Enhanced hepatic clearance of hyposialylated platelets explains thrombocytopenia in GNE-related macrothrombocytopenia

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Tessa Noordermeer (University Medical Center Utrecht, Netherlands) Ivar van Asten (University Medical Center Utrecht, Netherlands) Roger Schutgens (UMC Utrecht, Netherlands) Anke Lakerveld (University Medical Center Utrecht, Netherlands) Cornelis Koekman (UMC Utrecht, Netherlands) Kay Hage (University Medical Center Utrecht, Netherlands) Silvie Sebastian (University Medical Center Utrecht, Netherlands) Albert Huisman (University Medical Center Utrecht, Netherlands) Dave van den Heuvel (Utrecht University, Netherlands) Hans Gerritsen (Utrecht University, Netherlands) Suzanne Korporaal (University Medical Center Utrecht, Netherlands) Marc Bierings (University Medical Center Utrecht, Netherlands) Jasper van der Smagt (University Medical Centre Utrecht, Netherlands) Mariëlle van Gijn (University Medical Center Groningen, Netherlands) Rolf Urbanus (University Medical Center Utrecht, Netherlands)

Abstract:

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Enhanced hepatic clearance of hyposialylated platelets explains thrombocytopenia in GNE-related macrothrombocytopenia

Tessa Noordermeer1*, Ivar van Asten1,2*, Roger EG Schutgens1, Anke J Lakerveld2, Cornelis A Koekman2, Kay Y Hage2, Silvie AE Sebastian2, Albert Huisman2, Dave J van den Heuvel3, Hans C Gerritsen3, Suzanne JA Korporaal2, Marc Bierings4,5, Jasper J van der Smagt6, Mariëlle E van Gijn6, Rolf T Urbanus1

1 Center for Benign Haematology, Thrombosis and Haemostasis, Van Creveldkliniek, University Medical Center Utrecht, Utrecht University, Utrecht, The Netherlands
2 Central Diagnostic Laboratory, University Medical Center Utrecht, Utrecht University, Utrecht, The Netherlands
3 Department of Molecular Biophysics, Utrecht University, The Netherlands
4 Department of Stem Cell Transplantation, Utrecht University Children's Hospital, Utrecht University, Utrecht, The Netherlands
5 Princess Maxima Center for Pediatric Oncology, Utrecht, The Netherlands
6 Department of Genetics, University Medical Center Utrecht, Utrecht University, Utrecht, The Netherlands

* Equal contribution

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Correspondence: Rolf T. Urbanus, PhD, Heidelberglaan 100, room G03.550, 3584 CX, the Netherlands.
Tel. +31-88-7557769, Fax: +31-88-7555418, e-mail: r.t.urbanus@umcutrecht.nl
Abstract

Glucosamine (UDP-N-Acetyl)-2-Epimerase and N-Acetylmannosamine Kinase (GNE) is the rate-limiting enzyme in the synthesis of sialic acid. Mutations in the GNE gene are associated with adult-onset myopathy. Recently, four GNE variants were identified in patients with congenital macrothrombocytopenia. The relationship between GNE mutations and macrothrombocytopenia is incompletely understood, as most myopathy patients have normal platelet counts. Here, we investigated the molecular mechanism underlying GNE-related thrombocytopenia. We identified 2 female siblings with a severe congenital macrothrombocytopenia, a severe bleeding pattern and a homozygous p.R420Q mutation in the GNE gene. Functional characterization of the 420Q-GNE variant confirmed loss of function. Patient platelets showed hyposialylation, but plasma markers for glycosylation were normal. $^{111}$Indium-labeled autologous platelet scanning in one patient indicated hepatic platelet sequestration and a decreased platelet half-life. Consistent with these findings, uptake of patient platelets by THP-1 macrophages and HepG2 liver cells was strongly increased. One patient was treated with romiplostim, which increased the platelet count and ameliorated the bleeding tendency. In conclusion, this study shows that the 420Q-GNE variant results in hyposialylation of glycans on platelets, which leads to rapid clearance by the liver and causes thrombocytopenia. Bleeding symptoms can be treated with romiplostim.

