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MUTATION IN *GNE* IS ASSOCIATED WITH A SEVERE FORM OF CONGENITAL THROMBOCYTOPENIA

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Inherited thrombocytopenias are associated with bleeding of all types of severity depending on the reduction in platelet count and whether there is altered platelet function.[1] The normal range for platelet counts varies by up to threefold, but an individual's platelet count is normally maintained within a narrow range. This requires a constant balance between thrombopoiesis, and platelet senescence and consumption. Heritable forms of thrombocytopenia are frequently caused by genes which regulate megakaryocytic differentiation and/or platelet production. Next generation sequencing strategies, such as whole exome sequencing, are efficient in identifying gene mutations that cause Mendelian disorders.[2-4] In this study, we used a whole exome-sequencing approach, to elucidate the genetic basis of a severe form of congenital thrombocytopenia.

We present a UK consanguineous family of Pakistani origin with two cousins with severe thrombocytopenia (Figure 1B). The proband, III:5, was aged three years with a platelet count of $3x10^9$/l when entered into the Genotyping and Phenotyping of Platelets (GAPP) study. He was born by emergency caesarean section at 34 weeks gestation and had neurological symptoms shortly after birth and bilateral intraventricular haemorrhages. He had a ventriculo-peritoneal shunt inserted which required several revisions, with HLA platelet transfusion prophylaxis. He has developmental delay, skull abnormalities secondary to hydrocephalus and nystagmus. His baseline platelet count has remained at approximately 10 x 10^9/l. He received HLA matched platelet transfusions every 1-2 weeks for the first twelve months of life and his platelet count incremented well hereafter. There are no other abnormalities in the blood count. The bone marrow aspirate and trephine showed a normocellular specimen with normal megakaryocyte numbers and morphology and normal cytogenetics. Patient III:3 was aged seven years when recruited into the study. She has a baseline platelet count of 15-20 x 10^9/l and receives weekly HLA matched platelet transfusions to minimize symptoms from epistaxis and haematomata, previously causing hospitalisation. In both patients, coagulation parameters were normal and there were no anti-platelet autoantibodies or HLA antibodies. Blood (15 ml) from patients and healthy controls was taken in 10% by volume 3.8% trisodium citrate. Platelet rich plasma (PRP) was prepared and flow cytometry was conducted as previously described.[5] Transmission electron microscopy was performed as described[6] and examined using a JEOL 1200EX transmission electron microscope (Hertfordshire, UK). The number of α-granules per μm^2 was calculated for at least 40 platelets from each patient/control. The whole exome of the two affected individuals was sequenced with the SureSelect human All Exon 50Mb kit (Agilent Technologies) and sequencing on the HiSeq 2000 (Illumina) with 100 bp paired-end reads. The sequences were aligned to the reference genome (hg19 build).[5] To verify candidate

mutations, Sanger sequencing was performed using standard methods on an ABI 3730 automated sequencer.

Both patients have severe thrombocytopenia with platelet counts in platelet rich plasma (PRP) of :- patient III:5 1.5 x 10^7/ml and patient III:3 2.5 x 10^7/ml, reference range 2.1-7.1 x 10^8/ml [mean±2s.d.]. Mean platelet volume in patient III:5 and III:3 was 15.0fl and 10.4fl respectively (reference range 7.68-10.0fl). Parental platelet counts were normal. An extremely high Immature Platelet Fraction (IPF) of 87 and 83% (normal range 1.3-10.8%, n=40) was found in patients III:5 and III:3 respectively which suggests rapid production (possibly due to rapid clearance). Flow cytometry was used to assess platelet function in the two affected individuals, using an assay validated for activated platelets using dilutions of PRP from healthy volunteers. The levels of surface glycoproteins CD42b (GPIbα), CD41 (αIIb) and GPVI in patient III:3 were within the reference ranges established in healthy volunteers, whereas for patient III:5, the levels of CD42b and CD41 fell outside. This could suggest global platelet dysfunction, where loss of glycans can lead to failure of the receptor being transported to the surface, or to increased proteolysis (Figure 2A). Patient III:5 also showed a complete abolition of CD62P (P-selectin) expression and a very weak increase in binding of fibrinogen to ADP, CRP and PAR-1 (Figure 2B and 2C). Patient III:3 showed a slightly greater increase in fibrinogen binding than III:5 to most agonists and a recovery of CD62P expression to high concentrations of CRP and PAR1 peptide, although this was below the range of responses to healthy controls in all cases. Electron microscopy of patient platelets revealed that these are enlarged but with a similar number of α-granules per surface area, compared to controls (Figure 2D).

