Impaired glucose-1,6-bisphosphate production due to bi-allelic PGM2L1 mutations is associated with a neurodevelopmental disorder

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Summary

We describe a genetic syndrome due to PGM2L1 deficiency. PGM2 and PGM2L1 make hexose-bisphosphates, like glucose-1,6-bisphosphate, which are indispensable cofactors for sugar phosphomutases. These enzymes form the hexose-1-phosphates crucial for NDP-sugar synthesis and ensuing glycosylation reactions. While PGM2 has a wide tissue distribution, PGM2L1 is highly expressed in the brain, accounting for the elevated concentrations of glucose-1,6-bisphosphate found there. Four individuals (three females and one male aged between 2 and 7.5 years) with bi-allelic inactivating mutations of PGM2L1 were identified by exome sequencing. All four had severe developmental and speech delay, dysmorphic facial features, ear anomalies, high arched palate, strabismus, hypotonia, and keratosis pilaris. Early obesity and seizures were present in three individuals. Analysis of the children’s fibroblasts showed that glucose-1,6-bisphosphate and other sugar bisphosphates were markedly reduced but still present at concentrations able to stimulate phosphomutases maximally. Hence, the concentrations of NDP-sugars and glycosylation of the heavily glycosylated protein LAMP2 were normal. Consistent with this, serum transferrin was normally glycosylated in affected individuals. PGM2L1 deficiency does not appear to be a glycosylation defect, but the clinical features observed in this neurodevelopmental disorder point toward an important but still unknown role of glucose-1,6-bisphosphate or other sugar bisphosphates in brain metabolism.

Glycosylation defects are a major group of metabolic diseases comprising more than 100 different defects and a significant proportion of them are due to deficiencies in enzymes involved in the synthesis of nucleoside diphosphate sugars (NDP-sugars).1 In several instances, the synthesis of NDP-sugars starts with the conversion of a hexose-6-phosphate to a hexose-1-phosphate. This step is catalyzed by a phosphomutase and is followed by the addition of a nucleotidyl group to the phosphate on carbon 1, resulting in the activated sugars uridine diphosphate (UDP)-glucose, UDP-N-acetyl-glucosamine, and guanosine diphosphate (GDP)-mannose. The three phosphomutases that are needed to form glucose-1-phosphate (phosphoglucomutase 1 [PGM1]), N-acetyl-glucosamine-1-phosphate (phosphoglucosaminate 3 [PGM3]), and mannose-1-phosphate (phosphomannomutase 2 [PMMP2]) all require low concentrations of the corresponding hexose-bisphosphate (glucose-, N-acetyl-glucosamine-, and mannose-1,6-bisphosphate) as a cofactor.5-7 This cofactor participates in the reaction mechanism and is therefore indispensable.5,9 The main enzymatic reaction forming these hexose-bisphosphates uses the high-energy intermediate of glycolysis 1,3-bisphosphoglycerate as a phosphoryl donor and a hexose-1 or 6-phosphate as a phosphate acceptor.10,11 In vertebrates, two proteins have been shown to catalyze this reaction: PGM2 and PGM2L1. PGM2 is a multifunctional enzyme able to catalyze several phosphomutase reactions on ribose-phosphate, deoxyribose-phosphate, and glucose-phosphate and, at a much lower rate, the synthesis of hexose-1,6-bisphosphates and ribose-1,5-bisphosphate.11 PGM2L1 has similar catalytic activities, but its bisphosphate synthesizing activity is higher compared to its mutase activity, which makes it suitable to synthesize high concentrations of glucose-1,6-bisphosphate and other hexose- and pentose-bis phosphates.12 This is also consistent with the tissue distribution of these two enzymes: PGM2 is widely distributed in tissues, while PGM2L1 is almost restricted to the brain. Intriguingly, the brain is characterized by a very high concentration of glucose-1,6-bisphosphate (>100 μM), which is at least 100-fold more than the concentration needed for the functioning
of sugar phosphomutases\textsuperscript{12,13} These findings suggest that glucose-1,6-bisphosphate and PGM2L1 play a still unknown role in the brain. Here, we report the identification of four children with PGM2L1 deficiency sharing a common, largely neurological phenotype. More than 10 years after the molecular identification of PGM2L1,\textsuperscript{11} the discovery of this developmental disorder highlights the importance of glucose-1,6-bisphosphate in the brain.

