Three or more copies of the proteolipid protein gene PLP1 cause severe Pelizaeus–Merzbacher disease

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Summary
We describe five boys from different families with an atypically severe form of Pelizaeus–Merzbacher disease (PMD) who have three, and in one case, five copies of the proteolipid protein (PLP1) gene. This is the first report of more than two copies of PLP1 in PMD patients and clearly demonstrates that severe clinical symptoms are associated with increased PLP1 gene dosage. Previously, duplications, deletions and mutations of the PLP1 gene were reported to give rise to this X-linked disorder. Patients with PLP1 duplication are usually classified as having either classical or transitional PMD rather than the more severe connatal form. The clinical symptoms of the five patients in this study included lack of stable head control and severe mental retardation, with three having severe paroxysmal disorder and two dying before the first year of life. Gene dosage was determined using interphase FISH (fluorescence in situ hybridization) and the novel approach of multiple ligation probe amplification (MLPA). We found FISH unreliable for dosage detection above the level of a duplication and MLPA to be more accurate in determination of specific copy number. Our finding that three or more copies of the gene give rise to a more severe phenotype is in agreement with observations in transgenic mice where severity of disease increased with Plp1 gene dosage and level of overexpression. The patient with five copies of PLP1 was not more affected than those with a triplication, suggesting that there is possibly a limit to the level of severity or that other genetic factors influence the phenotype. It highlights the significance of PLP1 dosage in CNS myelinogenesis as well as the importance of accurate determination of PLP1 gene copy number in the diagnosis of PMD and carrier detection.

Keywords: Pelizaeus–Merzbacher disease; proteolipid protein (PLP); gene dosage; myelin; multiplex ligation-dependent probe amplification (MLPA)

Abbreviations: FISH = fluorescence in situ hybridization; MLPA = multiplex ligation-dependent probe amplification; PCR = polymerase chain reaction; PLP1 = proteolipid protein 1; PMD = Pelizaeus–Merzbacher disease


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Introduction

Pelizaeus–Merzbacher disease (PMD; OMIM 312080) is a rare X-linked recessive disorder presenting with nystagmus, hypotonia later evolving into spastic tetraparesis, dystonia, ataxia and, compared with the motor disability, mild cognitive delay (Boulloche and Aicardi, 1986; Garbern et al., 1999). Traditionally, PMD is divided into classical, transitional and connatal phenotypes, in order of increasing severity. In addition, the milder spastic paraplegia 2 (SPG2) is also caused by PLP1 mutations (Saugier-Veber et al., 1994; Cambi et al., 1996; Kobayashi et al., 1996). In connatal PMD, symptoms start shortly after birth and few, if any, milestones are reached. Additional signs such as seizures, stridor and severe dysphagia are typical (Boulloche and Aicardi, 1986; Scheffer et al., 1991). The main cause of PMD is alterations of the proteolipid protein (PLP1) gene on chromosome Xq22 (Gencic et al., 1989; Hudson et al., 1989; Ellis and Malcolm 1994). PLP1 together with DM20, a smaller isoform generated by alternative splicing, are the most abundant myelin proteins in the CNS (Baumann and Pham-Dinh, 2001). Duplications of PLP1 can be found in up to 70% of all patients with PMD, indicating that increased PLP1 dosage is deleterious for normal myelination (Sistermans et al., 1998; Mimault et al., 1999). These duplications lead to the classical or transitional type of PMD (Cailloux et al., 2000). Point mutations give rise to 10–25% of all cases of PMD with a wide clinical spectrum, ranging from the most severe connatal form to uncomplicated spastic paraplegia, depending on which part of the protein is affected (Cailloux et al., 2000). In cases where DM20 remains intact, or there is complete loss of PLP1/DM20 function, the phenotype is relatively mild (Raskind et al., 1991; Sistermans et al., 1996; Garbern et al., 1999, 2002).

