doi:10.1093/brain/awt013 Brain 2013: 136; 882–890 | 882

BRAIN
A JOURNAL OF NEUROLOGY

Exome sequencing reveals a novel Moroccan founder mutation in *SLC19A3* as a new cause of early-childhood fatal Leigh syndrome

Mike Gerards,[1,2] Rick Kamps,[1] Jo van Oevelen,[1] Iris Boesten,[1] Eveline Jongen,[1] Bart de Koning,[1] Hans R. Scholte,[3] Isabel de Angst,[4] Kees Schoonderwoerd,[5] Abdelaziz Sefiani,[6] Ilham Ratbi,[6] Wouter Coppieters,[7] Latifa Karim,[7] René de Coo,[4,*] Bianca van den Bosch[1,*] and Hubert Smeets[1,2,*]

1 Department of Clinical Genetics, Unit Clinical Genomics, Maastricht University Medical Centre, P.O. Box 616, 6200 MD Maastricht, the Netherlands
2 School for Oncology and Developmental Biology, Maastricht University Medical Centre, P.O. Box 616, 6200 MD Maastricht, the Netherlands
3 Department of Neuroscience, Erasmus Medical Centre, P.O. Box 2040, 3000 CA Rotterdam, the Netherlands
4 Department of Neurology, Erasmus Medical Centre, P.O. Box 2040, 3000 CA Rotterdam, the Netherlands
5 Department of Clinical Genetics, Erasmus Medical Centre, P.O. Box 2040, 3000 CA Rotterdam, the Netherlands
6 Department of Medical Genetics, National Institute of Health, Human Genomic Centre, University Mohammed V Souissi, B.P. 769 Rabat 11400, Rabat, Morocco
7 Department of Genetics, Faculty of Veterinary Medicine, University of Liège, 4000 Liège, Belgium

*These authors contributed equally to this work.

Correspondence to: Prof. Dr. H.J.M. Smeets,
Department of Genetics and Cell Biology,
P.O. Box 616, 6200 MD Maastricht,
the Netherlands
E-mail: Bert.Smeets@maastrichtuniversity.nl

Leigh syndrome is an early onset, often fatal progressive neurodegenerative disorder caused by mutations in the mitochondrial or nuclear DNA. Until now, mutations in more than 35 genes have been reported to cause Leigh syndrome, indicating an extreme genetic heterogeneity for this disorder, but still only explaining part of the cases. The possibility of whole exome sequencing enables not only mutation detection in known candidate genes, but also the identification of new genes associated with Leigh syndrome in small families and isolated cases. Exome sequencing was combined with homozygosity mapping to identify the genetic defect in a Moroccan family with fatal Leigh syndrome in early childhood and specific magnetic resonance imaging abnormalities in the brain. We detected a homozygous nonsense mutation (c.20C>A; p.Ser7Ter) in the thiamine transporter *SLC19A3*. *In vivo* overexpression of wild-type SLC19A3 showed an increased thiamine uptake, whereas overexpression of mutant SLC19A3 did not, confirming that the mutation results in an absent or non-functional protein. Seventeen additional patients with Leigh syndrome were screened for mutations in *SLC19A3* using conventional Sanger sequencing. Two unrelated patients, both from Moroccan origin and one from consanguineous parents, were homozygous for the same p.Ser7Ter mutation. One of these patients showed the same MRI abnormalities as the patients from the first family. Strikingly, patients receiving thiamine had an improved life-expectancy. One patient in the third family deteriorated upon interruption of the thiamine treatment and recovered after reinitiating. Although unrelated, all patients came from the province Al Hoceima in Northern Morocco. Based on the recombination events the mutation was estimated to have occurred 1250–1750 years ago.

Received July 17, 2012. Revised December 11, 2012. Accepted December 18, 2012
© The Author (2013). Published by Oxford University Press on behalf of the Guarantors of Brain. All rights reserved.
For Permissions, please email: journals.permissions@oup.com

Downloaded from https://academic.oup.com/brain/article/136/3/882/321679 by guest on 20 August 2022

Our data shows that *SLC19A3* is a new candidate for mutation screening in patients with Leigh syndrome, who might benefit from high doses of thiamine and/or biotin. Especially, Moroccan patients with Leigh syndrome should be tested for the c.20C>A founder mutation in *SLC19A3*.