The exome of both patients was sequenced and the alignment of the sequencing reads revealed 23,943 and 24,293 variations in patients III:3 and III:5 respectively. Comparisons within the EVS, 1000G and our in house GAPP database of over 1200 exomes identified two homozygous non-synonymous variants and 1 non-frameshift deletion that were present in both patients (Supplementary Tables 1 and 2), with all variations mapping to a tightly-linked homozygous region on chromosome 9p13.3 (Supplementary Table 2). The two non-synonymous variants were in genes *GNE* (p.G416R) and *FRMPD1* (p.A509V) and the non-frameshift deletion in *ANKRD18A* (p.Glu801del). Family studies using Sanger sequencing confirmed that all three variants segregated with disease status (Figure 1B). Pathogenicity was predicted using four separate *in silico* based pathogenicity prediction softwares (MutationTaster, SIFT, PROVEAN and PolyPhen-2) and conservation at the site of variation was determined by PhyloP and PhastCons. Together all three variants were classified as “unknown significance” when considering the ACMG consensus guidelines. Upon further

analysis within the ExAC database, only the variants within *ANKRD18A* and *GNE* were novel. Data from RNA sequencing of hematopoietic progenitors (blueprint.haem.cam.ac.uk), suggested that there was very low expression of *ANKRD18A* mRNA in hematopoietic progenitors. This is in contrast to *GNE* mRNA, which is expressed widely in hematopoietic progenitors.

In previous studies, two compound heterozygous variations in the gene encoding *GNE* have been noted to cause a disorder of progressive muscle weakness with a secondary symptom of thrombocytopenia.[7 8] Previous dominant mutations in *GNE* have been associated with sialuria.[9 10] It is important to note that the recessive patients presented with severe body myopathy as a primary symptom, while the patients in our study do not display signs of myopathy although this is possibly because of their age. Furthermore a previous study involving the whole exome sequencing of a single pedigree with severe thrombocytopenia and bleeding identified an apparent *PRKACG* variant but a strong candidate variant in this family was also a homozygous missense variant in the kinase domain of *GNE* (p.G559R) as shown in Figure 1C.[11]

GNE encodes Glucosamine (UDP-N-Acetyl)-2-Epimerase/N-acetylmannosamine kinase, a bi-functional enzyme involved in the sialic acid biosynthesis pathway and is expressed within all cells of the haematopoietic lineage. Thrombocytopenia is known to be associated with increased platelet desialylation in septic patients due to altered platelet production/survival.[12] Further, platelet counts are increased in a cohort of influenza patients treated with the sialidase inhibitor, oseltamivir (Tamiflu).[13] A platelet clearance system has been shown to exist for desialylated platelets involving macrophages and hepatocytes.[14] The Ashwell-Morell receptor binds platelets with reduced sialic acid expression[15] and removal of just 8-10% of sialic acid residues by neuraminidase treatment leads to increased platelet clearance rates *in vivo*.[16]

In summary, our results indicate that the *GNE* mutation described here leads to macrothrombocytopenia, possibly due to a reduction in sialic acid biosynthesis, which is expected to cause increased removal of platelets and altered platelet formation.