Clinical and radiological criteria for diagnosis of PMD have been published (Boulloche and Aicardi, 1986; Caro and Marks, 1990), but no biochemical test is available. Determination of PLP1 dosage is the first step in mutation screening and confirmation of the clinical diagnosis. Various methods have been used including Southern blotting (Ellis and Malcolm, 1994), quantitative polymerase chain reaction (PCR) (Inoue et al., 1996; Woodward et al., 1998) and fluorescent in situ hybridization (FISH) (Woodward et al., 1998; Inoue et al., 1999). Here we show the application of FISH and the novel technique of multiplex ligation-dependent probe amplification (MLPA) (Schouten et al., 2002) for the detection of increased PLP1 dosage in five male patients and three female carriers. Clinical presentation is severe, with three patients suffering from therapy-resistant seizures. Two boys died within their first year of life. These findings confirm that an increase in PLP1 gene dosage above two correlates with disease severity.

Patients and methods

This study was approved by the local ethical committees at the Institute of Child Health, London, Wayne State University School of Medicine, Detroit, MI and the Alfred I. duPont Hospital for Children, Wilmington, DE, and informed consent was obtained. Patient 1 was thought to have a possible triplication following quantitative PCR (Woodward et al., 1998) and quantitative Southern blot (Ellis and Malcolm, 1994), patient 2 by FISH, patients 3 and 4 were detected by routine quantitative PCR, and patient 5 by routine MLPA analysis for assessing PLP1 copy number. Patient clinical characteristics are shown in Table 1.

Table 1 Clinical characteristics of patients 1–5

<table>
<thead>
<tr>
<th>Patient 1</th>
<th>Patient 2</th>
<th>Patient 3</th>
<th>Patient 4</th>
<th>Patient 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at first symptom</td>
<td>Birth (nystagmus; stridor)</td>
<td>First weeks of life (nystagmus)</td>
<td>3 weeks (nystagmus)</td>
<td>Birth (nystagmus; hypotonia)</td>
</tr>
<tr>
<td>Age at death</td>
<td>9 months</td>
<td>7 months</td>
<td>Alive (4.7 years)</td>
<td>Alive (14 months)</td>
</tr>
<tr>
<td>Nystagmus</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Seizures</td>
<td>+ +</td>
<td>+ +</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Stridor</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Head control</td>
<td>None</td>
<td>None</td>
<td>Poor</td>
<td>None</td>
</tr>
<tr>
<td>Muscular hypotonia</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Spasticity</td>
<td>–</td>
<td>–</td>
<td>Mild</td>
<td>–</td>
</tr>
<tr>
<td>Nerve conduction studies</td>
<td>Normal</td>
<td>Not performed</td>
<td>Normal</td>
<td>Not performed</td>
</tr>
</tbody>
</table>

First weeks of life (nystagmus) (4.7 years) (15 months)

Life (nystagmus)
Patient 2

Patient 2 was the third child of unrelated, healthy parents; two sisters are healthy. A maternal uncle had died at 2 years of age with similar seizures and severe developmental delay. Labour was induced at 39 weeks of gestation; otherwise pregnancy and birth were normal. The parents observed rolling eye movements early on. Aged 3.5 months, he was hospitalized with seizures. Horizontal nystagmus and hypotonia were present, and deep tendon reflexes were brisk in all four limbs. He could fixate on a bright light but had rotatory nystagmus. Dysmorphic features were noted, including plagiocephaly, down-slanting palpebral fissures with prominent eyes, a small mouth and low-set ears. He was readmitted at 4 months of age with multifocal clonic movements and paroxysmal cyclic movements of all limbs associated with eye deviation. These episodes proved resistant to anticonvulsant therapy. The EEG showed occasional potentially epileptiform waveforms over the left hemisphere. A repeat EEG was normal. The child died at 7 months of age from bronchopneumonia following exacerbation of seizures and failure to thrive. At post-mortem examination, poor intensity staining of central myelin with preservation of axons was found.