We performed clinical whole-exome sequencing (WES) in all four families as WES trio analysis (supplemental subjects and methods) to explore the cause of an identified neurodevelopmental disease affecting young children. This disclosed bi-allelic variants in PGM2L1 in all four affected children (PT1, PT2, PT3, and PT4—see Table 1), which were connected via the web-based tool GeneMatcher.\textsuperscript{14} The PGM2L1 (MIM: 611610) variants identified (GenBank: NM_173582.4) were as follows: for PT1, homozygous variant c.1548_1549delTC (p.Pro517Lys*19); for PT2, compound heterozygous for the frameshift variants c.51delC (p.Tyr18Thrfs*36) and c.1115delA (p.Asn372Ilefs*8); for PT3, homozygous truncating variant c.172C>T (p.Arg58*); and for PT4, homozygous truncating variant c.1282G>T (p.Glu428*). In addition, microarray analysis did not identify any clinically relevant copy number variations in any of the affected individuals and none of the additional gene variants identified appeared to contribute to the phenotype (supplemental subjects and methods).

Clinically, all four individuals (three females and one male, between the ages of 2 and 7.5 years) had severe developmental and speech delay, dysmorphic facial features (Figure 1), ear anomalies, high-arched palate, strabismus, muscular hypotonia, and keratitis pilaris/dry skin. All pregnancies were uneventful and growth parameters were normal at birth. There was no significant failure to thrive. Three out of the four children developed significant overweight after the age of 2 to 3 years (BMI > 97\textsuperscript{th} percentile). Two out of the four children had primary ataxia. Three had seizures, including tonic-clonic and absence seizures. One child developed status epilepticus and severely regressed with this event, losing all motor milestones. One child was unable to sit independently or use words. Only one child learned to walk independently and developed speech; this individual had intellectual disability and behavioral abnormalities and demonstrated aggressive outbursts and pica (Table 1 and supplementary subjects and methods).

Two children developed early severe caries. None of the children had cardiac, urogenital, endocrine, or skeletal anomalies. Hearing was normal. Vision was impaired only in the affected individual who had a severe episode of status epilepticus. Brain MRI was unremarkable in two affected individuals and a third demonstrated frontal atrophy; in one child, the MRI showed symmetric supratentorial parenchymal anomalies, signal changes in the lower cerebellar hemispheres, in the nucleus lentiformis, and between the cortex and the white matter. One child, who had unremarkable MRI, had a magnetic resonance spectroscopy (MRS) examination, which showed mildly elevated lactic acid and decreased N-acetylaspartate (NAA) levels. Metabolic studies were performed and were unremarkable in all individuals. Transferrin glycoform analysis was performed in the blood of three out of the four affected individuals and was equally unremarkable (Table 1 and supplemental subjects and methods).

Given that all four children harbor predicted loss-of-function variants of PGM2L1, we anticipated that such variants would reduce the level of glucose-1,6-bisphosphate and other sugar-bisphosphates that are normally synthesized by PGM2L1. Therefore, it was of interest to investigate these and other metabolites in PGM2L1-deficient versus control fibroblasts. Accordingly, a skin biopsy was obtained for functional studies as part of the clinical workup in three out of the four children, and consent for research usage was obtained. Residual deidentified biological material was used for fibroblast-based functional assays (Mayo Clinic IRB 16-004682-19). Deidentified data on participating individuals were collected under the oversight of the respective institutional review boards or equivalent ethical committees. Inspection of the chromatograms derived from the liquid chromatography/mass spectrometry (LCMS) analysis of the fibroblasts’ extracts showed a peak corresponding to an ion with an m/z value of 338.9890, matching mono-deprotonated glucose-1,6-bisphosphate, which was strongly reduced in extracts of fibroblasts deficient in PGM2L1. Nonetheless, an accurate quantification of glucose-1,6-bisphosphate was difficult to achieve via its m/z value (338.9890) because of a partial overlap of the elution profiles of glucose-1,6-bisphosphate and fructose-1,6-bisphosphate, which have the same elemental composition. Fortunately, we noticed that fragmentation of glucose-1,6-bisphosphate during electrospray ionization gave rise to an ion with an m/z value of 241.0121, which matched a fragment produced by the elimination of H\textsubscript{3}PO\textsubscript{4} and was at least 10-fold more abundant in the case of glucose-1,6-bisphosphate than of fructose-1,6-bisphosphate. On the basis of the analysis of this fragment (m/z value 241.0121), we could conclude that PGM2L1 deficiency led to an approximate 90% decrease in the concentration of glucose-1,6-bisphosphate but not to its complete absence (Figure 2).