Keywords: Leigh syndrome; thiamine; exome sequencing; gene mapping; mitochondria

Introduction

Leigh syndrome (MIM 256000) is a progressive neurodegenerative disorder characterized by focal, bilateral lesions in one or more areas of the CNS, including basal ganglia, thalamus, cerebellum, brainstem and spinal cord. Leigh syndrome is an early onset mitochondrial disorder with extensive genetic heterogeneity and can be caused by mutations in the mitochondrial DNA and many nuclear genes. The most common cause of Leigh syndrome is a defect in one of the subunits or assembly factors of the oxidative phosphorylation complexes (complexes I, II, IV, V and coenzyme Q) (Tucker *et al.*, 2010). Additionally, mutations in components of the pyruvate dehydrogenase complex and in the gene encoding the leucine-rich PPR motif containing protein have been described (Matthews *et al.*, 1993; Grafakou *et al.*, 2003; Mootha *et al.*, 2003). In total, mutations in at least 35 genes have been reported to cause Leigh syndrome. Although, for example 25–75% of patients with Leigh syndrome and complex IV deficiency harbour mutations in the complex IV assembly factor SURF1 (Pequignot *et al.*, 2001), no clear candidate gene can be selected in the majority of patients despite small phenotypic differences. Because of the extreme genetic heterogeneity of this disorder, gene specific mutation detection is a time consuming and expensive approach. Additionally, the limited genotype–phenotype correlations, the small size of families and large number of isolated cases make classical family based approaches, like linkage analysis, less applicable. Recently, new sequencing techniques have been developed enabling the sequencing of large packages of candidate genes, the whole protein-coding region (exome) or even the whole genome at higher rate and at lower costs (Metzker, 2010). In particular, exome sequencing has become a valuable tool for identifying the genetic cause for different disorders. Over the past 3 years, exome sequencing enabled the identification of over 30 new genes involved in Mendelian diseases (Ku *et al.*, 2011).

Here we describe a new candidate gene for Leigh syndrome identified by whole exome sequencing in combination with homozygosity mapping in a consanguineous Moroccan family with Leigh syndrome and specific abnormalities on MRI.

Materials and methods

Patients

Family A is a Moroccan family with three healthy children and three deceased children, whose cause of death was a result of Leigh syndrome, who were born from healthy consanguineous parents (Fig. 1). The deceased children died 1 month after birth due to respiratory insufficiency. Patient II:1 was born at 41 weeks gestation without complications. He was admitted to the hospital for his opisthotonic posture, inconsolable crying and roving eye movements after 1 month of age. After 2 to 3 weeks he began experiencing seizures. To reduce the seizures, phenobarbital was prescribed. He was hypotonic with hyperreflexia in the extremities, including positive Babinski sign. Later he developed feeding difficulties. His blood tests showed a slightly increased lactic acid of 3.8 mmol/l and an alanine level that was in the upper normal range. Urine culture revealed a haemolytic *Streptococcus* group B. Biochemical analysis of respiratory chain complex activities in fibroblasts was normal. His brain CT scan demonstrated various hypodense areas, most pronounced in the basal ganglia. After 1 month and 7 days he died of Leigh syndrome, mainly due to apnoeas and Cheyne-Stokes type of breathing. The fifth child of this family (Patient II:5) was born at a gestational age of 40 weeks. After almost 5 weeks of age he presented with convulsions, nystagmus, hypotonia with positive Babinski sign and sun-set phenomenon. His convulsions responded well to phenobarbital. Soon after presentation a brain MRI was performed, which demonstrated abnormal signal intensity in the basal ganglia, thalamus, brainstem and cerebellum (Fig. 2A). Brain magnetic resonance spectroscopy showed a lactate peak in the basal ganglia. These findings were similar to the clinical course of his brother and therefore carnitine, riboflavin and vitamin E were prescribed. Blood tests revealed disturbance of liver function with gamma glutamyl transpeptidase 114 U/l, aspartate aminotransferase 109 U/l, alanine aminotransferase 70 U/l, increased lactic acid 4.4 mmol/l and pyruvate 247 µmol/l. In CSF increased levels of leucine and isoleucine were found. Biochemical analysis on muscle and skin biopsy showed no clear decreased activity of the respiratory chain complexes. After 1 month of age he died of respiratory failure. The youngest son (Patient II:6) was born at a gestational age of 41 weeks without complications and showed an identical disease course as his brothers. He was first admitted to the hospital at 3 weeks of age with feeding difficulties, opisthotonic posture and roving eye movements. Clinical examination showed hypotonic extremities and symmetric hyperreflexia with positive Babinski sign. Blood tests revealed a slightly increased lactic acid of 3.4 mmol/l. Neurotransmitters in CSF showed lowered levels of homovanillic acid and hydroxyindoleacetic acid. Brain specific proteins MBP, GFAP and phospho-tau were increased in liquor. Metabolic urine tests showed slightly increased levels 2-ketoglutaric acid, 2-hydroxyglutaric acid, glutaric acid, succinate and 2-ketoadipic acid. Biochemical analysis on skin biopsy revealed a slightly decreased activity of complex IV. Later he began experiencing breathing difficulties. Treatment was started with carnitine, vitamin E, riboflavin and CoQ_{10}. The patient died due to a progressive abnormal breathing pattern at 6 weeks of age.