Patient 3

This patient was born after 37 weeks gestation with a pregnancy complicated by pre-eclampsia and gestational diabetes. Apgar scores were 8 and 8 at 1 and 5 min, respectively. In the newborn nursery, he was noted to be occasionally ‘jittery’. Mild hypoglycaemia with blood glucose levels of ~50 mg/dl resolved during the second day of life. At the age of 3 weeks, his parents noted unusual eye movements. At the age of 6 months, poor head control and muscular hypotonia which had been present since birth were noted by his paediatrician, and the diagnosis of ataxoid cerebral palsy was made. An EEG at the age of 10 months was normal; there were no seizures reported. At the age of 3.1 years, neurological examination revealed pronounced hypotonia with poor head and truncal control. He was sometimes able to track a visual target, but did not reach for or grab objects. Stridor was absent. There were minimal flailing movements of the arms, more so than of the legs. Reflexes were brisk symmetrically with bilateral Babinski signs. At 4 years and 7 months, he was still unable to sit without support, had poor head and trunk control, and had moderately severe hypotonia. He was predominantly non-verbal but could occasionally speak a few words or short sentences, although he had fairly good receptive language in addition to appropriate socialization, including smile and laughter.

Patient 4

This patient, now a 14-month-old boy, was born 3 weeks early by normal spontaneous vaginal delivery. He was noted to be occasionally 'jittery'. Mild hypoglycaemia with blood glucose levels of ~5 s and seen only at night during sleep. On examination at 9 months of age, the patient exhibited laryngeal stridor that resolved with changing positions, occasional pendular nystagmus, minimal verbal output, mild plagiocephaly, severe axial hypotonia with increased tone in both arms and occasional spastic catch in both legs, poor head control, occasional opisthotonic posturing and constant choreoathetoid movements of all extremities. Reflexes were 2+ and symmetrical in the arms, pathologically brisk at the knees, and 2 at the ankles.

The patient’s Vietnamese mother had a history of a prior pregnancy that ended with spontaneous fetal demise at 4 months gestation. A brother of hers with similar eye movements, developmental delay and failure to thrive died at age 7 years.

Patient 5

This patient, now 15 months old, was born at 37 weeks gestation after an uncomplicated pregnancy and delivery. Roving eye movements were noticed at birth. Hypotonia was prominent, accompanied by opisthotonic posture. In the neonatal period, mild to moderate hypoglycaemia was found on three occasions which subsided thereafter. From day 3, hyperkinetic movements of his hands and feeding difficulties were noticed. At the age of 4 months, he had his first paroxysm which was interpreted as an epileptic seizure: a unilateral tonic contraction of facial muscles, head deviation to the same side, dystonic posturing of the extremities (flexion of the hands and trunk, extension of the legs) and blinking of one eye. The EEG showed no epileptiform discharges. Topiramate was tried, but both the paroxysms and the general status deteriorated. Clonazepam as well as carbamazepine had a temporary effect on the frequency of the episodes. At last examination, nystagmus was still present. Hypotonia was severe and head control was absent. His hands showed hyperkinetic choreoathetotic movements and his legs showed both hyperkinesia and dystonic posturing. Tendon reflexes were present, although decreased. The boy was able to fixate and smile; he recognized some people.

The patient’s older brother, a son from a previous marriage of his mother, had died at the age of 22 months of an undiagnosed disorder with nystagmus and seizures. Three maternal uncles had also died, one after premature birth, two in infancy. Both were reported to have suffered from seizures. There are two other maternal relatives said to have cerebral palsy and cerebral palsy with nystagmus.

MRI

MRI of patient 1 was performed at age 3.5 months on a 1.5 T Siemens Magnetom SP63, and of patient 2 at age 4 months on a General Electric Signa 1.5 T instrument. T1-weighted and fast spin-echo sequences were obtained. MRIs of patients 3 and 4 were done at age 11 months on a General Electric Signa 1.5 T scanner; spin-echo, fast spin-echo and fluid-attenuated inversion recovery sequences were obtained. Patient 5 did not undergo MRI.

PLP1 dosage analysis

FISH

Metaphase chromosomes and interphase nuclei were prepared from peripheral blood lymphocytes from the mother of patient 1, patient 2, his mother and maternal grandmother using standard protocols. A fibroblast cell line from patient 2 was cultured according to standard methods. Prior to harvesting, the cells were starved for 5–8 days to block them in the G0 phase of the cell cycle. The cells were harvested using a routine procedure generating metaphase chromosomes and interphase nuclei.

FISH was performed as described previously (Woodward et al., 2003) using the PLP1 and control cosmid DNA probes (Woodward et al., 1999). When using the fixed cell suspensions from blood cultures, both probes were hybridized to different sections of the same glass slide. When using the patient cell line, the probes were

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hybridized to two separate slides. The patterns of hybridization were scored for 100 nuclei from patient 2 and 50 nuclei for the mother of patient 1 and the mother and grandmother of patient 2. All slides except those using the cell line were scored by a second trained cytogeneticist, and the findings were the same (data not shown).