Key words: thrombocytopenia, sialic acid, platelet disorder, GNE-enzyme
Introduction
Platelets are produced by megakaryocytes in the bone marrow. Failure to produce enough platelets or enhanced clearance results in thrombocytopenia, and a bleeding tendency if the platelet count becomes too low.
Platelets contain many N- and O-linked glycans, which are normally capped by a sialic acid group. Loss of sialic acid from surface carbohydrates is a clearance signal for senescent platelets, but desialylation is also frequently observed in acquired thrombocytopenia. Recently, genetic variants in the Glucosamine (UDP-N-Acetyl)-2-Epimerase/N-Acetylmannosamine Kinase (GNE) gene were identified in patients with congenital macrothrombocytopenia. Two of the identified GNE-variants were associated with platelet hyposialylation, but platelet sialylation was not investigated in the context of the other variants.
GNE is the rate-limiting enzyme in the endogenous synthesis of SA. Mutations in GNE are associated with adult-onset myopathy. While low platelet counts have been reported in isolated cases of GNE myopathy, most patients have normal platelet counts. Hence, the relationship between GNE mutations and macrothrombocytopenia is incompletely understood. Here, we present two cases with congenital macrothrombocytopenia, a severe bleeding tendency and a mutation in the GNE gene. We provide evidence that the mutation results in impaired GNE activity and is associated specifically with hepatic clearance of hyposialylated platelets.

Case description
Two White European female siblings (P1 and P2) presented with spontaneous mucocutaneous bleeds and menorrhagia, which required frequent hospitalization. Their history showed a persistent severe macrothrombocytopenia from 1 week of age (table 1), with giant platelets in a peripheral blood smear (Fig 1A). Both patients received platelet transfusions on demand to restore hemostasis. Surface expression of the GPIb-V-IX complex was reduced (Fig 1B). Analysis of platelet reactivity towards agonists indicated normal responses (Fig 1C), although P-selectin expression was slightly reduced with two agonists. Their non-consanguineous parents did not have a bleeding tendency and had normal platelet counts (207x10^9/L and 341x10^9/L). Clinical quadruple whole exome sequencing indicated a homozygous missense variant (c.1259G>A; p. R420Q) in the GNE gene. The allele frequency of this variant is 8x10^-6 in the total population according to the gnomAD browser and it is predicted to be "probably damaging".

Methods
Participants

All patients provided informed consent in accordance with the declaration of Helsinki. P1 provided blood samples for additional analyses. Healthy subjects were recruited amongst employees and students of the UMC Utrecht. Institutional Ethics Review Board approval was obtained, and all blood donors gave written informed consent.

$^{111}$Indium-labeled autologous platelet scanning

$^{111}$Indium labeled platelet scanning was performed as described. Platelet signals in spleen and liver were quantified at 30min, 3h, 24h and 48h. The combined signals of liver and spleen were set at 100% and relative sequestration of radioactive platelets to liver or spleen was assessed as described. A spleen:liver ratio <0.8 indicates hepatic sequestration. Radioactivity in blood samples acquired at these time points was used to assess clearance rates, with radioactivity at 30 minutes set at 100%.

Flow cytometry

Platelet reactivity was determined as described. For measurement of surface glycans, whole blood or Human Embryonic Kidney (HEK)293 cells were labeled with either Fluorescein conjugated Ricinus Communis Agglutinin (RCA)-1 (Vector Labs, Burlingame, CA, USA) or fluorescein-conjugated Sambucus Nigra Lectin (SNA) (Vector Labs), fixated and analyzed on a BD FACS Canto II (BD Biosciences, San Jose, CA, USA). Platelets and cells were identified based on forward and side scatter. Glycan exposure on platelets and cells was expressed as median fluorescent intensity (MFI).

Wild-type and 420Q-GNE expression in HEK293 cells.

A guide (g)RNA targeting exon 2 of the GNE gene (5’-AAACCGATCATGGTTTGGCATTTAAC-3’) was cloned into the pSpCas9(BB)-2A-Puro (PX459) V2.0 plasmid, a gift from prof. Feng Zhang (Addgene plasmid #62988), as previously described. HEK293 cells were transfected with PX459-GNE plasmid. GNE knockout cells were selected with puromycin and were transfected with pcDNA6 containing either wild-type GNE (based on NM_005476.6) or the Q420-GNE variant. GNE overexpressing cells were selected with blasticidin.

In vitro platelet binding assays with HepG2 cells and THP-1 macrophages

Washed platelets were obtained as previously described. Platelets were labeled with CellTracker Deep Red (Invitrogen, Carlsbad, CA, USA). Human hepatocarcinoma (HepG2) cells were seeded and cultured
for 24 hours in a 24-well plate. Human mononuclear THP-1 cells were seeded and differentiated into macrophages with 0.1µM PMA for 24 hours. Serum-free medium was added to HepG2 cells or THP-1 macrophages and incubated for 30 minutes, before adding labeled platelets. Platelets were incubated for 30 minutes, unbound platelets were washed away, cells were harvested and analyzed with flow cytometry. HepG2 or THP-1 cells were gated based on forward and sideward scatter. Platelet binding was defined as the percentage Celltracker-positive HepG2 or THP-1 cells.

