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Supplemental Data

Mutations in *PIGU* Impair the Function of the GPI Transamidase Complex, Causing Severe Intellectual Disability, Epilepsy, and Brain Anomalies

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Supplemental Data

Clinical report of individuals with *PIGU*-associated GPIBD

Individual P-1, is the first daughter of healthy first-degree cousins. She was born after uncomplicated pregnancy at term with a birth weight of 3520 g (0.0 z, P 50), a birth length of 52 cm (0.02 z, P 51) and an occipitofrontal head circumference (OFC) of 33 cm (-2.00 z, P 2). APGAR score was 9/10/10 at one, five and ten minutes of life respectively. The first days of life were unremarkable, except phototherapy due to hyperbilirubinemia (max 27,3 mg/dl) at day four to six. Retrospectively parents noticed five to ten seconds of rhythmic myoclonic movements of the head, arms and less pronounced, the legs several times a day starting within the first month of life. At age three months, global developmental delay was documented. A first comprehensive examination took place at age seven months. The girl showed severe muscular hypotonia with reduced spontaneous movements, poor head control and inability to roll over. She interacted with reactive smiling, but she was not able to track objects with her eyes, to grasp or to make sounds. Tendon reflexes were weak except PSR. She showed repetitive myoclonic movements of the left hand and left leg. Measurements were within the normal range (weight 8.8 kg, 1.02 z, P 85; length 71 cm, 0.89 z, P 81; OFC 42 cm, -1.19 z, P 12). Electroencephalogram (EEG) revealed slowed basal activity, and polymorphic dysrhythmic delta waves with upstream high frequent sharp waves during sleep. MRI of the brain was unremarkable, as well as extensive metabolic work up. Eye examination revealed strabismus convergens at the age of one year. At age one year and 3 months she had still a severe global developmental delay, muscular hypotonia and persistent myoclonic seizures regardless of physiotherapy, early support and antiepileptic treatment. However, she could grasp and to roll over from back to belly. A second MRI of the brain revealed delayed myelination and a small periventricular focal gliosis on the left side. Reinforcement of antiepileptic therapy was able to reduce but not prevent seizures. At age two years she could roll over from back to belly and back, sit on all-fours positions and make sounds. Length and weight were normal, OFC was small (45.5 cm, -1.87 z, P 3). A further MRI of brain was normal except left sided periventricular gliosis.
Further course of disease was characterized by persistent focal myoclonic seizures up to 100 times a day, occurrence of generalized myoclonic-tonic seizures and the development of spasticity in all limbs leading to a slow regression and loss of all achieved skills. Due to dysphagia and dystrophy, a gastrostomy was implemented at age 8. Repeated extended metabolic analyses gave normal results except elevation of glutamine in plasma. A forth MRI of the brain at age 5 years showed atrophy of the white matter and an increase of lactate and a decrease of N-acetylaspartate (NAA) in the spectroscopy. X-ray of spine revealed osteopenia and scoliosis. Muscular biopsy was not suggestive for mitochondriopathy. Chromosomal analysis showed a normal female karyotype. Array-CGH revealed a 0,16Mb deletion in 11q14,1 (arr 11q14.1 (84,017,779-84,177,562)x1 (NCBI Build 36.1)), a CNV with unknown significance.

Individual P-2, a boy, is the third child of family 1 and the brother of individual P1. He was born at 41+3 weeks after normal pregnancy with normal measurements: birth weight of 3.5 kg (0.19 z, P42), birth length of 56 cm (1.44 z, P 93) and OFC of 37 cm (1.19 z, P 88) and good APGAR scores (9/10/10 at minute 1, 5 and 10 respectively). Parents reported normal postna-