MLPA
MLPA has been described recently (Schouten et al., 2002; Koolen et al., 2004) and was used for the detection of PLP1 copy number changes using probe mix SALSA P022 obtained from MRC Holland, Amsterdam, The Netherlands. The mix included a total of 32 probes, of which seven were for each of the PLP1 exons, eight were from different regions of the X chromosome, one was from the Y chromosome and the remainder were autosomal controls.

Details of probe sequences, gene loci and chromosome locations can be found at http://www.mrc-holland.com. The reactions were carried out in a thermal cycler using the recommended protocol.

Analysis for MLPA
Genotyper software allowed determination of the peak area for each PCR product, and these data were entered into a spreadsheet developed using Microsoft Excel. Data were normalized by dividing each individual peak area by the average of all the autosomal peak areas for the sample. For females, the X-chromosomal peak values were divided by 2, to compensate for the presence of a second X chromosome. The normalized peak value thus obtained was then divided by the average peak area of the same fragment of all control samples within the experiment. The relative peak area calculations should give a value of 1.0 for one copy of PLP1 (i.e. normal males), 2.0 for two copies of PLP1 (i.e. normal females or males with a duplication), 3.0 for three copies of PLP1 (i.e. a female carrier with a duplication or a male with a triplication) and 4.0 for four copies of PLP1 (i.e. a female carrier with a triplication).

Results
MRI
MRI of patient 1 displays no normal myelin signal in the supratentorial structures, with homogeneously hyperintense white matter signal in T2-weighted and hypointense signal in T1-weighted images. The corpus callosum is extremely thin. Infratentorily, only the brainstem and the cerebellar peduncles show some myelin signal, the cerebellar white matter lacking the normal dark myelin signal on T2-weighted images (Fig. 1). At the age of 3.5 months, when this MRI was performed, brainstem, cerebellar white matter, the posterior limb of the internal capsule and the central region should be myelinated. The MRI of patient 2 performed at approximately the same age shows similar findings, although there is plagiocephaly in addition (Fig. 1). MRIs of patients 3 and 4 at age 11 months display a comparable picture regarding myelination; patient 4 additionally shows mild atrophy (Fig. 1). At this age, myelination should have progressed on T2-weighted images to the anterior limb of the internal capsule, the corpus callosum, the optic radiation and the paracentral region; in the central white matter, the normal dark signal of white matter begins to appear.

PLP1 dosage analysis by FISH
Patient 1 was the first boy suggested to have more than two copies of the PLP1 gene and his mother was predicted to be a carrier. However, the triplication could not be supported by FISH on lymphoblastoid cell lines as only two hybridization signals were observed in the patient’s interphase nuclei (Woodward et al., 1998). The interphase FISH was repeated on peripheral blood lymphocytes from a fresh blood sample of the mother using a PLP1 cosmid probe and a control probe mapping distal to the PLP1 gene beyond the region frequently rearranged in other duplication patients (Woodward et al., 1998). Four hybridization signals for the PLP1 probe, one single and a cluster of three, could be clearly seen in many nuclei, representing the normal and the abnormal X chromosome, respectively (Fig. 2). However, the distribution of PLP1 hybridization signals gave confusing results as cells had either three, four, five or six signals (Fig. 3). This could not be simply explained by the presence of a PLP1 duplication and DNA replication in the region because the control probe gave clear results with the majority of cells having two hybridization signals. The results suggested a copy number >2 on the abnormal X chromosome and a possible triplication of the PLP1 gene in this family.