Family B is a consanguineous Moroccan family with seven children (Fig. 1) of which two girls died after 4 weeks of age due to a progressive neurological deterioration caused by Leigh syndrome. When Patient II:1 was admitted to the hospital, she was irritable, cried inconsolably and presented with jitteriness. She had drinking difficulties and frequent green defecation. Her extremities were hypertonic with clonic triceps surae reflexes including a positive Babinski sign. Shortly after hospitalization she became respiratory insufficient with

Downloaded from https://academic.oup.com/brain/article/136/3/882/321679 by guest on 20 August 2022

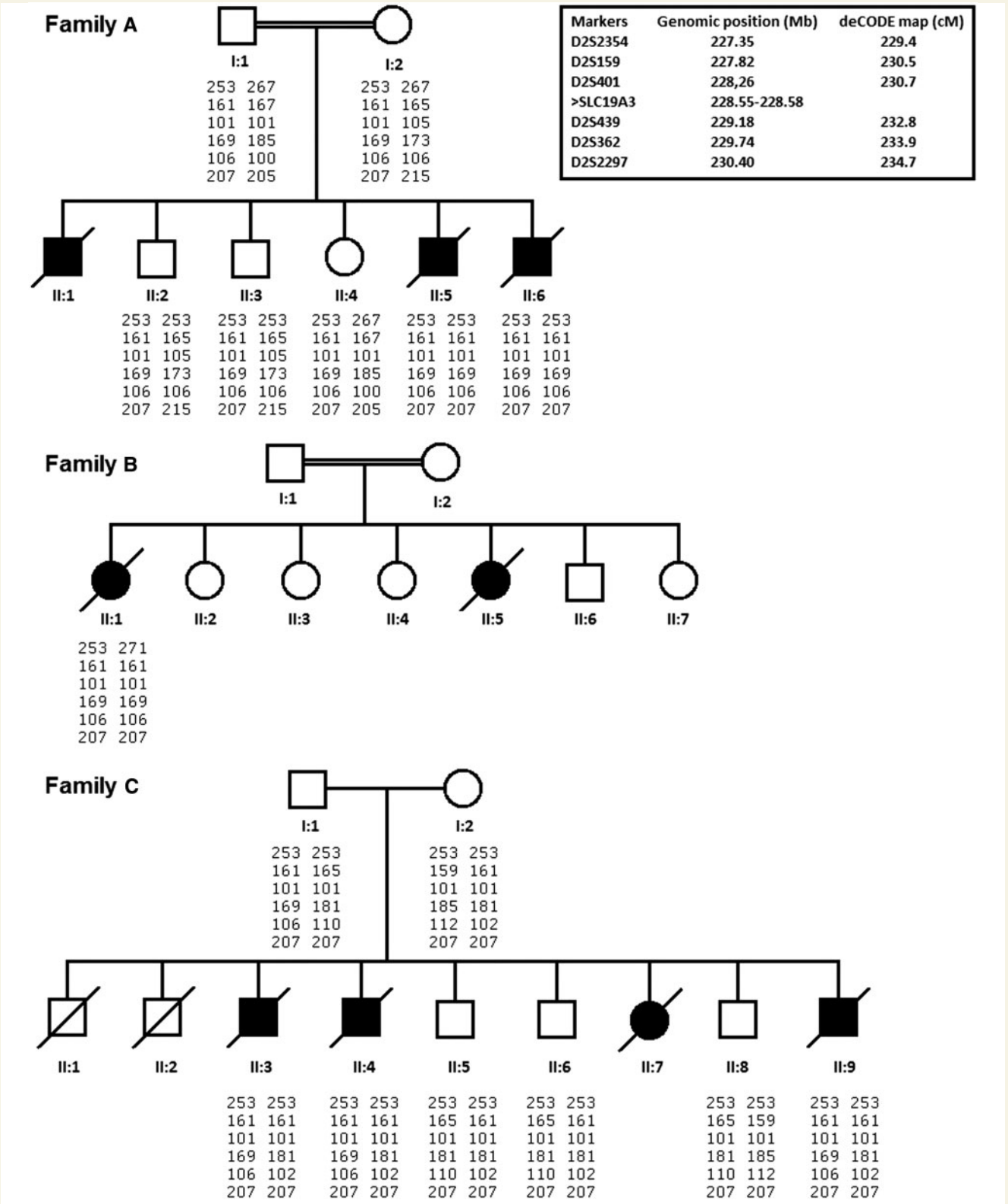

Figure 1 Pedigrees of three Moroccan families with Leigh syndrome showing the haplotype in the region surrounding the mutation.

Downloaded from https://academic.oup.com/brain/article/136/3/882/321679 by guest on 20 August 2022

