GNE-related thrombocytopenia: evidence for a mutational hotspot in the ADP/substrate domain of the GNE bifunctional enzyme

The *GNE* gene encodes UDP-N-acetylglucosamine (UDP-GlcNAc) 2-epimerase/N-acetylmannosamine (ManNAc) kinase (GNE), a bifunctional enzyme catalyzing the synthesis of a sialic acid called 5-acetylneuraminic acid (Neu5Ac).[1]

Mutations of *GNE* are responsible for GNE myopathy (OMIM #605820), an autosomal recessive late-onset progressive muscle disorder[1] and sialuria (OMIM #269921), an autosomal dominant disease characterized by a congenital impairment of sialic acid metabolism.[1] In a small set of patients, biallelic mutations of *GNE* have only recently been associated with thrombocytopenia, either isolated or combined with muscle weakness.[2-6] However, it is unclear why only a few of the almost 1,000 individuals carrying biallelic mutations of *GNE* show thrombocytopenia.

We studied two families with severe thrombocytopenia using whole exome sequencing (WES). Family 1 proband (P1) was an 18 months-old boy born to consanguineous Egyptian parents with a platelet count of $5x10^9$/L at birth. The proband (P2) of family 2 was a 4-year-old boy, the third child of consanguineous Moroccan parents, who had scattered petechiae associated with severe thrombocytopenia (platelet count $4x10^9$/L) in his first hours of life (Figure 1A). In both P1 and P2 allo- and auto-antibodies against platelet antigens were not found in the mother's serum. Parents and siblings were healthy with normal blood counts, and no family history of thrombocytopenia was reported in either family. Splenomegaly or dysmorphic features were not observed in either of the patients, and the neurological assessment showed normal psychomotor development. Creatine phosphokinase (CPK) level was average, and no sign of myopathy was detected. In P1, both karyotyping and comparative genomic hybridization (CGH) array did not reveal any chromosomal alteration.

The WES analysis allowed us to identify two novel homozygous variants (c.1546_1547delinsAG and c.1724C>G) of the *GNE* gene, leading to the p.Val516Arg and p.Thr575Arg missense substitutions, respectively (Figure 1A and B). Extensive analysis of the exome data did not yield any other potential pathogenic variant, not even in the inherited thrombocytopenia-causing genes (IT-related genes; *Online Supplementary Table S1*). Moreover, we analyzed the runs of homozygosity (ROH) shared by P1 and P2 searching for potentially deleterious variants. Candidate genes were regarded as those whose mutations are associated with thrombocytopenia (n=56 from *Online Supplementary Table S1*) and those enlisted in the gene ontology (GO) term "Hemopoiesis" (n=788). Except for the mutations in *GNE*, all the other homozygous variants were excluded based on pathogenicity and splicing bioinformatic predictions (*Online Supplementary Table S2*).

Substitutions p.Val516Arg and p.Thr575Arg are rare variants affecting well conserved residues during evolution (Figure 1C). Their potential deleterious effect was supported by segregation analysis, bioinformatics predictions (*Online Supplementary Table S2*), and significant reduction of the GNE protein expression, which was likely to maintain some residual activity due to the incompatibility of complete loss of the GNE function with life (Figure 1D).[7] Consistent with alteration of the GNE kinase activity, the transferrin serum glycoforms analysis revealed a higher level of the asialo, disialo and trisialo forms and a correspondent decrease in the tetrasialotransferrin form in both patients (Figure 2A).

The hematological and clinical features of P1 and P2 are strikingly similar. Except for petechiae and minor post-traumatic bruises (grade 1/5) occurring when the platelet count was below 10-$20x10^9$/L, no clinically significant bleeding was reported regardless of treatment. Consistent with data in the literature,[4] they had increased mean platelet volume (MPV) (MPV 11.9 fL in P1 and MPV 10.8 fL in P2) and high immature platelet fraction (IPF) (IPF 50-80% in P1 and IPF 39-89% in P2) (Figure 2B). In bone marrow aspirates of both the affected individuals the number of megakaryocytes was markedly increased, and several immature small-sized and hypolobulated megakaryocytes were observed (Figure 2C to E). Interestingly, this condition mimicked the pathophysiology of another inherited defect of platelet sialylation, namely SLC35A1 deficiency, which affects the same biochemical pathway,[8] and is partly reminiscent of the peripheral platelet destruction in immune thrombocytopenia (ITP).