**GPIbα clustering**

GPIbα clustering was measured as described.\(^1\) FRET efficiency was used as a measure of GPIbα clustering and was defined as: \(\frac{\tau^D - \tau^{D/A}}{\tau^D} \times 100\%\), with \(\tau\) as the lifetime of the donor fluorophore in the absence (\(\tau^D\)) or presence (\(\tau^{D/A}\)) of the acceptor.

**Statistical analysis**

Statistical analyses were performed in GraphPad Prism 9 (San Diego, CA, USA). Differences between conditions were analyzed with Student’s T-tests. P-values <0.05 were considered statistically significant.

**Results and discussion**

The 420Q-GNE variant affects the ATP binding region of the N-acetylmannosamine kinase domain of GNE and is predicted to disrupt phosphorylation of N-acetylmannosamine. To investigate functional consequences of the 420Q-GNE variant, both Wild-type (WT)-GNE and Q420-GNE were introduced in engineered GNE-deficient HEK293 cells. Compared with WT cells, GNE deficient cells had reduced sialic acid (\(P=0.01\)) and increased galactose surface expression (\(P=0.01\)) (Fig 1D). Overexpression of WT-GNE fully restored the glycosylation profile but overexpression of 420Q-GNE did not, confirming pathogenicity of the 420Q-GNE variant.

Both patients had normal glycosylation of transferrin and APOCIII, accepted markers for N- and O-linked glycosylation, respectively\(^1^5\) (table 1), similar to what was reported in GNE-related myopathy. Since both patients had severe macrothrombocytopenia, and platelet hyposialylation has been reported to induce platelet clearance,\(^1\) platelet glycosylation profiles were investigated (Fig 1E). Binding of SNA, which recognizes terminal sialic acid in \(\alpha6\)-galactose linkage on N-linked glycans,\(^1^6\) was normal. In contrast, binding of RCA-1 was strongly increased. Since RCA-1 recognizes terminal galactose on both N- and O-linked glycans with or without sialic acid in \(\alpha6\)-linkage but does not recognize galactose with sialic
acid in $\alpha_3$-linkage, these data suggest reduced expression of sialic acid in $\alpha_3$-linkage with galactose. Sialic acid in $\alpha_3$-linkage with galactose is predominantly found on O-linked glycans. Interestingly, abnormal O-linked glycosylation has been reported in GNE-myopathy.

$^{111}$In-labeled autologous platelet scanning was performed in P1, which confirmed enhanced platelet clearance and showed a very short platelet half-life of 16 hours (Fig 1F). In addition, there was hepatic sequestration of platelets, based on a spleen:liver ratio of 0.33 (Fig 1G). Consistent with clearance rather than decreased production as the cause of the macrothrombocytopenia, both patients had normal TPO levels (table 1). Hepatic clearance of desialylated platelets has been attributed to both hepatocytes and liver-resident macrophages.$^{19,20}$ In line with these reports, platelets from P1 showed increased binding to both THP-1 macrophages and HepG2 cells (Fig 1H). Platelet GPIb$\alpha$ contains abundant N- and O-linked glycosylation sites. We have previously reported that platelet desialylation results in clustering of GPIb$\alpha$, which leads to enhanced platelet clearance.$^{14}$ Indeed, platelets from P1 showed substantial GPIb$\alpha$ clustering (fig 1I).

To increase platelet production, P1 and P2 were treated with TPO-receptor agonist romiplostim (Fig 1J). While this caused an increase in platelet count and ameliorated bleeding symptoms substantially, the platelet count remained low and platelets remained giant sized.

In conclusion, data presented here show that platelets from patients with the 420Q-GNE variant are hyposialylated and show GPIb$\alpha$ clustering. The hyposialylated platelets are rapidly cleared in the liver, leading to thrombocytopenia. Bleeding symptoms due to thrombocytopenia can be successfully treated with romiplostim. Further mechanistic studies are required to investigate the association between platelet hyposialylation and GPIb$\alpha$ clustering in GNE macrothrombocytopenia, to unravel why platelets are giant sized in GNE macrothrombocytopenia and why only platelets seem affected by the 420Q-GNE variant.

**Data Sharing Statement**
For data sharing, please contact the corresponding author at r.t.urbanus@umcutrecht.nl.

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**Authorship Contributions**
RS and RU designed the study. TN, IvA, AL, CK, KH, SS, AH, DvdH, JvdS and MvG collected the data. TN, IvA, AH, HG, SK, MB, JvdS, MvG and RU wrote the manuscript.