FISH was also used to analyse increased PLP1 dosage in patient 2 and family members (Fig. 2). The distribution of multiple PLP1 signals in interphase nuclei was very different from that observed for the single copy control probe in each family member. In both a fresh blood sample and a fibroblast cell line from the patient, we found that the signal number per cell ranged from a single large diffuse signal to up to six separate smaller foci (Fig. 3). The FISH results for the mother and maternal grandmother indicated that they were carriers of the rearrangement as they both had a similar distribution of signal numbers ranging from three to eight. The majority of cells had four or five signals (Fig. 3D and E), which was higher than that detected in the mother of patient 1 who had mostly three or four signals (Fig. 3A). The FISH results were difficult to interpret but suggested that family 2 may have more than three copies of the PLP1 gene in affected males. On metaphase chromosomes, the additional PLP1 signals could not be simply explained by the presence of a PLP1 duplication and DNA replication in the region because the control probe gave clear results with the majority of cells having two hybridization signals. The results suggested a copy number >2 on the abnormal X chromosome and a possible triplication of the PLP1 gene in this family.

FISH showed two PLP1-related probe signals in patient 3 and three in his mother (data not shown). It was not performed for patients 4 and 5.

PLP1 dosage analysis by MLPA
All the samples were analysed using MLPA to determine accurate PLP1 gene copy number. PLP1 dosage was compared with controls including a normal male, normal female, duplication male and duplication female with one, two, two and three copies of PLP1, respectively (Fig. 4). Three copies of PLP1 were detected in patients 3, 4 and 5. All seven exons of the gene gave results suggestive of a triplication, whereas
other X-linked loci were single copy, and autosomal control probes had two copy numbers. The mother of patient 1 had four copies of \( PLP1 \) consistent with patient 1 having a triplication. This supported the interphase FISH results (Figs 2 and 3) and also the previous Southern blot and quantitative PCR data (Ellis and Malcolm, 1994; Woodward et al., 1998). Interestingly, the dosage detected for patient 2 was much higher than that of the other patients and greater than the four copies in the mother of patient 1. We concluded that patient 2 had five copies of \( PLP1 \) and, in accordance, his mother and grandmother had six copies. The MLPA results explained the confusing pattern of hybridization signals obtained by FISH in this family and gave a more accurate determination of \( PLP1 \) dosage.

**Discussion**

We describe five patients with PMD that have three, and in one case five, copies of the \( PLP1 \) gene. The accurate determination of \( PLP1 \) copy number is important for diagnosis of PMD and carrier detection. Our group and others have previously found interphase FISH to be very effective for \( PLP1 \) duplication detection as the copy number is based on hybridization signal number (Woodward et al., 1998; Inoue et al., 1999). However, in this study, we found that when the dosage of \( PLP1 \) increases above two, the precise determination of copy number by FISH analysis is more difficult. The triplication in patient 1 could not be seen by interphase FISH (Woodward et al., 1998). Difficulties also arose in defining the \( PLP1 \) copy number in patient 2, his mother

![Fig. 1 MRI of patients 1, 2, 3 and 4. (A) Infratentorial, (B) and (C) supratentorial axial T2-weighted images. Note the homogeneous high signal of unmyelinated white matter in the cerebellar and the cerebral hemispheres. (D) A sagittal T1-weighted image. The corpus callosum is thin and the cerebellum is of normal size.](image)
and grandmother. In addition, patient 3 had been diagnosed previously with a duplication rather than a triplication. Detection using FISH will depend upon the structure of each rearrangement, and our work analysing PLP1 duplications suggests that these may be unique, with different proximal and distal breakpoints in each family (Hobson et al., 2003). In this study, we found the novel technique of MLPA to be a more efficient methodology for detection of PLP1 dosage combining sensitivity and robustness with high-throughput and low cost. However, it must be noted that there are cases where the PLP1 gene has duplicated and integrated into a new position in the genome. FISH would be required for identification of these rare events (Hodes et al., 2000; Inoue et al., 2002). Therefore, direct PLP1 gene quantification by MLPA or quantitative PCR is recommended for initial dosage evaluation of a PMD patient, followed by FISH analysis of any positive cases.

