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ADAMTS13 inhibition causes TTP in baboons

Thrombotic thrombocytopenic purpura directly linked with ADAMTS13 inhibition in the baboon (*Papio ursinus*)

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Supplementary information available online
Abstract

Thrombotic thrombocytopenic purpura (TTP) is the prototypical microangiopathy characterized by disseminated microthromboses, hemolytic anemia, and ultimately organ dysfunction. A link with deficiency of the von Willebrand factor (VWF) cleaving protease (ADAMTS13) has been demonstrated but additional genetic and/or environmental triggers are believed to be required to incite acute illness. Here we report that four days of ADAMTS13 functional inhibition is sufficient to induce TTP in the baboon (Papio ursinus), in the absence of inciting triggers, since injections with an inhibitory monoclonal antibody (mAb) consistently (n=6) induced severe thrombocytopenia (<12x10^9/L), microangiopathic hemolytic anemia and a rapid rise in serum lactate dehydrogenase. Immunohistochemical staining revealed the characteristic disseminated platelet-and VWF-rich thrombi in kidney, heart, brain and spleen but not lungs. Prolonged inhibition (14 days, n=1) caused myocardial ischemic damage and asplenia but not death. Control animals (n=5) receiving equal doses of a non-inhibitory anti-ADAMTS13 mAb remained unaffected. Our results provide evidence for a direct link between TTP and ADAMTS13 inhibition and for a mild disease onset. Furthermore, we present a reliable animal model of this disease as an opportunity for the development and validation of novel treatment strategies.
Introduction

Von Willebrand factor (VWF) is a multimeric glycoprotein that bridges platelets to injured arterial vessels through interactions with both subendothelial collagen and platelet membrane receptors. Unusually large VWF multimers (UL-VWF) are released as VWF precursors into the bloodstream by stimulated endothelial cells.1 These high molecular weight proteins are abnormally adhesive being able to bind and cross-link platelets in circulation even in the absence of endothelial injury.2 Normally, UL-VWF are rapidly cleaved by circulating VWF cleaving protease (ADAMTS13)3, which generates VWF multimers of sizes seen in normal plasma.4 The inability to process UL-VWF in cases of ADAMTS13 deficiency can cause disseminated platelet-rich thrombi which block terminal arterioles1,5, leading to hemolytic anemia with ischemic organ failure and ultimately death in patients with thrombotic thrombocytopenic purpura (TTP). Diagnosis is based on signs of concurrent thrombocytopenia with hemolytic anemia and fragmented red blood cells (schistocytes) in the absence of other identifiable primary causes.6

ADAMTS13 deficiency can be hereditary by mutations in the ADAMTS13 gene3 or acquired by inhibiting autoantibodies to ADAMTS13.7 The former is currently treated by infusion of fresh frozen plasma which contains donor ADAMTS13 to overcome the deficiency. The latter often requires plasma exchange to both replenish the diminished proteolytic activity and remove inhibitors. These plasma therapies could effectively reduce mortality to ~20%,8 but morbidity still is considerable and not seldom as a consequence of the plasma therapy.9,10 Safer therapeutic strategies are therefore required11 and could focus on the inhibition of the platelet-VWF interaction12 or on the reconstitution of enzyme by infusion of a recombinant ADAMTS13 preparation.13 However, the current Adamts13−/− mouse model does not consistently present with TTP features14,15 hampering preclinical evaluation of novel potential therapeutic approaches.

Motto et al. have indeed demonstrated that TTP can be triggered by injection of Escherichia coli derived shigatoxin in a subset of Adamts13−/− mice provided the ‘susceptible’ genetic background CASA/Rk.15 The authors concluded that unidentified genetic and environmental modifiers contribute significantly to the development of acute disease. This was strengthened by the variable onset of TTP in the CASA/Rk Adamts13−/− animals, i.e. while a small number of mice spontaneously developed TTP symptoms, others did not present with TTP even following stimulation with shigatoxin.
In man, triggers are not always distinctively identifiable, although some observations on pregnancy\textsuperscript{16,17}, infection\textsuperscript{18} and surgery\textsuperscript{19} as precipitating factors of acute illness in the background of ADAMTS13 deficiency, have been reported. On the other hand, some patients with congenital ADAMTS13 deficiency present with recurrent TTP from childhood on\textsuperscript{20} without reports of a trigger (e.g. proband C and proband D in Matsumoto \textit{et al}\textsuperscript{21} and patient 05 I-1 in Schneppenheim \textit{et al}\textsuperscript{22}), so-called Upshaw-Schulman syndrome (MIM #274150). Hence, identification, classification, as well as pathophysiologic explanation of the exact role and mechanism of triggers remains obscure.