tal adaptation and development in the first weeks of life. At the age of 7 weeks first generalized seizures occurred, followed by frequent focal myoclonic seizure series, which did not respond to different therapies. In the first weeks seizures were accompanied by apneas, but apneas occurred also independently and frequently required stimulation and oxygen insufflation. In addition to epilepsy severe muscular hypotonia and an insufficient eye contact were noted. Extended metabolic analyses at the age of two months gave normal results except a slight elevation of glutamine in plasma and glycoseaminoglycans (GAGs) in urine. EEG showed multifocal spikes and slow basal activity. ECG revealed an incomplete right bundle branch block (RBBB), and an ASD type II. Visual evoked cortical potentials (VECPs) were absent on the right eye and very small on the left eye. A fundoscopy of both eyes were inconspicuous as well as an MRI of brain. The development in the next years was poor. The boy did not achieve any milestones in motor development: he was never able to roll over, nor sit or walk. He never grasped. In toddlerhood he showed eye contact and he could follow with eyes, but these abilities were lost in the late preschool age. Optic atrophy was diagnosed at the age of 15 months. At age of 16 months acute hydrocephalus malresorptivus occurred requiring a placement of a ventriculo-peritoneal shunt system. Due to feeding difficulties the boy required PEG at age of three years. The boy was affected by recurrent upper airway infections which were often associated with an aggravation of seizures. At the age of 4 years he showed supraventricular tachycardia (heart frequency of 240/minute) for the first time, which was followed by further episodes of tachycardia requiring hospitalization and medical cardio version at different times.
Last examination took place at age of 10 years and 9 months. We saw a severe disabled boy with normal measurements (length 142 cm, -0.52 z, P 30; weight 35 kg, -0.42 z, P 34) who was not able to walk, sit unaided, nor to crawl or roll over. He had a severe truncal muscular hypotonia and hypertonia of all limbs. His spontaneous movements were very limited and he suffered from scoliosis. He gave sounds but did not speak syllables or words. He was blind due to severe optic atrophy. Feeding was completely by GI tube. Karyotyping revealed a normal male karyotype (46, XY). Array CGH revealed a 0,8Mb duplication in chromosome 5 (arr 5p15.33 (770,367-1,603,192)x3 (NCBI Build 36.1)) and a 0,16Mb deletion in 11q14,1 (arr 11q14.1 (84,017,779-84,177,562)x1 (NCBI Build 36.1)), both CNVs are of unknown clinical significance.

Individual P-3 was the first boy of non-consanguineous healthy parents of European descent (family 2), born at 42 weeks of gestation with a secondary caesarian section after a normal pregnancy with a birth weight 4.2 kg (1.18 z, P 88). He was hypotonic but there were no feeding problems. At 6 months of age he was examined by the ophthalmologist because of nystagmoid eye movements. At the age of 10 months he was admitted to the hospital because of pneumonia. One month later he had his first epileptic seizure. He presented with tonic and myoclonic seizures, thereafter mainly myoclonic seizures and absences treated with Valproate and Colbazam, but not fully controlled. A brain MRI showed progressive cerebellar atrophy. At the age of 6 years he showed dysmetric movement disorder. His height was 119.5 cm (0.31 z, P 62), weight was 20.5 kg (-0.38 z, P 35) and OFC was 53 cm (0.63 z, P 74). At this age he was not able to sit, stand or walk without help. He had no speech. There were no facial dysmorphisms noted. A variety of investigations followed including metabolic tests in blood, muscle biopsy and liquor, and genetic tests including chromosomal analyses, MLPA subtelomeric regions, genome wide array, DNA tests for *MECP2, FRAX, ATRX*, Pelizaeus-Mertzbacher *POLG, ARX, OPHN1*, mtDNA, all with normal results. He was followed in clinic regularly, at the age of 12 years he had no seizures during the daytime, and only at night a few times a week.

He was wheelchair bound, needed plasters for heel contractures, he had still not developed words and only used some sounds. His height was 144.5 cm (-1.03 z, P 15) and weight was 33.4 kg (-1.35z, P 9). At current age of 17 years he is a friendly boy and surgically corrected for his scoliosis.