**Disclosure of Conflicts of Interest**
The authors have nothing to declare.
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Table 1: clinical parameters probands

|                        | Proband 1 | Proband 2 | Reference |
|------------------------|-----------|-----------|-----------|
| Current Age (y)        | 20        | 17        |           |
| ISTH-BAT               | 29        | 27        | <6        |
| platelet count (10^9/L) | 13        | 14        | 150-450   |
| MPV (fl)               | 18        | 21.2      | 7.0-9.5   |
| TPO (E/mL)             | 8         | 17        | 4-32      |
| vWF:RCo (IU/dL)        | 137       | 121       | 40-150    |
| Hb (mmol/L)            | 8.5       | 8.3       | 7.4-9.6   |
| RBC (10^{12}/L)        | 4.6       | 4.8       | 3.7-5     |
| WBC (10^9/L)           | 6.5       | 4.7       | 4-10      |
| Urinary Sialic Acid    | 30        | 35        | 19-43     |
| (mmol/mol creatinine)  |           |           |           |
| Sialylation APOCIII    | Normal    | Normal    |           |
| Sialylation Transferrin| Normal    | Normal    |           |

ISTH-BAT: Bleeding assessment tool of the International Society on Thrombosis and Haemostasis; MPV: Mean platelet volume; TPO: Thrombopoietin; VWF:RCo: Von Willebrand factor-ristocetin cofactor activity; Hb: Hemoglobin; RBC: Red blood cell count; WBC: Leukocyte count. *Platelet counts were measured with the CD61 method on a CELL-DYN Sapphire hematology analyzer (Abbott Laboratories, Abbott Park, IL, USA).
Figure 1. The 420Q-GNE variant is associated with platelet hyposialylation and enhanced hepatic clearance. (A) Peripheral blood smear of P1 (B) Relative expression of the Glycoprotein Ib-V-IX complex (GPIbα), fibrinogen receptor αIIbβ3 (αIIb) and collagen receptors glycoprotein VI (GPVI) and α2β1 (α2) in P1 (Red dots) and 49 healthy controls measured with flow cytometry. Data of healthy controls (Green) are expressed in box and whiskers. Average median fluorescent intensity values in controls were set at 100%. Whiskers represent 2.5th and 97.5th percentiles. (C) Platelet reactivity measured with flow cytometry in P1 (Red dot) and 49 healthy controls (Green box and whiskers). Platelets were stimulated with 60 μM ADP, 25 μM protease-activated receptor (PAR)-1 activating peptide (PAR1-AP) SFLLRN, 250 μM PAR4-AP AYPGKV, 1 μg/mL cross-linked collagen-related peptide (CRP-XL) or 5 μM U-46619, fixated and analyzed for expression of P-selectin as marker for granule secretion and fibrinogen binding as marker for αIIbβ3 activation. Average median fluorescent intensity values in controls were set at 100%. Whiskers represent 2.5th and 97.5th percentiles. (D) Wild-type (WT), GNE-deficient (KO), and GNE-deficient HEK293 cells overexpressing either recombinant WT-GNE, or recombinant 420Q-GNE (RQ) were incubated with fluorescein-conjugated SNA lectin for analysis of sialic acid expression, or fluorescein-conjugated RCA-1 lectin for analysis of galactose expression. Lectin binding was assessed with flow cytometry. Data were normalized on lectin binding in WT HEK293 cells (n=3). * indicates P-value <0.05, error bars represent the standard deviation. (E) Platelet sialic acid exposure was measured with fluorescein-SNA lectin and galactose exposure was measured with fluorescein-RCA-1 lectin on a flow cytometer in both P1 and P2, their parents, 4 thrombocytopenic patients and in 68 healthy controls. MFI: median fluorescent intensity. (F) Autologous platelets were labeled with 111Indium-tropolone and injected into P1. Platelets were collected at different time points to determine platelet half-life. The fraction 111Indium labeled platelets at 30 minutes post injection was set at 100%. Data represent the relative proportion of 111-Indium-labeled platelets at each time point. (G) Anterior static SPECT scans of the abdomen of P1 were made with a Symbia T2 gamma camera (Siemens, Erlangen, Germany) at indicated time points to quantify platelet sequestration. Radioactivity in liver and spleen regions (thick black lines) was assessed as percentage of total radioactivity. Spleen:liver radioactivity ratio <0.8 indicates hepatic sequestration. (H) Celltracker Deep Red-labeled platelets of P1 (Red) (n=2) and healthy controls (Green) were incubated with THP-1 macrophages (n=4) and HepG2 hepatocytes (n=3). Data are normalized on the number of platelet-binding cells with control platelets. (I) Platelets from P1 (Red) (n=3) or healthy controls (Green) (n=3) were labeled with fluorophore conjugated anti-
GPIbα Fab'-fragments (6B4). Fluorophore lifetime in presence and absence of an acceptor was assessed and used to calculate FRET efficiency. (J) Change in platelet count in P1 and P2 after initiation of treatment with romiplostim (black arrow).
Figure 1