High-dose intravenous immunoglobulin and steroid treatment resulted in no improvement of the patients' platelet count, therefore both probands required regular platelet transfusions in the first year of life (Figure 2F). The response to platelet transfusion and the megakaryocytes features suggest that the patients' platelets are more rapidly removed from circulation for intrinsic cellular defects, such as sialylation reduction, rather than a decreased platelet production.[4]

Both patients were treated with romiplostim to reduce the need for transfusions. P1 responded at low doses (4 µg/kg/week) and his platelet count was higher than $25x10^9$/L during treatment, which is currently ongoing (Figure 2G), while P2 required a high dose of romiplostim (up to 10 µg/kg/week) to obtain a substantial, extremely unstable, response (Figure 2G), thus discouraging the continuation. Fluctuating response to romiplostim was also reported in patients with ITP further supporting the similarity with GNE-related thrombocytopenia.[8] Currently, despite a low platelet count (5-$10x10^9$/L), patient P2 did not experience any bleeding up to the last follow-up.

The absence of severe bleeding despite extremely low platelet counts was in line with other cases reported in the literature (*Online Supplementary Table S3*). Among these, only two (P6 and P17) experienced severe or life-threatening hemorrhages. The mild bleeding diathesis might be attributed to the abundance of young enlarged platelets, which have a prothrombotic potential.[9] These data suggested that prophylactic treatment might be needed only in specific conditions (e.g., neonatal period; severe hemorrhage; surgical procedures), and an approach aimed at treating only acute events with platelet transfusion might be considered in most patients.

Several lines of evidence supported the hypothesis that *GNE* mutations can cause thrombocytopenia. At least to our knowledge, 20 patients from nine unrelated families of the nearly 1,000 individuals with alterations of this gene have been reported to have thrombocytopenia (*Online Supplementary Table S3*).[2-4,10,11] Of the 15 different variants identified in these patients, including P1 and P2, seven have been previously reported in patients with GNE myopathy (Figure 3). Except for p.His188Tyr, which is in cis with a known mutation (p.Asn550Ser) associated with myopathy,[4] the others (p.Asp444Tyr,

p.Gly447Arg, p.Gly506Phe, p.Val516Arg, p.Leu517Pro, p.Thr575Arg, and p.Gly578Ser) were regarded as specific variants associated with isolated thrombocytopenia. Of note, these seven variants spread over a region of approximately 130 amino acids of the ManNac kinase domain (Figure 3).

We analyzed the structure of N-acetylmannosamine kinase in complex with N-acetylmannosamine and ADP (2yhy)[12] to assess the potential effect of the mutations on the structure and function of the enzyme (Figure 3). Residues Asp444, Gly447 and Thr575 in the ADP pocket, and Gly506 and Gly578 close to the substrate domain