Glucose-1,6-bisphosphate was consistently found to be reduced by about 10-fold in PGM2L1-deficient fibroblasts, regardless of whether the m/z values of ions 241.0121 (Figure 3A) or 338.9890 (Figure 3B) were used for the quantification, while fructose-1,6-bisphosphate (which is not formed by PGM2L1, but by phosphofructokinase) was unchanged. We similarly found that the concentrations of N-acetyl-glucosamine-1,6-bisphosphate and ribose-1,5-bisphosphate, two other products previously shown to be formed by PGM2L1 in vitro,\textsuperscript{11} were also markedly reduced in the PGM2L1-deficient fibroblasts, yet they were still significantly present (Figures 3C and 3D).

Hexose-/pentose-bisphosphates are activators of phosphomutases, which are required for the synthesis of NDP-sugars.\textsuperscript{8,9} Thus, a major question was whether the decrease in hexose- and pentose-bisphosphates might lower the level
Table 1. Clinical features of four PGM2L1-deficient individuals

| Individuals     | PT1                      | PT2                      | PT3                      | PT4                      |
|-----------------|--------------------------|--------------------------|--------------------------|--------------------------|
| Age/sex         | 7 years/male             | 7.5 years/female         | 7 years/female           | 30 months/female         |
| Ethnic background| Turkish                  | French-Canadian (Cajun)  | Middle Eastern           | Pakistani                |
| Genetic variants| c.1548_1549delTC (homozygous) | c.[51delC]; [1115delA] | c.172C>T (homozygous)   | c.1282G>T (homozygous)  |
| Protein         | p. Pro517Lysfs*19        | p.[Tyr18Thrfs*36]; [Asn372Ilefs*8] | p.Arg58*                | p.Glu428*                |
| Global development delay | +            | +                        | +                        | +                        |
| Intellectual disability | +            | +                        | +                        | not applicable           |
| Gross motor skills | 18 months sitting, 34 months walking | 10 months sitting, 18 months walking | 7 years can sit/stand with support; no targeted hand activity | 8 months sitting, 30 months walking |
| Speech delay    | single words (4–5) at 34 months | severe speech delay      | severe, no verbal language | 30 months babbling, no speech |
| Abnormal behavior | no meaningful contact[b] | severe pica and aggressive behavior | –                        | –                        |
| Neurologic features | +            | +                        | +                        | +                        |
| Hypotonia       | +                        | + (truncal)              | shifting tonus, with hypertonia | +                        |
| Muscle weakness | +                        | +                        | –                        | –                        |
| Seizures        | +                        | +                        | –                        | +                        |
| Pyramidal signs | +[^b]                    | –                        | –                        | –                        |
| Ataxia          | –                        | +                        | –                        | –                        |
| MRI             | dilation of the frontal interhemispheric cleft | unremarkable (MRS showed decreased NAA and mildly elevated lactic acid) | symmetric infra/supra tentorial parenchymal anomalies; signal changes[^c] | unremarkable |
| Dysmorphic features | +                        | +                        | +                        | +                        |
| Ears            | over-folded helices, large earlobes, pronounced antitragus | prominent ears, large ear lobes, and over-folded helices | large earlobes | prominent ears |
| Eyes            | long eyelashes, down-slanting palpebral fissures | epicanthal folds, down-slanting palpebral fissures | – | – |
| High arched and narrow palate | + | + | + | – |
| Nose            | flat nasal bridge, broad and bulbous nasal tip, a long philtrum | prominent nasal tip, flat nasal bridge, under-developed nasolabial fold | prominent nose, flat philtrum | – |
| Thin upper lip  | +                        | +                        | –                        | –                        |
| Pointy chin     | +                        | –                        | +                        | –                        |
| Teeth           | early caries             | early caries             | –                        | –                        |
| Skin abnormalities | +                      | +                        | +                        | +                        |
| Dry skin        | + (keratosis pilaris)    | + (keratosis pilaris)    | + (keratosis pilaris)    | + (eczema)               |