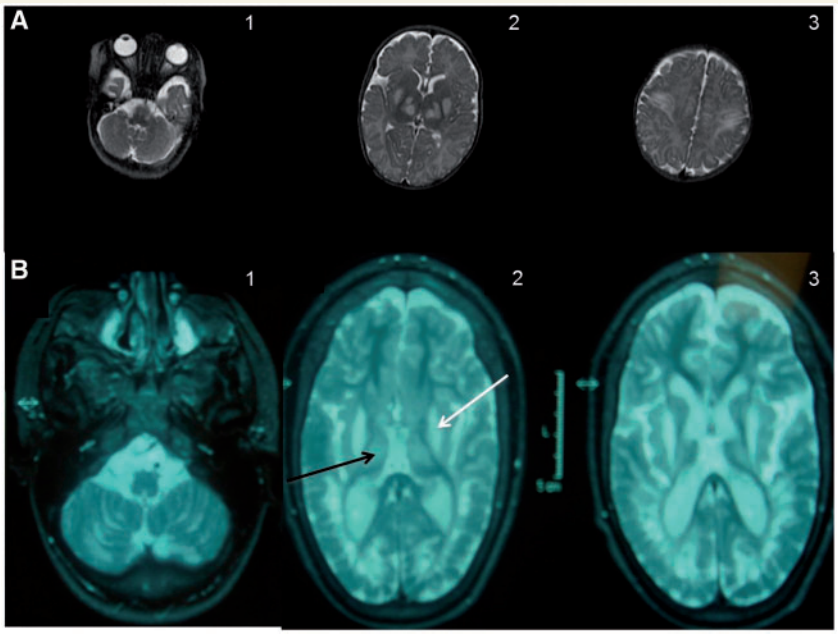

Figure 2 Brain MRIs (T_2-weighted images, axial view). (**A**) *Upper* row, Patient II-5 of Family A at age 1 month. Cerebellum and brainstem show minor changes. Increased signal intensities in putamen and mediocaudal and ventrolateral thalamic nuclei. The caudate nuclei are spared. Image **A3** shows an abnormal intensity and swelling of the rolandic area. (**B**) *Lower* row, Patient II-9 of Family C at the age of 6 years. (**B1**) Infratentorial increased signal intensities in the periaquaductal and cerebellar region. (**B2** and **B3**) Supratentorial loss of white matter increasing in antero-posterior direction with subcortical cysts; relatively sparing of directly periventricular situated white matter and thin corpus callosum. Pathological hyperintensity of the putamen (white arrow) and less so of the globus pallidi and thalamic nuclei (black arrow).