Individual P-4 was the second boy of family 2 and the younger brother of P3, born at 39 weeks of gestation by primary SC after a normal pregnancy with a birth weight 4.7 kg (2.12 z, P 98). He was hypotonic and an MRI made at the age of 4 months showed frontal atrophy and a Dandy Walker variant. At the age of 8 months he was not able to roll. His length was 74 cm (0.73 z, P77), weight 9.4 kg (0.45 z, P 67) and OFC 49.5 cm (3.17 z, P 99). In general, his development was better than his brother. At the age of 5 years he was able to speak 2-3 word sentences and to walk few steps. His height at that age was 111.2 cm (-0.05 z, P 48), weight 18.5 kg (-0.26 z, P40) and OFC 53.2 cm (1.12 z, P 87). His first myoclonic seizures started at the age of 6 years which were generally well controlled with Levetiracetam. An MRI at this age showed progressive vermis hypoplasia. At the current age of 12 years he is a friendly boy, able to walk independently for short distances and to speak a few word sentences. He also developed scoliosis.

Individual P-5 was the first girl of non-con-sanguineous healthy parents of European descent (family 3), born at 42 weeks of gestation by normal delivery after a normal pregnancy with a birth weight 4.8 kg (2.70 z, P 99). She was hypotonic and had feeding problems right from the start. At the age of six weeks ophthalmologic examination revealed nystagmoid eye movements. At the age of three months she was admitted to the hospital due to poor head control and muscular hypotonia. EEG and brain MRI performed at the age of 5 months showed no pathological finding. A brain MRI performed at the age of 10 months showed a thin corpus callosum and an enhanced ventricular system without signs of hydrocephalus. At the age of 2 years she still showed hypotonia and general developmental delay. Her height was 91 cm (1.30 z, P 90), weight was 12.5 kg (0.35 z, P64) and OFC was 50.5 cm (1.84 z, P97). At this age she was not able to sit, stand or walk and she had no speech. Some facial dysmorphisms were noted, a broad forehead, a short and anteverted nose, a short philtrum and dysmorphic low set ears. A variety of investigations followed including metabolic tests in blood and liquor without pathological findings. Genome wide array-CGH results were negative. At the age of 3.5 years she developed focal onset seizures with impaired awareness and was successfully treated with Valproate.
She was followed in clinic regularly; at the age of 5 years she had no seizures. She was wheelchair bound and had developed only a few words and she is considered for a surgically corrected for her scoliosis. Her height was 116 cm (1.12 z, P 87), weight was 20 kg (0.04 z, P 66) and OFC was 53 cm (1.60 z, P 95).