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Authorship Contributions

GCL, SPW and NVM designed the research. JM and MW provided patient samples and clinical data. GCL and NVM undertook the research governance of the study. JF, AD, GCL, BJ, MS and NVM performed the research and analyzed data. NVM and AD wrote the paper and all authors critically reviewed and edited the paper.

Disclosure of Conflicts of Interest

The authors declare no competing conflicts of interest.

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Figures

Figure 1. Identification of a homozygous missense substitution in *GNE*. (A) Filtering strategy of whole exome sequencing results to identify candidate variants in patients III:3 and III:5 of the same family. (B) Segregation analysis of the exome candidates in family members where DNA was available. The three variants (in the genes GNE, FRMPD1 and ANKRD18A) were shared by both children and were located within a region of homozygosity on chromosome 9p13.3. Double lines linking parents signify first cousin unions. (C) Linear domain organisation of GNE encoding the enzyme UDP-GlcNAc 2-epimerase/ManNAc kinase. Experimental allosteric sites are based on *in vitro* studies (AS), region of unknown function (UF). The approximate position of amino acid substitutions (p.G416R and p.G559R) found in the family in this study and in an independent study[11] respectively are indicated and based on transcript NM_005476.

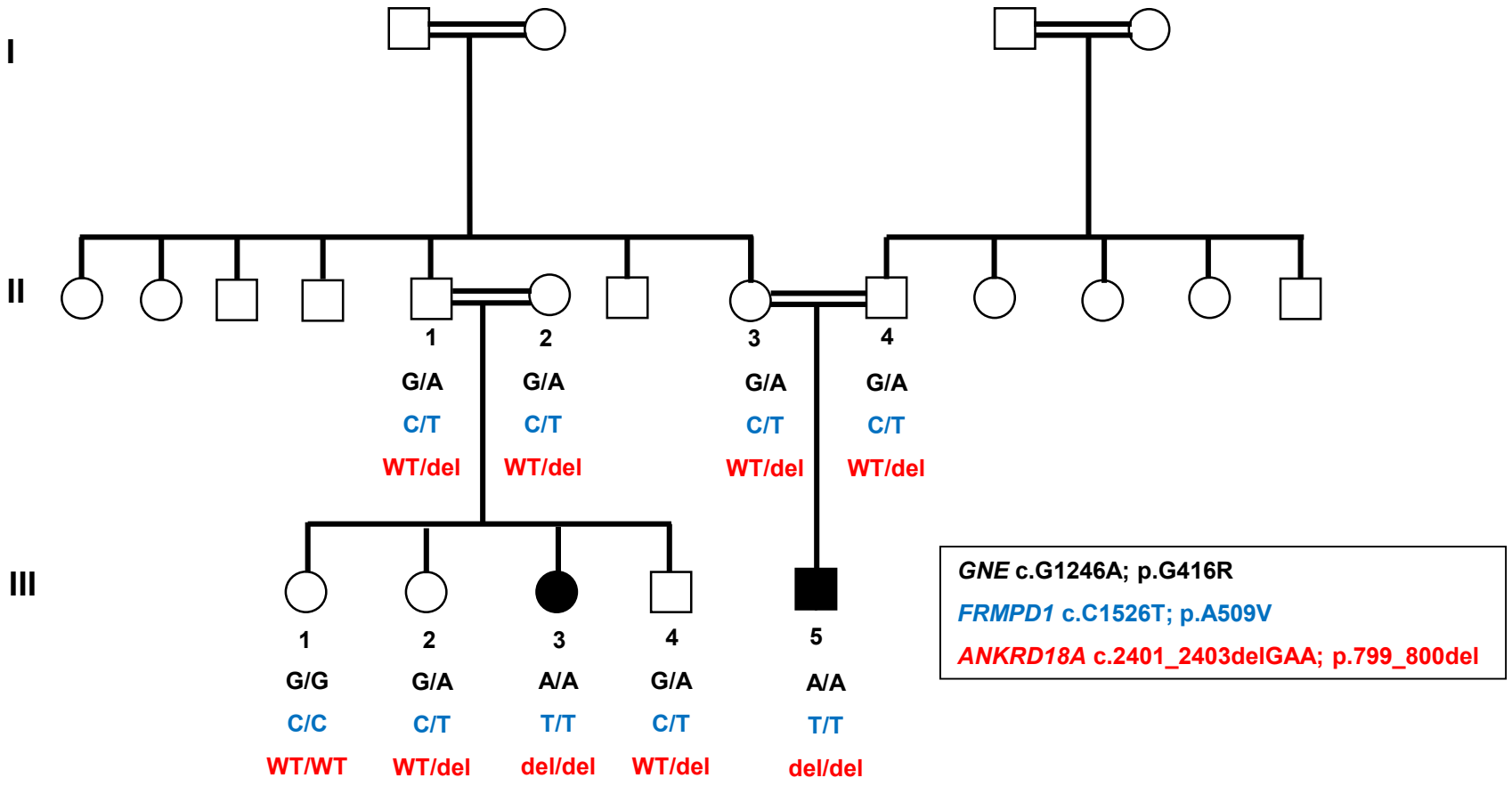
Figure 2. Flow cytometry and transmission electron microscopy assessment of platelet function in patients III:3 **and III:**5**.** Flow cytometry assessment of platelet function in patients III:3 and III:5 assessed on Accuri C6 flow cytometer. (A) Platelet glycoprotein receptors. (B) CD62P expression and (C) fluorescent fibrinogen binding following platelet stimulation by various agonists for 2 min. The platelet rich plasma from healthy controls was diluted 1 in 10 with phosphate buffered saline and served as a control range. Data for healthy volunteers shown as mean $\pm$ 1 s.d. (n= 9, except for GPVI where n=2)). (D) Transmission electron microscopy image of the platelets from patient III:5 and a healthy control platelets. Arrow indicates α granule, graph shows number of alpha granules/surface area, scale bar = 2μm.