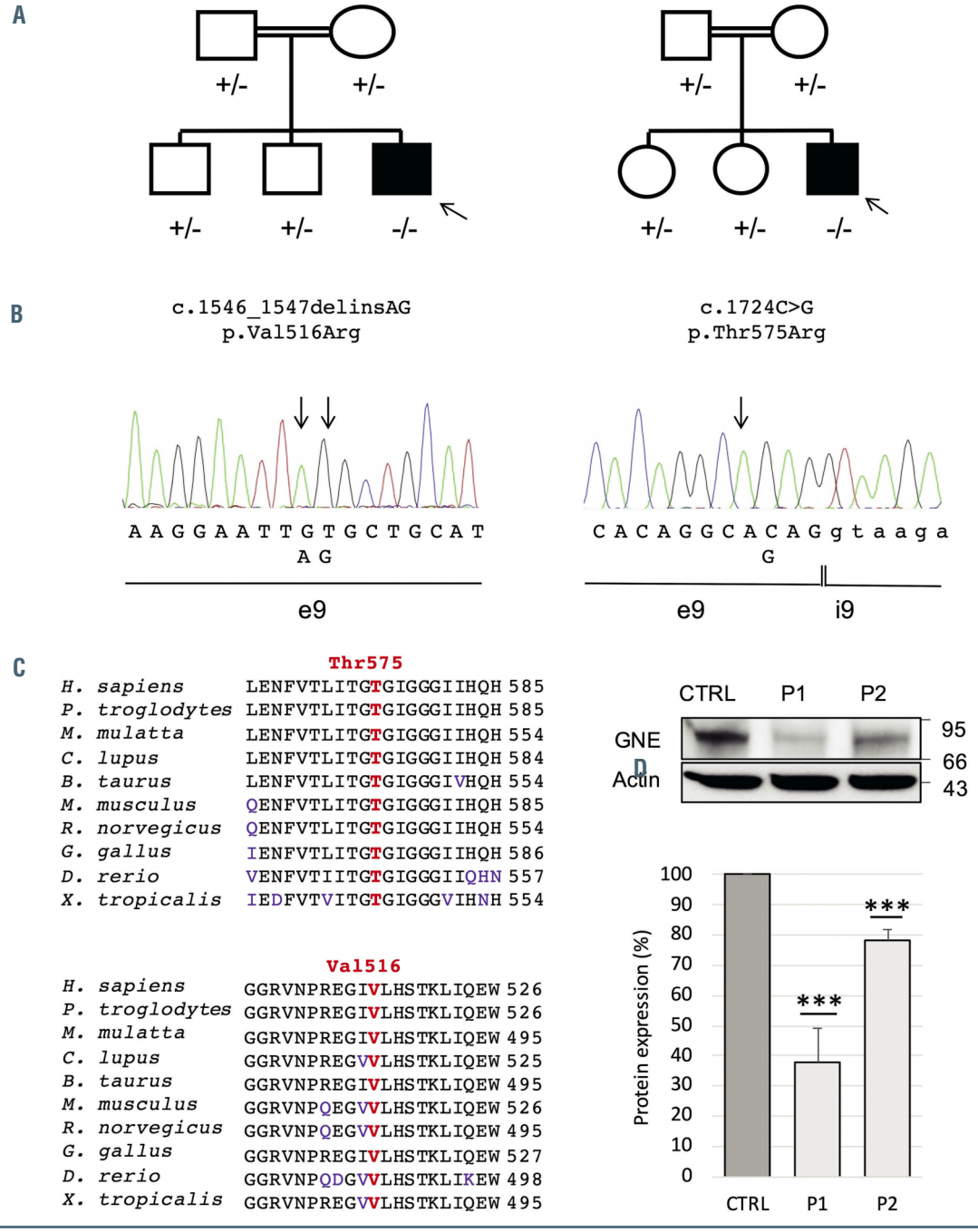
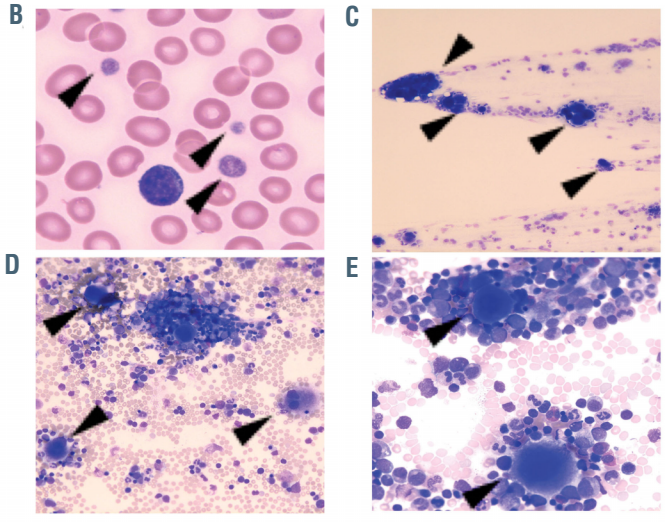