(Continued on next page)
| Individuals | PT1 | PT2 | PT3^ | PT4 |
|-------------|-----|-----|------|-----|
| Abnormal hair growth | hypertrichosis of the back | excessive arm hair growth | – | – |
| Ocular abnormalities | + | + | + | + |
| Exotropia | + | + | + | + |
| Hypermetropia | – | – | + | – |
| Vision loss | +^b | – | – | – |
| GI abnormalities | + | – | – | – |
| Swallowing difficulties | +^b | – | – | some feeding difficulties |
| Early obesity (at age 2 to 3) | + | + | – | + |
| Current obesity; BMI/centiles | +; 19.3/99th | +; 25.1/99th | –; 17.4/85th | +; 22.2/99th |
| Extremities | – | + | – | + |
| Joints | – | hypermobility | – | hypermobility |
| Hands and feet | – | small hands and feet and short toes | – | deep palmar creases |

### Laboratory investigations

| Transferrin glycosylation in blood | normal | normal | normal | not performed |
| Plasma acyl carnitine pattern | normal | normal | normal | normal |
| Urinary organic acids | normal | normal | normal | normal |

All four pregnancies were unremarkable and birth parameters were normal, except for PT1 in which a relative microcephaly was noted; no cardiac abnormalities and no failure to thrive were detected.

*Parents are first cousins.

^Symptoms appeared after status epilepticus and resuscitation.

^Signal changes observed were in lower cerebellar hemispheres, nucleus lentiformis, and scattered on the verge between cortex and white matter.
of NDP-sugars in these cells and thereby compromise protein glycosylation. However, no significant change was observed in the level of UDP-hexose (sum of UDP-glucose and UDP-galactose), UDP-glucuronate, UDP-GlcNAc/GalNAc, cytidine monophosphate (CMP)-sialic acid, GDP-mannose/GDP-glucose, GDP-fucose (Figures 3E–3J), and cytidine diphosphate (CDP)-ribitol (see note in the legend of Figure 3), indicating that the level of none of the precursors required for glycosylation reactions was affected. We also found that there was no change in the concentration of ATP and of ADP, two major nucleotides involved as substrate or product for a multitude of enzymes (Figures 3K and 3L). Experiments that were designed to rescue the PGM2L1 defect in fibroblasts from three PGM2L1-deficient individuals as well as a control individual showed that the level of the various hexose-bisphosphates were increased by 10- to 20-fold by complementation with PGM2L1 (Figures S1A and S1B), reaching levels above the ones observed in control cells. In contrast, UDP-hexose, UDP-glucuronate, UDP-GlcNAc/GalNAc, CMP-sialic acid, ADP, and ATP were not changed (Figures S1C–S1H). Accordingly, LCMS analysis of extracts from HEK293T cells overexpressing either a glucose-1,6-bisphosphate degrading enzyme (PMM1) or two glucose-1,6-bisphosphate forming ones (PGM2 or PGM2L1) showed a strong decrease or increase in intracellular glucose-1,6-bisphosphate (Figures S2A and S2B), N-acetyl-hexosamines-1,6-bisphosphate (Figure S2C), and ribose-1,5-bisphosphate (Figure S2D) without impacting the levels of NDP-sugars, CMP-sialic acid, ADP, or ATP (Figures S2E–S2J). Taken together, these data indicated that the loss of PGM2L1 activity in fibroblasts from PGM2L1-deficient individuals caused a significant yet incomplete loss of the hexose-/pentose-bisphosphates, activators of phosphomutases, but that this did not affect the level of the NDP-sugars, suggesting that protein glycosylation was most likely not affected.