Figure 1

A

	Patient III:3	Patient III:5
Total variants	23,943	24,293
Novel or Rare variants	583	607
Homozygous	77	83
Overlapping variants	15	
In linkage regions	3	
Predicted damaging	2	

B



C

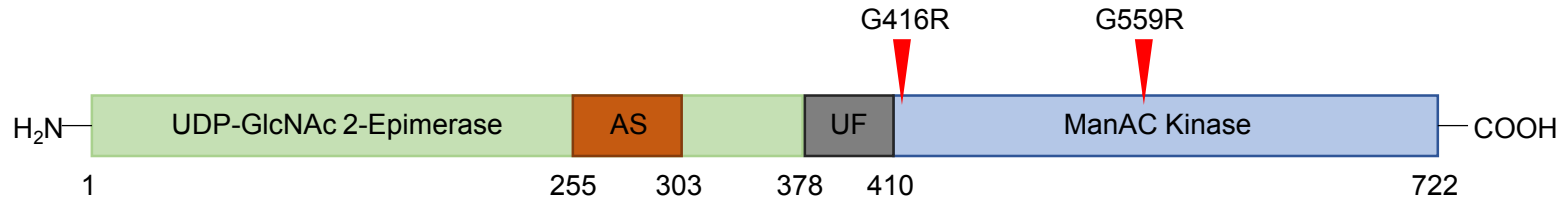
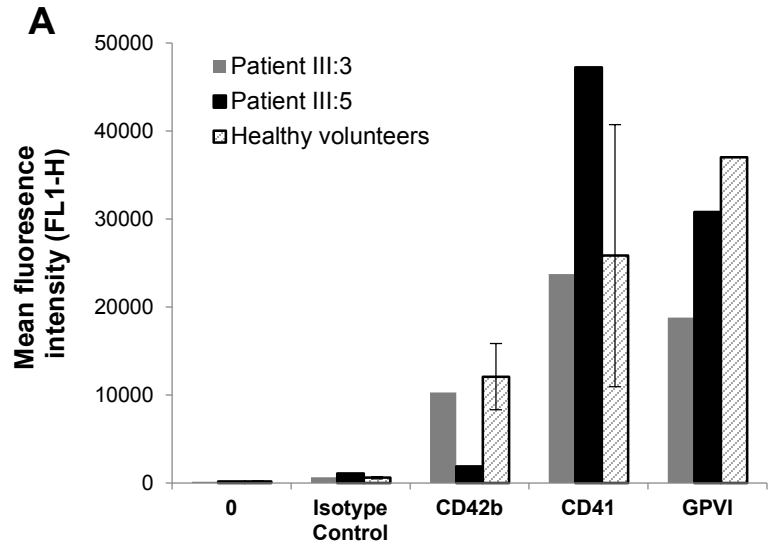
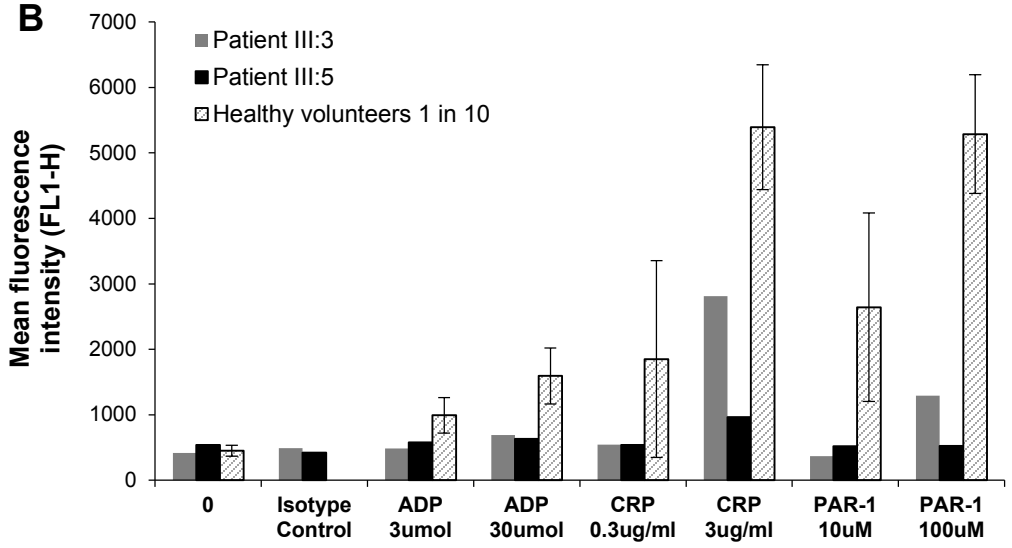
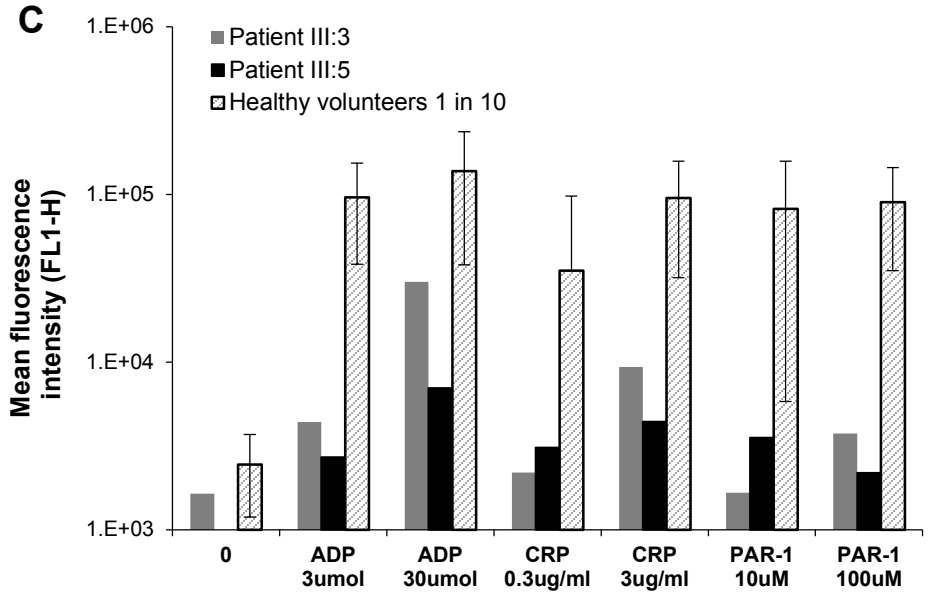