In this work we have examined the effect of ADAMTS13 inhibition on primate hematophysiology \textit{in vivo} to determine whether additional triggers would be required to provoke TTP and to develop a reliable animal model for this disease.
Materials and methods

Monoclonal antibody characterization

Hybridomas were generated from mice primed with 10 μg purified human recombinant (full length) ADAMTS13 (rADAMTS13) and mAbs were produced on large scale in a bio-incubator (Celline CL 350, Integra Biosciences, Chur, Switzerland). The proteins were purified using protein A coupled Sepharose FF (GE Healthcare, Waukesha, WI), dialyzed to phosphate buffered saline, pH 7.4. All preparations were controlled for purity by sodium dodecyl sulphate polyacryl amide gel electrophoresis (SDS PAGE) and coomassie staining, for inhibitory activity against baboon plasma ADAMTS13 by FRETS-VWF73 assay and for endotoxin content using the LAL precipitation assay following instructions from the provider (Lonza, Basel, Switzerland).

Two mAbs were selected for further study, 3H9 as an inhibitor to ADAMTS13 and 5C11 as a non-inhibiting control. Measurement of ADAMTS13 activity in the presence of varying concentration of either mAb was with 2μM FRETS-VWF73 and baboon plasma as a source of ADAMTS13 at 3% (v/v) as described. Reactions contained Pefabloc SC (Roche, Basel, Switzerland) to inhibit serine proteases. Animals injected with the ADAMTS13 inhibitor 3H9 are in the so-called ‘subject’ group and animals injected with control 5C11 will be in the ‘control’ group.

Binding of rADAMTS13 to 5C11 and 3H9 was assessed by immunosorbent assay. mAbs were immobilized onto an 96-well plate followed by incubation with serial dilutions of rADAMTS13. Detection of bound enzyme was with peroxidase labeled anti-V5 (anti-V5-HRP, Invitrogen, Carlsbad, CA) and o-phenylenediamine as chromogenic substrate. All rADAMTS13 proteins contain a V5 epitope fused to the C-terminus. Apparent dissociation constants (K_D) were calculated by iterative fitting to the quadratic binding equation using Prism 5 software (GraphPad, La Jolla, CA).

3H9 and 5C11 epitopes were mapped to rADAMTS13 metalloprotease domain and a rADAMTS13 mutant lacking the second thrombospondin-1 repeat respectively using immunoprecipitation. Hereto, 10nM of recombinant protein was mixed with 5nM of mAb. Recombinant protein A coupled to Sepharose beads was used to precipitate the complexes by centrifugation. Bound domains were visualized by SDS PAGE followed by western blotting with anti-V5-HRP and chemiluminscence.

Baboon treatment, injections and sampling
Housing, treatment, phlebotomy and care for the Cape baboons (*Papio ursinus*) as well as the final protocol was approved by the Control Committee on Animal Experimentation of the University of the Free State (Bloemfontein, South Africa) in accordance with the National Code for Animal Use in Research, Education, Diagnosis and Testing of Drugs and Related Substances in South Africa prior to engaging any experimentation. All animals were male, weighed between 10 and 20 kg and were disease-free for at least 6 months. Animals were anaesthetized by intramuscular delivery of 10mg/kg ketamine hydrochloride. Injections and blood sampling were performed by venepuncture of the femoral vein while under anesthesia. All antibody injections were at 600μg/kg in physiologic phosphate buffer.

**Blood analysis**

All common blood parameters were determined blindly by automated and standardized methods in the Tertiary National Health Laboratory Service, Universitas Hospital, Bloemfontein, South Africa. Schistocytes were counted by an expert hematologist not involved in this study. Samples were blinded and counting was done manually. When less than 1% schistocytes were observed, the smear was considered normal. ADAMTS13 antigen was measured by ELISA as described using normal human pooled plasma (NHP, n=20) as a reference set at 100%. The concentration of murine mAb in baboon plasma was determined by a sandwich ELISA as previously described. Residual ADAMTS13 activity in baboon plasma was determined by FRETS-VWF73 as described with modifications. ADAMTS13 activity at baseline (before mAb injection) was taken as a standard set to 100%. To control for antibody-antigen dissociation as a consequence of diluting the samples, measurements were carried out at room temperature and the initial velocity (first 20 minutes) was measured every 30 seconds. Effects of antibody-antigen dissociation were however never observed as both antibodies have a small $k_{off}$.