Figure 1. Identification of novel mutations of the *GNE* gene. (A) Pedigrees and segregation analysis in the two families F1 and F2. (B) Electropherograms of exon 9 showing the c.1546_1547delinsAG and c.1724C>G substitutions in probands P1 and P2, respectively. Sanger sequencing was performed using the following primers: 9F/5'-TTCTAGAAATCTTTAAGGTGCTATGG-3' and 9R/5'-CCACCTGACCATGTTGAAGA-3'. (C) Protein multiple alignments, showing conservation through different species at residues (in red) affected by the p.Val516Arg and p.Thr575Arg mutations. *H. sapiens* (NP_001121699.1), *P. troglodytes* (XP_003312121.1), *M. mulatta* (XP_001082113.2), *C. lupus* (XP_003431623.1), *B. taurus* (NP_001178072.2), *M. musculus* (NP_056643.3), *R. norvegicus* (NP_446217.1), *G. gallus* (NP_001026603.2), *D. rerio* (NP_957177.1), and *X. tropicalis* (NP_001072728.1). (D) Western blot and of total lysates from lymphoblast cells of P1 and P2. Total protein lysates were prepared from these cells using M-PER™ Mammalian Protein Extraction Reagent (Thermo Fisher Scientific). Protein quantification shows only a partial expression (39% and 79%, respectively) of GNE expression compared to wild-type (CTRL) (***P<0,002). Actin was used as a loading control for protein quantification. The antibodies were used as follows: anti-GNE (Santa Cruz Biotechnology, sc-376057, 1:500) and anti-β-actin (Santa Cruz Biotechnology, sc-47778, 1:4,000) as primary antibodies, anti-mouse immunoglobulin conjugated with horseradish peroxidase (HRP) (Bethyl, A90-116P, 1:10,000) as a secondary antibody. Statistical analysis was performed using the *t*-test. Error bars represent the standard deviation of 4 independent experiments.

A

	P1	P2	Normal value
Asialo-transferrin	absent	0.2% ↑	absent
Disialo-transferrin	12.18% ↑	5.8% ↑	0.3 – 2.4%
Trisialo-transferrin	22.9% ↑	14.9% ↑	1.3 – 8.9%
Tetrasialo-tranferrin	59% ↓	66.8% ↓	67.2 – 80.3%
Pentasialo-transferrin	5.88%	10.60%	5 – 17%



F

Timepoint after transfusion	Median platelet count (P1-P2) [x10^9/L]
Before platelet transfusion	13 (3-22)
Day 1	74 (66-88)
Day 2	91 (76-106)
Day 3	55 (34-71)
Day 4	40 (35-60)
Day 5	33 (26-53)
Day 7	26 (8-36)