Facial gestalt of individuals with *PIGU* mutations

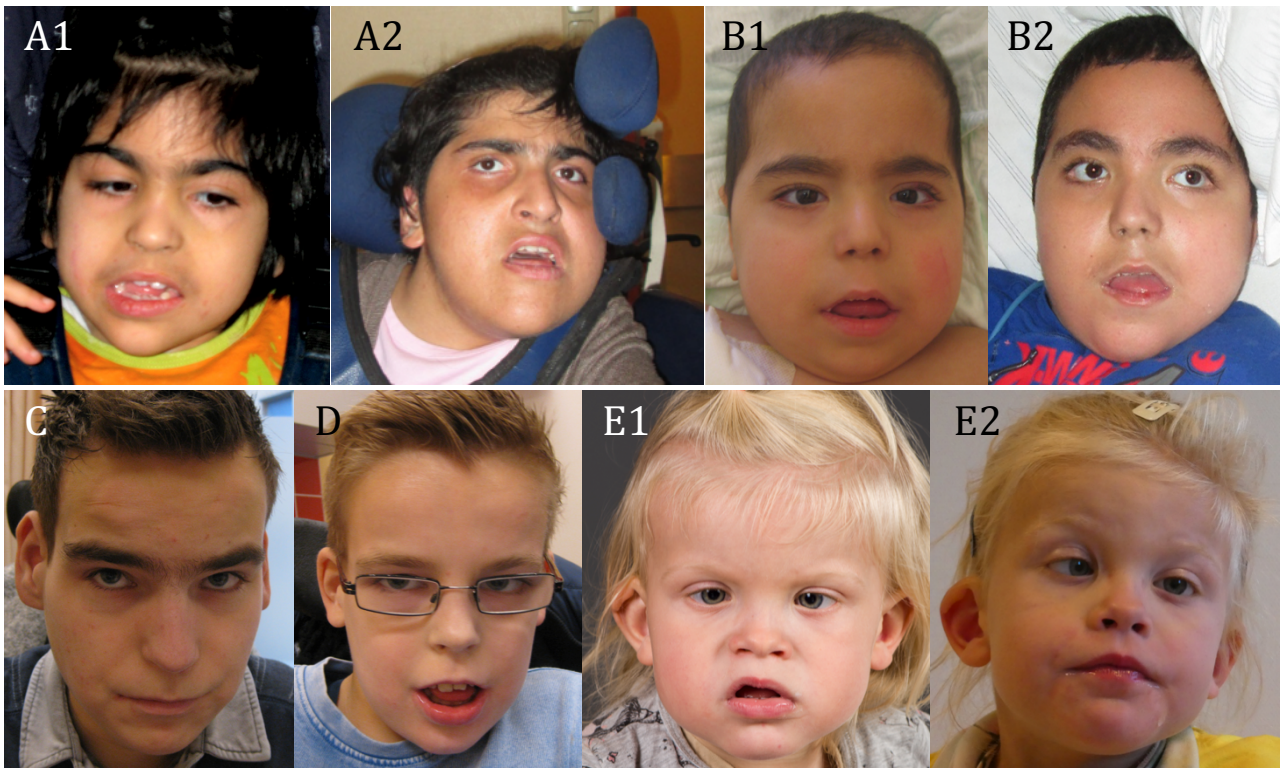

Figure S1. Facial appearance of individuals with biallelic PIGU-mutations. Individual P-1 at the age of 8 (A1) and 19 years (A2). Individual P-2 at the age of 3 (B1) and 12 years (B3). Individuals P-3 at the age of 17 years (C) and P-4 at the age of 12 years (D). Individual P-5 at the age 3 (E1) and 5 years (E2).