**Tissue preparation and analysis**

Animals were euthanized by intravenous injection of 200mg/kg pentobarbitone (Euthapent, Kyron Laboratories, Johannesburg, South Africa) immediately followed by dissection and macroscopic inspection of lung, liver, heart, brain, kidney and spleen. Wedges of lung, heart, brain, kidney and spleen were dissected and fixed in 10% buffered formaldehyde for 24h. Liver sections were not
included for microscopic analysis. A Tissue Tek microtome/cryostat device (Bayer Healthcare LLC, Leverkusen, Germany) was used for processing and embedding. H&E staining was by standard techniques. Staining for VWF and platelets was with a polyclonal anti-VWF (P0226) and the mAb anti-human glycoprotein IIIa clone Y2/51 (M0753), respectively, both peroxidase labeled (Dako, Glostrup, Denmark). 3-3’-diaminobenzidine was used as a chromogenic substrate. The Martius Scarlet Blue (MSB) technique was used for staining fibrin. This stain colors erythrocytes yellow, connective tissue blue, nuclei black to violet, muscle and fibrin red.

Statistics

Significance between datasets was assessed by the Student’s $t$-test or the Mann-Whitney U-test depending on the result of the Kolmogorov-Smirnov test for normality. Welch’s correction to the Student’s $t$-test was performed if parametric datasets were found not to have equal variances by F-test (Prism 5, GraphPad). $P$-values were calculated comparing mean values of 3H9 with 5C11-injected animals using 0.05 as a cut off for significance.
Results

3H9 is an inhibitory anti-ADAMTS13 monoclonal antibody.

A series of monoclonal antibodies (mAb) was developed using purified full length human recombinant ADAMTS13 (rADAMTS13) as an antigen in Balb/c mice. The anti-ADAMTS13 mAb 3H9 binds an epitope in the metalloprotease domain of ADAMTS13 (Figure S1). 3H9 inhibits human ADAMTS13 function (not shown) and cross-inhibits ADAMTS13 in baboon plasma (Figure 1A). The non-inhibitory anti-ADAMTS13 mAb 5C11\textsuperscript{28} binds to the second thrombospondin type 1 repeat (Figure S2) and serves as a control. Both mAbs have comparable affinity for rADAMTS13 with apparent dissociation constants of 2.4±0.4nM and 2.2±0.4nM (mean±s.d., n=3) for 3H9 and 5C11, respectively (Figure 1B).

Systemic inhibition of ADAMTS13 in baboons injected with 3H9

Two boluses of 600 μg/kg 3H9 and 5C11 were administered intravenously to six and five baboons, respectively, over the course of two days with a 96h follow-up regime (Figure 1C). Antibody injections resulted in a mean initial plasma mAb concentration of >50nM which declined similarly for both mAbs (Figure 1D). Consequently, plasma ADAMTS13 activity fell below detection limit (<5%, Figure 2A), and antigen decreased to ~40% in subject animals (Figure 2B). ADAMTS13 activity was reduced to ~50% in control animals despite normal antigen levels and no measurable functional inhibition, in vitro (Figures 1 and 2). Neither VWF antigen (Figure S3) nor multimer patterns (Figure S4) changed significantly over the course of the study in either group.