The clinical courses of all five children are unusually severe compared with patients in whom PMD is caused by a duplication of PLP1 (Sistermans et al., 1998; Inoue et al., 1999; Cailloux et al., 2000). Of 45 patients carrying a PLP1 duplication which we recently evaluated, 37 were able to sit unsupported. Of the 40 patients over the age of 4 years, all but six were able to speak at least a few words, and verbal

![Fig. 2 FISH on interphase nuclei (A–C) and metaphase chromosomes (D) using a PLP1 cosmid probe (green signal) and an X centromere probe (red signal). (A) Mother of patient 1, (B) patient 2, (C) mother of patient 2, (D) grandmother of patient 2.](image)

![Fig. 3 Distribution of hybridization signals for the PLP1 and control cosmid probes in (A) the mother of patient 1, (B) and (C) patient 2, and (D) the mother and (E) grandmother of patient 2. (F) A patient with a PLP1 duplication for comparison.](image)
comprehension was present in all. Two patients developed epilepsy, one in late childhood, the other in adulthood; both of them were readily treated with antiepileptic medication. One child developed extensor spasms after the age of 10 years. None of the children had significant respiratory problems such as stridor. In contrast, among the five children carrying a PLP1 triplication, none achieved stable head control or other motor milestones, and mental retardation is severe. Two of them died in infancy. Three of the five children suffered from severe epilepsy, although it could not be finally proven whether the paroxysmal episodes observed in these children represented true seizures or other paroxysmal events. MRI of the brain revealed almost complete absence of the normal myelin signal. Patients with PLP1 duplications usually suffer from the classical or transitional form of this disease, not from the connatal presentation or from early-onset seizures (Inoue et al., 1999; Cailloux et al., 2000). This connatal presentation has been described previously only for children with point mutations in the PLP1 gene, but even in this group, seizures are rare and easy to treat (Cailloux et al., 2000). In contrast, three of our patients (1, 2 and 5) had severe epilepsy, which may also be an important symptom in children with a PMD-like disorder thought to have an autosomal-recessive inheritance pattern (Cassidy et al., 1987; Scheffer et al., 1991; Wolf et al., 2004). Altogether, our data support the hypothesis that the level of PLP1 expression correlates with disease severity. This correlation has also been shown in animal models (Kagawa et al., 1994; Readhead et al., 1994). However, we did not find any significant difference in clinical signs between patient 1 with three copies of PLP1 and patient 2 with five. Our findings suggest that either severity increases only with dosage from two to three copies or that other factors in addition to PLP1 dosage influence clinical presentation. Our observations that patient 3, although bearing a PLP1 triplication, is less severely affected than the other three patients, and that patient 2, despite carrying a PLP1 quintuplication, is not more severely affected than patient 1, favour the latter hypothesis. Higher copy numbers of PLP1 seem to be rare. In one of the laboratories participating in this study, 51 families had been diagnosed with duplication and only two with triplication of PLP1.

Another myelin disorder, Charcot–Marie–Tooth disease 1a, affects exclusively peripheral myelin. The most frequent cause of disease is the duplication of a gene, PMP22 on chromosome 17p11.2, coding for a myelin protein (Lupski et al., 1991; Wise et al., 1993; Boerkoel et al., 1999). While missense mutations generally result in a more severe phenotype, PMP22 deletions lead to a different, milder disease. Hence, there are many parallels to PMD. Most patients with PMP22 duplications have three copies instead of two, but there are single cases who are homozygous for the duplication and suffer from a more severe disease (Kaku et al., 1993; LeGuern et al., 1997; Boerkoel et al., 1999). The same is true for the animal model in this disorder. A third neurological disorder has been associated with altered expression levels of a structural protein: a subtype of familial Parkinson’s disease is caused by triplication of α-synuclein (Singleton et al., 2003; Farrer et al., 2004). Carriers of this alteration have four copies of this gene instead of two, which seems to lead to more detrimental consequences than do point mutations (Farrer et al., 2004).

These examples show that the level of expression of certain proteins is critical for both the CNS and PNS. We have shown that three or more copies of PLP1 result in a severe PMD phenotype. Hence, in humans as well, disease severity of PMD correlates with gene dosage of PLP1 as animal models have already suggested. Given that PLP1 dosage can have such a dramatic effect on phenotype, this measurement of PLP1 gene copy number has very important implications for prognosis and genetic counselling.

Fig. 4 Dosage detection of the PLP1 gene using MLPA. CNM = normal male control; CNF = normal female control; CDM = duplication male; CDF = female duplication carrier; P2, P3, P4, P5 = patients 2, 3, 4 and 5; MP1 and MP2 = mother of patient 1 and 2; GMP2 = grandmother of patient 2.

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