MRI of individuals with *PIGU*-associated GPIBD

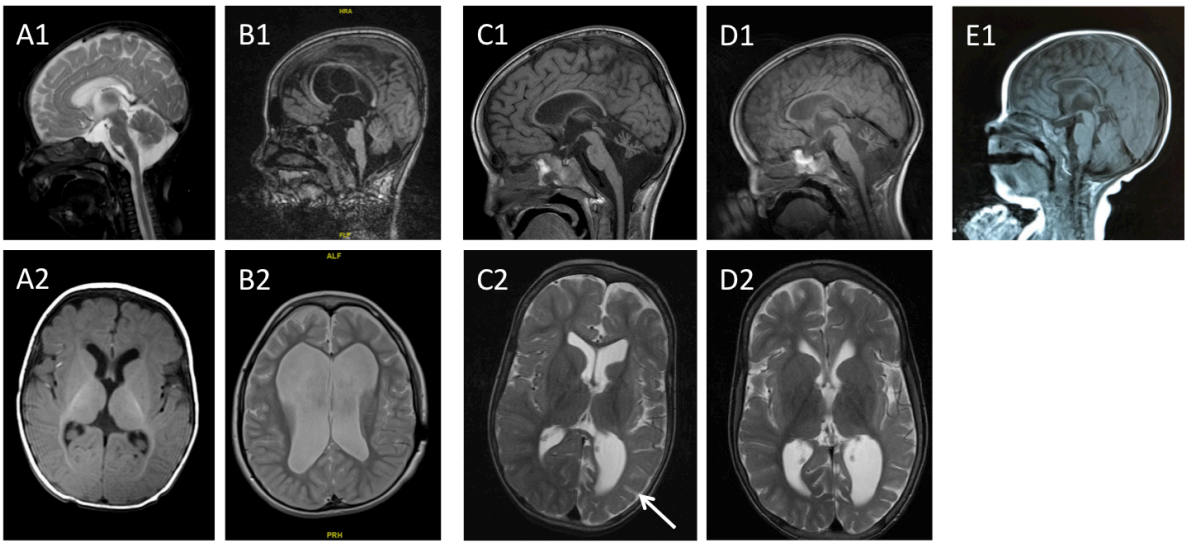

Figure S2. MRI scans of individuals with PIGU-associated GPIBD: MRI of individual P-2 at the age of 2 months was normal (A1, A2). At the age of 12 years he showed a hydrocephaly (B1, B2). MRI of individual P-3 revealed thin corpus callosum and vermis hypoplasia (C1) accompanied by asymmetric enlarged ventricles (left < right) and asymmetric myelinisation (C2, arrow) with no changes over time (D1, D2). MRI of individual P-5 showed a thin corpus callosum, vermis hypoplasia, and enlarged ventricles the age of 10 months (E1).

Clinical features of five individuals with *PIGU*-associated GPIBD

		Family 1		Family 2		Family 3
Individual		P-1	P-2	P-3	P-4	P-5
Genetic result	PIGU (NM_080476.4)	c.209T>A; p.Ile70Lys homozygous		c.1149C>A; p.Asn383Lys homozygous		c.1149C>A; p.Asn383Lys homozygous
Ethnicity		Turkish		European		Norwegian
Sex		Female	Male	Male	Male	Female
Genetic investigation		Sanger sequencing	WES	WES	WES	WES
Prenatal/ Neonatal						
Pregnancy		Normal	Normal	Normal	Normal	Normal
Birth at		40 weeks	40 weeks	42 weeks	39 weeks	42 weeks
Birth weight		3520 g/ P 54	3540 g/ P 35	4260 g/ P 84	4740 g/ P 98	4800 g/ >P 99
Birth length		52 cm/ P 56	56 cm/ P 94	Normal	Normal	50.5 cm/ P 19
Birth OFC		33 cm/ P 7	37c m/ P 86	Normal	Normal	37 cm/ P 90
Disease manifestation						
Age at manifestation of disease		First month	7 weeks	Birth	Birth	Birth
Presenting symptoms		Myoclonic seizures	Myoclonic seizure	Muscular hypotonia	Muscular hypotonia	Muscular hypotonia, feeding problems
Age at last examination		19 years	12 years	12 years	7 years	5 years
Weight at last examination		58 kg/ P 36	35 kg/ P 15	30.3 kg/ P 6	25.1 kg/ P 15	20 kg/ P 66
Length at last examination		155 cm/ P 2	142 cm/ P 5	146 cm/ P 6	124 cm/ P 50	116 cm/ P 87
OFC at last examination		Normal	Normal	Normal	Normal	53 cm/ P 95
Dysmorphic features						
		Malar flattening, smooth philtrum, thin upper lip, pointed chin, high arched palate, posteriorly rotated ears, hypertrichosis	Long face, high forehead, bitemporal narrowing, malar flattening, smooth philtrum, high arched palate, posteriorly rotated ears, hypertrichosis	Long face, high forehead, deep set eyes, long lose, malar flattening, smooth philtrum, thin upper lip	Long face, high forehead, malar flattening, smooth philtrum, thin upper lip	Broad forehead, epicanthus, telecathus, short nose, depressed nasal bridge, malar flattering, short philtrum, large mouth with downturned corners, low set dysplastic ears
Neurological features						
Developmental delay		Profound	Profound	Profound	Severe	Profound
Intellectual disability		Profound	Profound	Profound	Severe	Profound
Seizures		Yes	Yes	Yes	Yes	Yes
Seizure onset		1 months	2 months	10 months	6 years	3.5 years
Seizure characteristics		Initially focal myoclonic, now generalized	Generalized and focal myoclonic with apnoa	Myoclonic and absences, grand mal, tonic-clonic	Myoclonic	Focal
Seizure outcome		Intractable	Intractable	Relatively well controlled	Relatively well controlled	Well controlled
Muscular hypotonia		Yes	Yes	Yes	Yes	Yes, severe
Spasticity in limbs		Yes	Yes	No	No	No
Cortical visual impairment		Yes	Yes	No	No	Yes
Brain imaging						
Cerebral anomalies		Global cerebral atrophy	Hydrocephalus	Hydrocephalus	Frontal lobe atrophy	Slightly increased extracerebral fluid