Inhibition of ADAMTS13 consistently causes thrombocytopenia and schistocytic hemolytic anemia

All subject animals developed thrombocytopenia within 24h, with platelet counts decreasing further to 12±7x10⁹/L after 48h (Figure 3A). As a consequence, some animals had minor bleeding events which spontaneously resolved (Figure S5). Intravascular hemolysis became apparent as haptoglobin decreased to below the detection limit of the assay 72h post injection (Figure 3B) coinciding with a decrease in hemoglobin below 70% of baseline (Figure 3C). Schistocytes were observed in peripheral blood smears confirming the presence of fragmentation hemolysis (Figure 4A). The schistocyte count increased over time (Figure 4B) indicating that fragmentation was taking place continuously. Serum lactate dehydrogenase (LDH) levels, as a marker for tissue damage\textsuperscript{29} and hemolysis, increased ~3-fold (Figure 3D) with no signs of intravascular coagulation (Table S1). None of the animals had signs
of renal failure as evidenced by normal urea and creatinine levels (Table S2). One baboon had signs of myocardial ischemia by elevated serum troponin-T at 72h (0.08μg/L) and 96h (0.26μg/L). All animals behaved normally throughout the study with no noticeable signs of discomfort as evaluated by expert caretakers. Control animals had normal platelet counts, no bleeding (not shown), no evidence of intravascular hemolysis, no elevated LDH (Figure 3) and no schistocyte formation (Figure 4A).

**Baboons injected with 3H9 have immunohistochemical features characteristic of TTP**

Histological sections of kidney, heart, spleen and brain revealed numerous eosinophilic (hyaline) inclusions in small vessels of a subject but not control animal euthanized after 96h of treatment (Figure 5 and Figure S6). Immunohistochemical staining confirmed the thrombi were characteristic of TTP as these were enriched in VWF and platelet antigen CD61, while fibrin was lacking. Thrombi were not detected in lungs which is consistent with post-mortem pathological studies of human TTP. The combination of the hematological and histological findings provide sufficient evidence for TTP in all subject animals, suggesting a direct link between inhibition of ADAMTS13 activity and TTP.

**Advanced TTP by continuous injections of 3H9**

The above experiment induced TTP with limited organ damage except to the myocardium in one of the animals, but without evidence of discomfort. We hypothesized that prolonged inhibition of ADAMTS13 would increase the chance of advanced ischemic organ damage and raise the model to advanced or end-stage. One animal continuously received injections of 3H9 every 48h for twelve days with blood analysis every 24h. The immunoglobulin titer showed a cyclic pattern corresponding to the bolus infusions but without accumulation (Figure S7). As expected, all initial hematological indications characteristic of TTP gradually increased during the study. The animal remained thrombocytopenic with an average platelet count of 34x10^9/L (Figure S8A), serum LDH levels increased further to ~5-fold, and haptoglobin remained undetectable (Figure S8B). Severe anemia set in from day six but then leveled at 5 g/dL (Figure S8C). Blood smears became increasingly indicative for severe schistocytic hemolysis with a large number of reticulocytes and eventually Howell-Jolly bodies (Figure S8D). The latter were thought to indicate functional hyposplenia most likely due to widespread microvascular thrombosis of the spleen with autosplenectomy. At day six, a sudden but transient increase in troponin-T (0.41μg/L with cut off <.03, Figure S9) suggested some myocardial ischemic necrosis. Cardiac ischemia is observed in the majority of TTP patients and is associated with increased mortality.
and morbidity according to a recent study.\textsuperscript{31} However, this animal never became noticeably ill, and four days following cessation of injections all parameters returned to baseline with no further complications.
Discussion

Our data show that inhibition of ADAMTS13 in baboons consistently causes the characteristic hematological picture of TTP without the requirement of additional inciting triggers. Consequently, ADAMTS13 function is essential for maintaining microvascular integrity in non-human primates. This is in contrast to common laboratory mice where congenital deficiency of ADAMTS13 is prothrombotic but insufficient to cause spontaneous TTP. Differences between murine and primate physiology may account for this. For instance, certain modifiers, including other proteases that can substitute for ADAMTS13 activity, may process VWF more efficiently in mice than in primates.