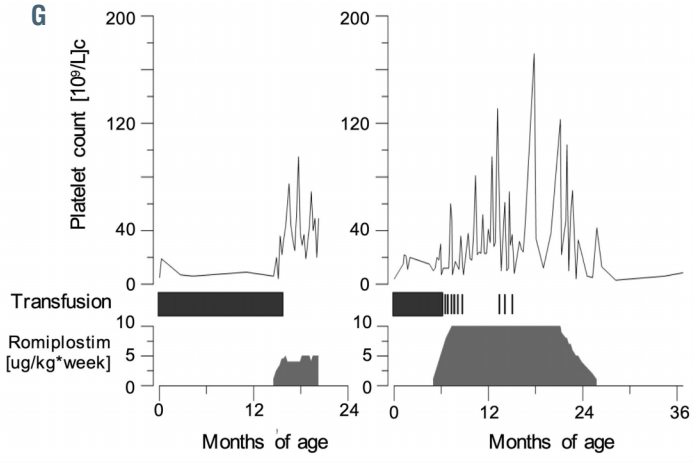


Figure 2. Blood, bone marrow features, and response to transfusion and treatment. (A) Sialotransferrin profile determined by ion-exchange chromatography using a commercial kit (CDT in Serum, Recipe München). The different isoforms were pointed out by UV detection at 460 nm and quantified by the "area percent method" (i.e., the relative abundance of each isoform is expressed as the percentage ratio of the peak area compared to the sum of the areas of all the peaks). (B) Peripheral blood smear of P2 showing enlarged platelets. (C to E) Bone marrow aspirates with an increased number of megakaryocytes at different stages of maturation. May-Grünwald-Giemsa staining; original magnification 100X (B), 10X (C), 20X (D), and 40X (E). (F) Response to platelet transfusion. Median platelet count before platelet transfusion and up to 7 days following transfusion are shown for both patients (P1 and P2's specific values are indicated in brackets). (G) Time course of platelet count in response to treatments for P1 (left) and P2 (right) in response to romiplostim administration at different dosages. The dark grey bar indicates a period of complete transfusion dependency, with transfusion every 5-7 days. Values of platelets measured within 7 days after platelet transfusion are not shown.

may directly affect the enzyme active site, impairing ADP or substrate binding, respectively (Figure 3). Otherwise, p.Val516 and p.Leu517 are localized in the hydrophobic core and when mutated into an arginine or proline, respectively, may destabilize the fold and the conformation of the entire protein. Accordingly, *in vitro* mutagenesis of the highly conserved Asp444 residue in the ADP binding pocket resulted in the complete loss of the kinase function, though retaining the epimerase activity.[13] Therefore we could hypothesize that megakaryocytes and platelets are more sensitive than other cells to defective kinetic activity or substrate-binding affinity, thus explaining the occurrence of thrombocytopenia.

Nevertheless, whether *GNE* mutations are responsible for thrombocytopenia either isolated or in combination with muscle wasting remains to be elucidated. Indeed, considering that the GNE myopathy typically appears in the third decade of life, we cannot exclude that patients with only thrombocytopenia will develop myopathy later in their life.[2-4] Patients carrying the p.Asp444Tyr (F3), p.Gly447Arg (F5), p.Val516Arg (F1), p.Leu517Pro (F7), and p.Thr575Arg (F2) mutations were neonates or in their first/second decade of life. Instead, among the six individuals homozygous for p.Gly506Phe (F6) or p.Gly578Ser (F4) and all between 24-42 years of age, only two have subclinical features of myopathy, suggesting that, in addition to a low platelet count, this mutation could correlate with a mild form of muscle wasting of late onset.

Trying to explain why only few patients with *GNE* mutations have a low platelet count, we cannot exclude that *GNE* variants cause thrombocytopenia only when co-segregating with other genetic factors. Whereas WES analysis did not provide any other plausible candidate in our families, the recessive transmission of variants in other

