelial and ADAMTS13 phenotypes.** (A-C) Serum levels of vascular cell adhesion marker-1 (VCAM-1, A), intercellular adhesion molecule-1 (ICAM-1, B), and P-selectin (C) were measured using ELISA tests in control (closed circles, ●)
and Hyal2⁻/⁻ (open circles, ○) mice. Each point represents one mouse. Horizontal lines represent means ± SEM. ***, P<0.001 (Mann-Whitney tests). (D-G) Representative images of small vessels with fibrin staining (in brown). Renal vessels are shown in glomeruli (D & E) and medulla (F & G), in both control (D & F) and Hyal2⁻/⁻ (E & G) mice. The bars indicate 50 µm. Positive stainings were found exclusively in Hyal2⁻/⁻ mice. (H) Percentage of plasma activity of ADAMTS13 in control (closed circles, ●) vs Hyal2⁻/⁻ (open circles, ○) mice, calculated according to the assay manufacturer’s instructions. Each point represents one mouse. Horizontal lines represent means ± SEM. *, P<0.05 (Mann-Whitney test). (I) Representative analysis of vWF multimers using 2% SDS agarose gels and Western blots in 4 wild-type inbred mice (1-4) and 4 Hyal2⁻/⁻ inbred mice (5-8). NP indicates the vWF multimeric pattern of normal human plasma. There was no difference between genotypes. Similar results were obtained for outbred mice.
SUPPLEMENTAL DATA

SUPPLEMENTAL METHODS

*Platelet phenotype.* Platelet-rich fractions were prepared as described\(^{34}\) using ACD (acid, citrate, dextrose) and apyrase (0.5 U/ml) as anticoagulants. ADP (10 µM) and thrombin (1 U/ml) were used to produce platelet partial and total activation, respectively. Platelet morphology was examined using electronic microscopy (JEOL and Philips Tecnai 10) observation as described elsewhere.\(^{34}\) In addition, the amount of platelet microparticles was measured using a BD Accuri C6 flow cytometer as described elsewhere.\(^{34}\) The following reagents were used: Megamix Beads (Biocytex), PE-rat anti mouse CD41 (BD Pharmingen, 558040), and FITC-Annexin V (BD Biosciences).

*Splenectomy.* Control and *Hyal2-* mice were anesthetized intraperitonally using a mixture of medetomidin (Medetor\(^ {®}\) 1mg/ml, Virbac Animal Health Belgium, Wavre, Belgium; 0.25 mg/kg) and ketamine HCl (Ceva\(^ {®}\), Brussel, Belgium; 40 mg/kg). The mice were placed on a heating table to maintain body temperature during all the procedure. After asepsis, a small incision was made in the left flank region, and the spleen was gently removed. One month after splenectomy, mice were injected with sulfo-NHS-LC-biotin to measure RBC survival.

*Anti-C5 administration.* Mice received biweekly intraperitononal injections of 750 µg anti-mouse C5 monoclonal antibodies (BB5.1) or control isotype-matched antibodies (both a kind gift of Dr Paul Tamburini, Alexion Pharmaceuticals, Cheshire, CT, USA) for 3 weeks. Blood samples were then collected to evaluate blood parameters and to confirm that treatment with anti-C5 led to decreased complement activity, using a hemolytic assay as described.\(^ {20}\)
**Glycocalyx staining in myocardial microvessels.** Endothelial glycocalyx was fixed and stained with Alcian blue 8GX as described elsewhere.\textsuperscript{35} Briefly, anesthetized mice were cannulated retrogradely at the aorta/brachiocephalic trunk bifurcation and the vena cava was transected. Three successive solutions were perfused at a constant pressure of 33 ± 5 mmHg: first, calcium-free cardioplegic solution (BSA 0.1% for 3 min); second, phosphate buffered fixative (pH 7.4) containing 30 mM MgCl\textsubscript{2} for 2 min; third, the same solution + 0.05% Alcian Blue 8GX (Sigma) for 30 min. Left ventricular wall was then cut into 2-mm segments. Samples were fixed as usual for transmission microscopy which was performed using a Tecnai instrument.

**Additional serum assays.** Serum creatinine was measured using Crea+ (Roche Diagnostics), urine creatinine using Urine Mouse Creatinine Assay Kit (Crystal Chem Inc., Downers Grove, IL, USA), and urine albumin using Mouse Albumin Elisa Kit (Aviva Systems Biology, San Diego, CA, USA).