The consistency of the response to inhibition of ADAMTS13 in baboons is rather unexpected, since TTP in human cases with congenital ADAMTS13 deficiency as well as in the susceptible Adamts13−/− mouse strain (CASA/Rk) has a variable time of onset. One possible explanation for this heterogeneity in human congenital TTP is that numerous ADAMTS13 mutations may result in diverse plasma ADAMTS13 activities below the detection limit of currently available assays (~5%), which would still be sufficient to prevent spontaneous recurrent TTP. This is substantiated by the observation that women with a specific hereditary ADAMTS13 deficiency get acute TTP only upon pregnancy, while their equally deficient brothers remain asymptomatic. Furthermore, a subset of TTP patients attains remission following plasma exchange treatment despite ADAMTS13 levels <5%. In addition, some polymorphisms drastically decrease ADAMTS13 activity with no reported medical consequences. Our findings are in line with this, since the control animals injected with 5C11 have reduced ADAMTS13 activity as well, yet without signs of disease. Contrary to this, Upshaw-Schulman syndrome is the rare congenital TTP variant which spontaneously sets in immediately after birth and is characterized by a chronic relapsing course requiring prophylactic infusion of donor plasma to constantly replenish the deficient enzyme. Taken together, this implies that a threshold ADAMTS13 activity, likely below 5%, underlies spontaneous precipitation of disease, i.e. in the absence of an obvious external inciting trigger. We assume ADAMTS13 inhibition in the subject baboons is below this critical level because the 3H9 plasma concentrations achieved in these animals exceed the Kd of the antibody-antigen interaction by far, indicating all ADAMTS13 molecules are occupied with inhibitor. This does however not rule out that TTP can be incited when ADAMTS13 activity levels are above threshold, since patients with measurable ADAMTS13 activity may still develop TTP. Though in that context, triggers like pregnancy or infection are likely to play a
substantial role by precipitating acute illness through a currently unknown mechanism. Therefore, our results do not exclude supplementary roles for triggers, they rather situate them in a specific context.

Another significant difference with the current *Adams13*−/− mouse model is that half of the shigatoxin treated CASA/Rk mice died15 while all baboons in our experiment survived. Even systemic inhibition of ADAMTS13 for as long as two weeks in a selected animal did not cause death. Prior to plasma exchange treatment, the average duration of hospitalization of TTP patients was fourteen days before death45,46, not taking into account a potential presymptomatic period. This could mean that the human and primate organs tolerate the process of ongoing thrombosis for a relatively long time. This may be because thrombi in TTP are disseminated in multiple organs47,48 and there allocate to terminal arterioles rather than to large afferent vessels. It is therefore possible that additional exogenous triggers accelerate the disease lifting it to an advanced level by acting synergistically with the ADAMTS13 deficiency rendering organs more susceptible to the consequences of microangiopathy. This has been observed in a patient homozygous for the 4314insA *ADAMTS13* mutation who presented with mild TTP symptoms which however exacerbated during pregnancy.49 In that respect certain triggers may not only function to merely incite disease and it therefore remains difficult to speculate how much longer the one animal with long-term ADAMTS13 inhibition could have survived the ongoing TTP.

Our model particularly reflects the early stages of acute acquired idiopathic TTP. In man this phase is poorly described, mostly because patients who seek medical assistance generally already have signs of organ damage (i.e. advanced stage TTP).50 Our data confirm that the early stages do not cause considerable discomfort which is in line with the minor signs of organ dysfunction in the baboons with TTP. Also in man, TTP has been suggested to present mildly36,50,51 prior to precipitating as an acute perceptible bout with symptoms of failing organs. Our study thus confirms and justifies the newly adapted diagnostic standard for TTP6,45 including thrombocytopenia with schistocytic hemolytic anemia in the absence of a primary illness but no longer requiring former criteria of advanced disease like neurologic and renal dysfunction. Consequently, our baboon model of TTP is ideally suited for preclinical testing of new treatment strategies since it provides consistency without animal discomfort. Moreover, the model can be used to address the role, the effect and nature of reported triggers.
Taken together, our results show that ADAMTS13 activity is essential for microvascular integrity in primates as TTP spontaneously and quickly occurs following ADAMTS13 inhibition in the absence of additional triggers. We are the first to demonstrate the initial stages of acquired idiopathic TTP suggesting these may go by relatively unnoticed as measurable organ failure and/or death are not seen before 96h of ADAMTS13 inhibition. Consequently, our model offers an opportunity for clinical and basic research on TTP by providing a framework for alternative therapies and novel insights.
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Author contributions