Corpus callosum hypoplasia	No	Yes	Yes	No	Yes
Cerebellar atrophy/ vermis hypo-plasia	Cerebellar atrophy	No	Vermis and cerebellar hypoplaisa	Vermis hypoplasia, megacisterna magna	No
Neuronal migration defect	Delayed myelination	Delayed myelination	Focal abnormal gyri	Delayed myelination	No
Other	Increased lactate in spectroscopy	Hydrocephaly at age of 1 year	No	No	No
Ophthalmologic features					
Strabismus	Yes	Yes	Yes	Yes	Yes
Nystagmus	No	No	Yes	No	Yes
Hyperopia	No	No	No	Yes	Yes
Myopia	No	No	No	No	No
Other	-	Optic atrophy	-	-	-
Audiologic features					
Hearing loss	No	No	No	No	No
Cardiologic features					
Patent ductus arteriosus	No	No	No	No	No
Increased atrial load	No	No	No	No	No
Rhythm anomalies	No	Right bundle branch block, supraventricular tachycardia	No	No	No
Respiratory problems					
Recurrent infections	Yes	Yes	No	No	No
Gastro-intestinal problems					
Feeding problems	Yes, PEG tube	Yes, PEG tube	No	No	No
Urologic/Renal features					
Nephrocalcinosis	No	No	n.a.	n.a.	No
Urine calcium	Normal	Normal	n.a.	n.a.	No
Ureteral dilation	n.a.	No	No	No	No
Skeletal anomalies					
Slender long bones	Yes	Yes	No	No	n.a.
Scoliosis	Severe	Severe	Severe	Severe	Severe
Pectus excavatum	No	No	No	No	Yes
Joint hypermobility	No	No	No	No	Yes
Osteopenia	Yes	n.a.	n.a.	n.a.	n.a.
Blood and CSF analysis					
Plasma alkaline phosphatase	Normal	Normal	Normal	Normal	Normal
Plasma calcium	Normal	Normal	Normal	Normal	Normal
Plasma phosphate	Normal	Normal	Normal	Normal	Normal
Parathyroid hormone values	n.a.	Normal	n.a.	n.a.	n.a.
Hypertriglyceridemia	No	No	No	No	No
CSF analysis	Normal	Normal	Normal	Normal	Normal
CSF albumin & albumin quotient	n.a.	n.a.	Normal	Normal	Normal

Abbreviation:
n.a. not analyzed
PEG: Percutaneous endoscopic gastrostomy
WES: Whole exome sequencing

Methods:

Multicolor flow cytometry

Blood samples of affected individuals, parents and healthy unrelated controls were collected in BCT CytoChex (Streck®) tubes. Erythrocytes from 50µl blood were removed using hypo-osmotic lysing buffer. The cells were washed and stained with fluorescently labeled antibodies against a cluster of differentiation (CD) marker for cell population gating (CD45 and CD19) and GPI-APs (CD16, CD24, CD55, and CD59), as well as with fluorescein-labeled proaerolysin (FLAER), which binds to the GPI anchor itself. Detection of free GPI anchors was accomplished using T5 4E10 antibody[1–3] and AF647 labed anti-IgM secondary antibody. The antibody stating was incubated for 30 min at room temperature, cells were washed in excess of flow cytometry buffer and re-suspended in 250µl FACS buffer for analysis on a MACSQant VYB (Miltenyi Biotec). Gating for living cells was based on forward and side scatter (FSC-A vs. SSC-A). Single cells were gated on a diagonal (FSC-A vs. FSC-H). Granulocytes were identified as granular (SSC-A high) and CD45-positive cells, B-cells as non-granular (SSC-A low) and CD19-positive cells. Flow cytometry data analysis was carried out using FlowJo V9.66 and Microsoft Excel.