In agreement, analysis by immunoblotting of the migration pattern of the heavily glycosylated protein LAMP2, whose glycans contribute to about 40% of its mass, shows that LAMP2 present in the extracts prepared from PGM2L1-deficient fibroblasts has the same normal migration pattern as the protein from control cells (Figure 4A), which also argues against a glycosylation defect in the PGM2L1-deficient cells. We and others have shown in previous work that when glycosylation was impaired in cells, the migration pattern of LAMP2 was altered. Because the changes in glucose-1,6-bisphosphate seen in LCMS (Figures 2 and 3) did not provide an absolute quantification, we also quantified glucose-1,6-bisphosphate in fibroblast extracts by using an enzymatic assay, which is based on the observation that phosphoglucomutase (PGM1) is activated by glucose-1,6-bisphosphate with a Ka (the concentration of enzyme activator at which half-maximal stimulation of the enzymatic activity is reached) below 1 μM. Thus, to determine the absolute concentration of glucose-1,6-bisphosphate in the PGM2L1-deficient cells, we used a spectrophotometric assay that is based on the stimulation of rabbit muscle phosphoglucomutase by glucose-1,6-bisphosphate. The use of appropriate dilutions of the extracts indicated that the intracellular concentration of glucose-1,6-bisphosphate amounted to 80 μM in control fibroblasts and 13 μM in PGM2L1-deficient ones (Figure 4B). Consequently, even in PGM2L1-deficient cells, the concentration of glucose-1,6-bisphosphate was more than one order of magnitude above the Ka of PGM1 for glucose-1,6-bisphosphate, explaining that PGM2L1 deficiency does not affect the formation of UDP-glucose (Figure 3E). A similar conclusion also applies to other phosphomutases because NDP-sugars dependent on the catalytic activity of PMM2 (GDP-mannose; Figure 3I) and PGM3 (UDP-N-acetyl-hexosamine; Figure 3F) were also not decreased.
Because PMM1 is the enzyme that serves to degrade glucose-1,6-bisphosphate and PGM2 is a multifunctional enzyme that also catalyzes the synthesis of glucose-1,6-bisphosphate and other hexose-bisphosphates, although less well than PGM2L1, we tested the impact of overexpressing these enzymes in HEK293T cells on their level of glucose-1,6-bisphosphate. As expected, overexpression of PMM1 caused a marked decrease in the concentration of glucose-1,6-bisphosphate from 137 mM to 5 mM and confirmed that PGM2 is indeed able to form glucose-1,6-bisphosphate (Figure 4C). PGM2 overexpression in HEK293T cells increased the concentration of this bisphosphate ester by about 2-fold, while that of PGM2L1, the enzyme dedicated to make sugar bisphosphates, including glucose-1,6-bisphosphate, increased it by about 20-fold (Figure 4C). These findings are consistent with data published in the literature. As initially reported for PGM2 purified from human erythrocytes and confirmed with the recombinant enzyme, PGM2 also displays some glucose-1,6-bisphosphate synthase activity, despite its main phosphopentomutase activity. Furthermore the glucose-1,6-bisphosphate synthase activity of PGM2 is more powerfully inhibited by glucose-1,6-bisphosphate than that of PGM2L1, indicating that PGM2L1 is more suitable to form the elevated concentrations of glucose-1,6-bisphosphate (about 100 nmol/g) that are found in mouse brain.