Contribution: H.B.F. and J.R. designed research, performed experiments and interpreted data. H.B.F. provided essential reagents and wrote the paper. J.R., and V.J.L. were responsible for the animal study design and supervised animal experimentation. J.R. coordinated the animal experimentation. K.V. designed research, provided essential reagents, interpreted data, coordinated tool development and wrote the paper. H.D. designed research and provided essential reagents. I.P., N.V., S.L. and W.J.V.R. performed experiments. V.J.L. and P.N.B. assisted as experts in clinical hematology and pathology. P.J.A. provided essential reagents and revised the manuscript. All co-authors critically reviewed the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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Figure 1. **3H9 inhibits baboon ADAMTS13.** (A) Enzyme activity was measured using FRETS-VWF73 as a substrate and baboon plasma as a source of ADAMTS13 in the presence of increasing concentrations of either 3H9 (●) or 5C11 (■). Change in fluorescence in function of time is depicted in function of the mAb concentration. (B) Binding of rADAMTS13 to immobilized 3H9 (●) and 5C11 (■) is shown as an increase in absorbance (A (490 nm)) in function of free rADAMTS13 concentration (n=3). Non-linear regression to the quadratic binding equation was performed (solid line). Error bars represent standard deviation. (C) Baboons were injected with boluses of 600 μg/kg of mAb at the time-points indicated by the arrow. Blood was sampled at the time-points indicated by the vertical dashed lines. (D) Concentration of mAb was determined ex vivo in citrated plasma samples from subject (●, n=6) and control baboons (■, n=5) and expressed as means ± s.d in function of time. The dashed lines indicate the rise in murine immunoglobulin titer following the second injection. Arrows indicate moments of injection.

Figure 2 **ADAMTS13 is inhibited and partially cleared in baboons injected with 3H9.** (A) The residual mean ADAMTS13 activity (Ac) and (B) antigen (Ag) levels were measured in citrated plasma from subject (●, n=6) or control baboons (■, n=5) relative to baseline (0h) and are shown in function of time. Error bars represent s.d. and dashed horizontal lines indicate the lower detection limit of the performed assay. Arrows indicate moments of injection.

Figure 3 **Hematological parameters indicate acute thrombocytopenia and intravascular hemolysis following inhibition of ADAMTS13.** (A-D) Hematological parameters were determined in blood samples from subject (●, n=6) or control animals (■, n=5) and mean values ± s.d are depicted. (A) Subject animals were thrombocytopenic from 24h onwards, dropping further below 12×10⁹/L at 48h. (B) In subject animals, haptoglobin levels fell below detection limit from 72h onwards. Regular statistics could no longer be performed (ND) for these time-points. (C) Hemoglobin levels were significantly lower in the subject group from 72h onwards and (D) total serum LDH activity increased significantly at 48h indicating organ and erythrocyte damage. Arrows indicate time-points of injection.

Figure 4 **Peripheral blood smears indicate thrombocytopenia, schistocytes and reticulocytes in baboons with functional inhibition of ADAMTS13.** (A) Peripheral blood smears from a representative animal from each group are shown. Blood was taken and prepared at the moments
indicated above the panel. A decrease in platelet numbers as well as the gradual appearance of schistocytes (▲) and reticulocytes (▼) is seen in smears from subject but not control animals. Preparation and staining was automated and according to the May Grunwald/Giemsa protocol. The original magnification was 400X for all panel. (B) Red blood cells were counted manually and blindly in peripheral blood smears from all subject animals (n=6). The mean percentage (± s.d.) schistocytes is plotted in function of time. On both panels arrows indicate time-points of mAb injection.

**Figure 5** Histopathological analysis confirms disseminated platelet-and VWF-rich aggregates in multiple organs. Tissue sections of kidney, heart, brain, lung and spleen (indicated above the panel) were prepared and stained as shown to the right; hematoxylin and eosin (H&E), immunohistochemical staining for platelet antigen CD61 and for VWF as well as a Martius yellow, Brilliant crystal scarlet 6R, and Soluble blue (MSB) stain for fibrin. The MSB stain colors both muscle and fibrin bright red and erythrocytes yellow/orange, nuclei and connective tissue stains blue/purple. Arrows (↓) and arrowheads (▲) indicate thrombi and open vessels, respectively. The original magnification was 400X for all panels.
Figure 3

(A) Platelet count (x10^9/L) over time (hours).
(B) Haptoglobin levels (g/L) over time (hours).
(C) Hemoglobin levels (g/dL) over time (hours).
(D) LDH levels (U/L) over time (hours).

Significance levels: P=0.019, ND, ND, P=0.201, P=0.007, P<0.001, P=0.032, P=0.026, P=0.005.
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