**SUPPLEMENTAL REFERENCES**


### SUPPLEMENTAL TABLES

**Supplemental Table 1. Corpuscular indices in inbred mice.**

<table>
<thead>
<tr>
<th></th>
<th>Control (N=5)</th>
<th>Hyal2−/− (N=5)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>RBC (10^6 cells/µl)</strong></td>
<td>9.05 ± 0.23</td>
<td>7.23 ± 0.48</td>
<td>*</td>
</tr>
<tr>
<td><strong>Hb (g/dl)</strong></td>
<td>12.8 ± 0.38</td>
<td>11.4 ± 0.68</td>
<td>NS</td>
</tr>
<tr>
<td><strong>HCT (%)</strong></td>
<td>38.4 ± 1.06</td>
<td>34.3 ± 1.23</td>
<td>*</td>
</tr>
<tr>
<td><strong>Ret (%)</strong></td>
<td>3.75 ± 0.28</td>
<td>16.5 ± 2.87</td>
<td>**</td>
</tr>
<tr>
<td><strong>MCV (fL)</strong></td>
<td>42.4 ± 0.57</td>
<td>48.0 ± 1.73</td>
<td>*</td>
</tr>
<tr>
<td><strong>MCH (pg)</strong></td>
<td>14.2 ± 0.23</td>
<td>15.9 ± 0.26</td>
<td>*</td>
</tr>
<tr>
<td><strong>MCHC (g/dl)</strong></td>
<td>33.4 ± 0.40</td>
<td>33.2 ± 1.02</td>
<td>NS</td>
</tr>
<tr>
<td><strong>CHCM (g/dl)</strong></td>
<td>34.2 ± 0.56</td>
<td>34.6 ± 0.98</td>
<td>NS</td>
</tr>
<tr>
<td><strong>CH (pg)</strong></td>
<td>14.6 ± 0.13</td>
<td>16.5 ± 0.18</td>
<td>*</td>
</tr>
<tr>
<td><strong>RDW (%)</strong></td>
<td>13.8 ± 0.29</td>
<td>15.4 ± 0.93</td>
<td>NS</td>
</tr>
<tr>
<td><strong>HDW (g/dl)</strong></td>
<td>2.60 ± 0.07</td>
<td>3.40 ± 0.12</td>
<td>*</td>
</tr>
</tbody>
</table>

*RBC,* red blood cell count; *Hb,* hemoglobin; *HCT,* hematocrit; *Ret,* reticulocytes; *MCV,* mean corpuscular volume; *MCH,* mean corpuscular hemoglobin; *MCHC,* mean corpuscular hemoglobin concentration (calculated); *CHCM,* corpuscular hemoglobin concentration mean; *CH,* corpuscular hemoglobin content; *RDW,* red cell volume distribution width; *HDW,* hemoglobin concentration distribution width. Comparisons using Mann Whitney test. *NS,* non significant; *P <0.05; **P <0.01.*
**Supplemental Table 2. Weight of various organs in male mice.**

<table>
<thead>
<tr>
<th>Animal (g)</th>
<th>Control (N=10)</th>
<th>Hyal2^-/- (N=10)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Animal (g)</td>
<td>33.1 ± 1.8</td>
<td>33.8 ± 1</td>
<td>NS</td>
</tr>
<tr>
<td>Heart (g)</td>
<td>0.158 ± 0.007</td>
<td>0.182 ± 0.006</td>
<td>*</td>
</tr>
<tr>
<td>Liver (g)</td>
<td>1.560 ± 0.098</td>
<td>1.553 ± 0.046</td>
<td>NS</td>
</tr>
<tr>
<td>Spleen (g)</td>
<td>0.095 ± 0.007</td>
<td>0.274 ± 0.022</td>
<td>***</td>
</tr>
<tr>
<td>Left Kidney (g)</td>
<td>0.220 ± 0.012</td>
<td>0.215 ± 0.015</td>
<td>NS</td>
</tr>
<tr>
<td>Right Kidney (g)</td>
<td>0.224 ± 0.011</td>
<td>0.227 ± 0.012</td>
<td>NS</td>
</tr>
</tbody>
</table>

Each value represents the mean ± SEM. P values for the differences between control and Hyal2^-/- mice were calculated using unpaired Student’s t-tests. NS, non significant; *, P<0.05; ***, P<0.001. Female Hyal2^-/- mice had similar alterations compared with male Hyal2^-/- mice (data not shown).
### Supplemental Table 3. Effect of anti-C5 antibodies.