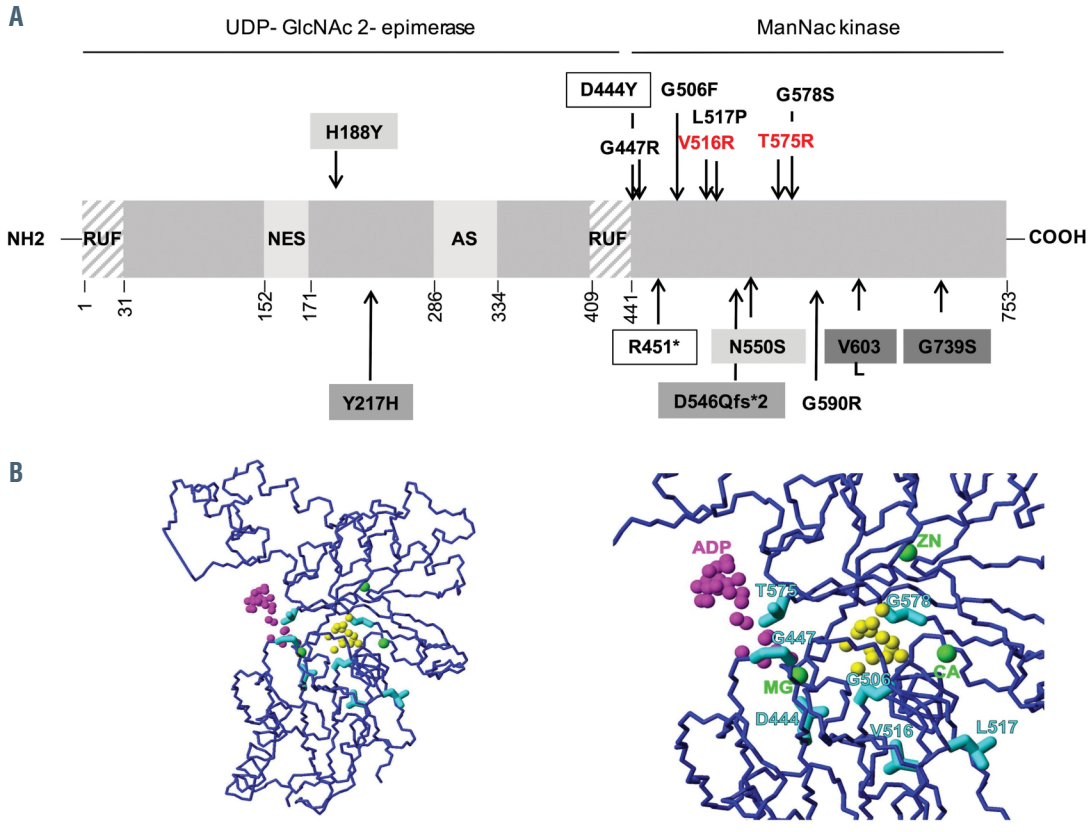


Figure 3. Localization of the *GNE* mutations associated with thrombocytopenia and three-dimensional structure of the GNE enzyme. (A) Among the 15 mutations (*Online Supplementary Table S3*), the 7 associated with myopathy are indicated below the schematic representation of the protein structure; the other 8 are depicted above the protein (in red, the novel mutations reported in this paper). All the mutations identified in patients are in a homozygous state except those represented in matched colored boxes that are heterozygous biallelic *GNE* mutations found in a single patient. H188Y and N550S (light grey boxes) are homozygous mutations identified in the same patient. UDP-GlcNAc 2-epimerase: UDP-N-acetylglucosamine 2-epimerase; ManNAc kinase: N-acetylmannosamine; NH2: NH2-terminus; COOH: COOH-terminus; RUF: a region with unknown function; NES: putative nuclear export signal; AS: allosteric site. Nomenclature of mutations was referred to the NM_001128227.3 transcript. (B) The overall structure (left) and the zoom of the enzymatic pocket (right) of GNE. The side chains of the positions affected by the mutations discussed in this article are explicitly indicated. The structure (Protein Data Bank [PDB] entry 2yhy) corresponds to the GNE complex with ManNAc and ADP. The structure was analyzed by PyMOL and MOLMOL graphic support tools. The degree of exposure of the residues affected by mutations was established by DSSP (Define Secondary Structure of Proteins) analysis.

genes, such as *ANKRD18A*, *FRMPD1*, *FLNB*, *PRKACG*, have been reported in other cases.[2,3,5,14] However, their potential impact was not further investigated, except for p.Ile74Met in *PRKACG*, whose functional studies demonstrated its effect in thrombocytopenia.[14]

In summary, although the role of *GNE* mutations is well-documented in GNE myopathy and sialuria, we identified two novel *GNE* variants, which together with a few other mutations reported in the literature could explain thrombocytopenia and extend the clinical phenotype of the GNE defects. In both patients, as well as in those of families F3 and F5 from a literature review, severe thrombocytopenia was reported since the first days of life, when the differential diagnosis of thrombocytopenia was broad and included thrombocytopenia secondary to sepsis and critical care, neonatal allo- and auto-immune thrombocytopenia, or ITP, such as congenital amegakaryocytic thrombocytopenia.[15] Therefore, evaluating the sialotransferrin profile in patients with suspected inherited thrombocytopenia, large platelets and increased reticulated fraction might provide an important diagnostic clue.

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