Again, we noted that reduction of the glucose-1,6-bisphosphate pool due to PMM1 overexpression did not appear to impact glycosylation of LAMP2. By contrast, LAMP2 glycosylation was markedly affected in neutrophils from individuals with G6PC3 deficiency, owing to a block in glucose metabolism due to accumulation of the hexokinase inhibitor 1,5-anhydroglucitol-6-phosphate (Figure 4D). In this context, it is remarkable that the clinical features of our PGM2L1-deficient children are very similar to those observed in older individuals with MAN1B1-CDG (MIM: 614202), a Golgi-related congenital disorder of glycosylation, but all four PGM2L1-deficient children had normal transferrin glycosylation in blood unlike MAN1B1-CDG-affected individuals. Taken together, these findings suggest that PGM2L1 deficiency is not a glycosylation defect due to the defective production of NDP-sugars. Yet, while our analyses in fibroblasts and the normal transferrin glycosylation in PGM2L1-deficient individuals do not reveal changes in glycosylation, one could argue that PGM2L1 deficiency might lead to a specific glycosylation defect in the brain where PGM2 expression is low. However, even if this was true, it does not explain the need to have such elevated concentrations of hexose-/pentose-bisphosphates (and particularly glucose-1,6-bisphosphate) in the brain together with the high expression of PGM2L1, an enzyme that is dedicated to their synthesis.
made by PGM2L1 may have a regulatory role in sugar metabolism in the brain. Indeed, besides their actions as activators of phosphomutases, glucose-1,6-bisphosphate and ribose-1,5-bisphosphate have, in the range of concentrations observed in the brain, potential regulatory effects on several enzymes of sugar metabolism. In *vitro*, glucose-1,6-bisphosphate has also been described to be an inhibitor of hexokinase 1 and 2 (only at concentrations that are much higher than those needed to saturate phosphomutases) and of phosphogluconate dehydrogenase. Additionally, both glucose-1,6-bisphosphate and ribose-1,5-bisphosphate are stimulators of phosphofructokinase, although not as potent as fructose-2,6-bisphosphate, but more studies are needed to understand the physiological relevance of these effects or to discover the real target of glucose-1,6-bisphosphate’s action.

Interestingly, degradation of glucose-1,6-bisphosphate is regulated by inosine monophosphate (IMP), suggesting that it may have some role in the control of energy metabolism. The concentration of IMP increases in energy shortage as a result of enhanced deamination of AMP by AMP-deaminase. Glucose-1,6-bisphosphate is indeed degraded by PMM1, a protein homologous to PMM2, which in the presence of micromolar concentrations of IMP, efficiently dephosphorylates glucose-1,6-bisphosphate. The effect of IMP is not only impressive by its magnitude, the low \( K_a \) for IMP (approximately 1.5–3 \( \mu \)M), and the specificity for this nucleotide but also by the fact that mechanistically it involves conserved residues that are specific for PMM1 and not found in PMM2. The occurrence of this regulation in *vivo* is supported by the observation that the decrease in glucose-1,6-bisphosphate that occurs during anoxia in mouse brain does not occur in PMM1-deficient mice. These findings, and the fact that the presence of this IMP-dependent phosphatase in the brain parallels that of the glucose-1,6-bisphosphate synthesizing activity...
enzyme, plead for a regulatory role of glucose-1,6-bisphosphate in response to energy shortage. Would such a role be compatible with the phenotype that is observed in PGM2L1 deficiency? It may be interesting in this respect to remember that the phenotype of cerebral creatine deficiency syndrome 1 (CCDS1 [MIM: 30052]), an X-linked disease due to creatine transporter (SLC6A8) deficiency, shares several features with PGM2L1 deficiency, including intellectual disability, speech delay, and hypotonia, with only occasionally reported microcephaly. Creatine phosphate serves as an energy reservoir and for the intracellular transport of high-energy phosphates. The similarity of the clinical findings in these two metabolic diseases supports a potential role of glucose-1,6-bisphosphate in energy metabolism.

In summary, we report a neurodevelopmental disorder related to a deficiency in PGM2L1, a glucose-1,6-bisphosphate synthesizing enzyme that associates a neurological syndrome with dysmorphism. Studies on fibroblasts from affected and control individuals and cell lines overexpressing recombinant proteins clearly indicate that PGM2L1 deficiency causes a decrease, but not a disappearance, of the sugar bisphosphates needed for the formation of NDP-sugars and that there is no evidence that this leads to a glycosylation defect. This indicates that there must be an important role of glucose-1,6-bisphosphate and/or other hexose-/pentose-bisphosphates in the brain that is still unknown.

Data and code availability
The WES datasets have not been deposited in a public database because of privacy and ethical limitations but can be made available on request.

Supplemental information
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Declaration of interests

The authors declare no competing interests

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Web resources

GenBank, https://www.ncbi.nlm.nih.gov/genbank/
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