The relative change of GPI-AP expression was determined by a ratio of the staining index (SI) of a patient to the SI of healthy controls (parents or unrelated individuals): The SI is calculated as the ratio of median fluorescent intensity (MFI) of a stained cell population (MFI_{pos}) minus the MFI of unstained (or antibody isotype control stained) cell population (MFI_{neg}) to twice the standard deviation of MFI_{neg}.

Functional analysis of p.Asn393Lys variant in *PIGU* by flow cytometry in CHO cells

PIGU deficient CHO cell (C311PA16)[4] were transiently transfected with wild type or mutant (p.Asn383Lys) pMEhPIGU-FLAG. Restoration of the surface expression of CD59, DAF, and urokinase plasminogen activator receptor (uPAR), were assessed 2 days later by flow cytometry. Wild-type PIGU efficiently restored the surface expressions of CD59, DAF, and uPAR whereas p.Asn383Lys mutant rescued less efficiently (Figure S3 A). Experiments were performed in duplicates.

Lysates from transfectants in Figure S3 A were applied to SDS PAGE and western blotting were performed. Mutant PIGU expression was similar compared to wild type protein, normalized by the intensity of GPADH as loading control. Luciferase acitivity was used for evaluation of transfection efficiency (Figure S3 B).

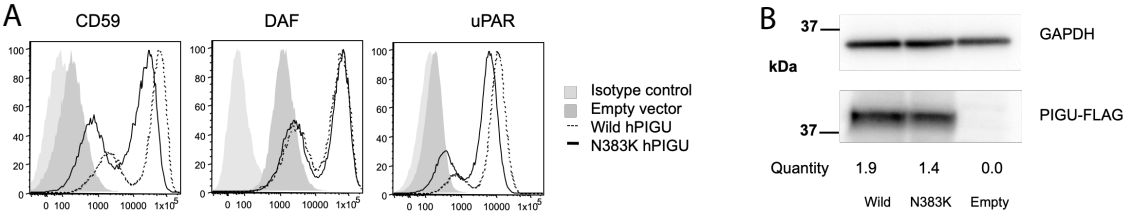

Figure S3. Functional analysis of p.Asn383Lys mutation in PIGU. (A) Restoration of CD59, DAF, and uPAR expression after transient transfection of *PIGU* deficient CHO cell lines. Grey shadow, empty vector; light grey shadow, isotype control. (B) Western blot analysis of p.Asn383Lys mutant and wild type PIGU of transiently transfected PIGU deficient CHO cells.

Facial Analysis

Frontal facial images of the five individuals with PIGU mutations were collected with informed consent of their parents or legal guardians. 128-dimensional facial embeddings vectors for each of the 112 patients (25 PIGA, 15 PIGN, 13 PIGT, 5 PIGU, 5 GPAA1, 24 PIGV, and 25 PGAP3 cases) were extracted using a pre-trained FaceNet model[5] (version 20170512-110547). The similarity of faces correlates with the distance between facial embeddings after an L2 norm (Euclidean norm) is calculated. In order to compare the faces in a gene cohort manner, the facial embeddings of four random samples from a gene cohort were selected and averaged to generate one self-reference point. The four facial embeddings were removed from the gene cohort and the process was iterated. When less than four embeddings remained for a gene cohort, all former embeddings were added. This process was iterated over the number of samples present in each gene cohort (4*25 for PIGA, 4*15 for PIGN, 4*13 for PIGT, 4*5 for PIGU, 4*5 for GPAA1, 4*24 for PIGV, and 4*25 for PGAP3) yielding 448 self-reference points that form the GPIBD space. In order to represent the position of each patient in the GPIBD space, Euclidean distances from all cases to all the self-reference points were computed. Only the shortest distance between all self-reference points for a gene was considered, thus the final distance matrix contains seven distance values representing each case. Thereafter, the centroids for each gene cohort were calculated and the Euclidean distances between the centroids were computed and hierarchically clustered using the single linkage method (Figure 5).

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