<table>
<thead>
<tr>
<th></th>
<th>Before injections</th>
<th></th>
<th>After injections</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Hyal2^/-</td>
<td>Control</td>
<td>Hyal2^/-</td>
<td>Control</td>
<td>Hyal2^/-</td>
</tr>
<tr>
<td></td>
<td>(N=6)</td>
<td>(N=14)</td>
<td>(N=3)</td>
<td>(N=7)</td>
<td>(N=3)</td>
<td>(N=6)</td>
</tr>
<tr>
<td><strong>WBC</strong></td>
<td>4.97 ± 0.90</td>
<td>6.54 ± 0.87</td>
<td>6.47 ± 1.34</td>
<td>5.27 ± 0.57</td>
<td>5.85 ± 0.76</td>
<td>5.99 ± 0.35</td>
</tr>
<tr>
<td>(10^3/µl)</td>
<td>NS</td>
<td></td>
<td>NS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>RBC</strong></td>
<td>9.57 ± 0.18</td>
<td>7.83 ± 0.25</td>
<td>9.72 ± 0.46</td>
<td>7.52 ± 0.52</td>
<td>10.02 ± 0.25</td>
<td>7.26 ± 0.32</td>
</tr>
<tr>
<td>(10^6/µl)</td>
<td>**</td>
<td>**</td>
<td>*</td>
<td></td>
<td>*</td>
<td></td>
</tr>
<tr>
<td><strong>PLT</strong></td>
<td>1237 ± 57</td>
<td>416 ± 40</td>
<td>1174 ± 75</td>
<td>507 ± 90</td>
<td>1459 ± 193</td>
<td>426 ± 101</td>
</tr>
<tr>
<td>(10^3/µl)</td>
<td>***</td>
<td></td>
<td>*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Ret</strong></td>
<td>2.57 ± 0.13</td>
<td>10.48 ± 0.69</td>
<td>2.61 ± 0.12</td>
<td>12.00 ± 1.58</td>
<td>2.72 ± 0.30</td>
<td>11.74 ± 0.76</td>
</tr>
<tr>
<td>(%)</td>
<td>***</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Blood parameters before and after 6 biweekly ip injections of control (isotype-matched) or anti-C5 monoclonal antibodies (mAb) in control and Hyal2^-/- mice. One mouse was excluded from the active group because its plasma hemolytic activity was not decreased by the anti-C5 mAb. WBC, white blood cell count; RBC, red blood cell count; PLT, platelet count; Ret, reticulocytes. Each value represents the mean ± SEM. P values for the differences between control and Hyal2^-/- mice were calculated using Mann-Whitney test; NS, non significant; *, P<0.05; **, P<0.01; ***, P<0.001.
**Supplemental Table 4. Serum creatinine and urine albumin-to-creatinine ratio in outbred mice.**

<table>
<thead>
<tr>
<th></th>
<th>6 months (N=8)</th>
<th></th>
<th>11 months (N=6)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Hyal2^−/−</td>
<td>P</td>
<td>Control</td>
</tr>
<tr>
<td>Serum creatinine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(mg/dl)</td>
<td>0.41 ± 0.02</td>
<td>0.39 ± 0.02</td>
<td>NS</td>
<td>0.41 ± 0.03</td>
</tr>
<tr>
<td>Urine alb/creat</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(g/mol)</td>
<td>3.80 ± 0.29</td>
<td>4.43 ± 0.78</td>
<td>NS</td>
<td>4.66 ± 0.36</td>
</tr>
</tbody>
</table>

Statistical comparisons using unpaired t test. NS, non significant.
SUPPLEMENTAL FIGURE LEGENDS

**Supplemental Figure 1. Platelet Phenotype.** (A-F) Platelet morphology observed using scanning electronic microscopy. (A-B) Morphological discoid appearance of washed platelets in control (A) and Hyal2⁻/⁻ (B) mice. (C-F) Morphological changes of washed platelets during ADP (C-D) or thrombin (E-F) induced aggregation in control (C and E) and Hyal2⁻/⁻ (D and F) mice. (G) Flow cytometric measurement of platelets microparticules in control (N=5) and Hyal2⁻/⁻ (N=6) mice. Means ± SEM are shown. There is no difference (NS) between genotypes.

**Supplemental Figure 2. Effect of splenectomy on RBC half-life.** Control and Hyal2⁻/⁻ outbred male mice were splenectomized. One month post-surgery, they were injected with sulfo-NHS-LC-biotin and 5 µl of blood were taken at the indicated time points. The percentage of biotinylated RBCs (biotin+ RBCs) was calculated as the ratio of positive cells to all RBCs in flow cytometry. The RBC survival of splenectomized control mice (▲) and Hyal2⁻/⁻ mice (△) is very close to the survival of endogenous RBCs in the respective, non-operated animals (control, ●; Hyal2⁻/⁻, ○). Each value represents the mean ± SEM of 3 mice in each group. There was no significant difference between RBC survival in splenectomized and non-splenectomized mice (two-way ANOVA).

**Supplemental Figure 3. Glycocalyx morphology in microvessels.** Endothelial glycocalyx (stained in black using Alcian Blue 8GX) was observed using transmission electronic microscopy in myocardial microvessels. Glycocalyx was structurally intact in Hyal2⁻/⁻ (B & D) compared with control (A & C) mice.
SUPPLEMENTAL FIGURES

Supplemental Figure 1

A

B

C

D

E

F

G

Platelet microspheres/μl

150

100

50

0

Control

Hyp2−

NS