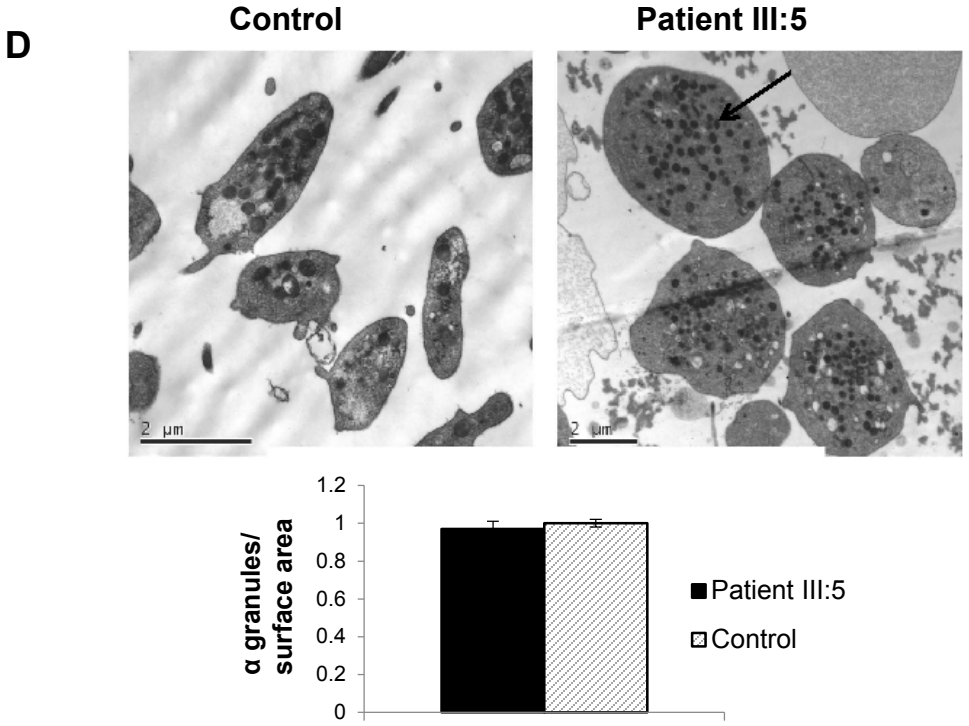


Figure 2


A
Mean fluorescence intensity (FL1-H)
50000
40000
30000
20000
10000
0
Patient III:3
Patient III:5
Healthy volunteers
0
Isotype Control
CD42b
CD41
GPVI


B
Mean fluorescence intensity (FL1-H)
7000
6000
5000
4000
3000
2000
1000
0
Patient III:3
Patient III:5
Healthy volunteers 1 in 10
0
Isotype Control
ADP 3umol
ADP 30umol
CRP 0.3ug/ml
CRP 3ug/ml
PAR-1 10uM
PAR-1 100uM


C
Mean fluorescence intensity (FL1-H)
1.E+06
1.E+05
1.E+04
1.E+03
Patient III:3
Patient III:5
Healthy volunteers 1 in 10
0
ADP 3umol
ADP 30umol
CRP 0.3ug/ml
CRP 3ug/ml
PAR-1 10uM
PAR-1 100uM


D
Control
Patient III:5
2 µm
2 µm
α granules/ surface area
1.2
1
0.8
0.6
0.4
0.2
0
Patient III:5
Control