progressive neurological symptoms. Her EEG was normal. Her brain CT scan showed symmetric hypodense areas in pons, mesencephalon, vermis and thalamus. Medication existed of phenobarbital. Before she died, she was hypotonic with hyperreflexia in the extremities. Patient II:5 had sucking difficulties and could not open her eyes for 3 days. Because of the progressive disease course of her older sister, she was admitted to the hospital for observation. At presentation she had an abnormal breathing pattern and increasing convulsions confirmed by EEG. The epileptic activity was treated with phenytoin. Clinical examination showed hyperreflexia in the extremities including a positive Babinski sign. Apart from that, the extremities were hypertonic with an axial hypotonia. Lactic acid was found slightly increased in liquor as well as in blood. Her MRI demonstrated abnormal signal intensity in basal ganglia, thalami, mesencephalon and brainstem. The lesions were bilateral and symmetrical. After 1 month her clinical condition deteriorated rapidly. She began experiencing a Cheyne-Stokes respiration, which eventually led to her death.

Moroccan Family C consists of nine children, of which six died due to a Leigh-like syndrome (Fig. 1). All children were born to healthy non-consanguineous parents. The children who were clinically investigated had a similar disease course and features. Three of the children died at 1 month of age, one at 3 years, one at 15 years and one at 20 years of age.

The proband, Patient II:3, was born at a gestational age of 39 3/7 weeks. Besides meconium in the amniotic fluid there were no further complications. Because his brother died of a progressive encephalopathy he was hospitalized for observation during the first month of life. At 1 month of age he appeared to be alternating between hypo- and hypertonic and was irritable. His clinical picture and failure to thrive indicated the possibility of a mitochondrial disease. Oxidative phosphorylation was measured in a fresh muscle biopsy of the patient. Polarographic measurements were performed in isolated muscle mitochondria by a Clarke electrode as previously described (Scholte *et al.*, 1990) with pyruvate + malate, succinate + rotenone and ascorbate + TMPD. Oxygen consumption in the isolated mitochondria of the patient with pyruvate as the substrate was only 12% of normal oxygen consumption and with succinate and ascorbate as substrate 50% of normal oxygen consumption. When normalized for citrate synthetase, oxygen consumption with pyruvate was 25% compared with normal and the oxygen consumption with succinate and ascorbate was normal. Biochemical measurement of complex I and IV activities in the muscle homogenate were normal. Almost the same biochemical result was found in proband II:7 with normal activities of the respiratory chain complexes and a lowered oxygen consumption of 25% compared with normal with pyruvate + malate. The activities of pyruvate dehydrogenase and α-ketoglutarate dehydrogenase measured in the muscle homogenate *in vitro*, after addition of thiamine pyrophosphate, were increased to 441% and 156%, respectively, compared with controls. In the 1990's, treatment with vitamin cocktails for mitochondrial disease was recommended. Patient II:3 was treated with thiamine and riboflavin, which were later discontinued because he was developing well. Shortly after this change of treatment regimen he became hyperactive and irritable again, so treatment with these vitamins was resumed and the irritability disappeared. The following year he experienced three periods with relapses presented with an opisthotonic posture, inconsolable crying and loss of motor functions. His brain CT scan showed hypointense lesions in the thalami and basal ganglia, suggestive for Leigh disease. A metabolic analysis for a

Downloaded from https://academic.oup.com/brain/article/136/3/882/321679 by guest on 20 August 2022

wide range of metabolic disorders was normal. Biotin was added to his standard medication. At the age of 11, he presented with a status epilepticus for which he was treated with sodium valproate and phenytoin. When he was 12, he was severely mentally retarded. In that same year a gastrostomy was performed because of swallowing difficulties. The patient eventually died due to respiratory insufficiency and an abnormal breathing pattern at the age of 20. Patient II:9 was born at a gestational age of 42 weeks without complications. Soon after birth, treatment was started with riboflavin, thiamine and carnitine. At 1 month of age he became hypertonic and cried inconsolably. After 1 year he presented with seizures. His EEG demonstrated a severely distorted picture with epileptogenic phenomena. Therefore he received phenobarbital combined with carbamazepine. At 6 years of age severe psychomotor retardation was confirmed and he began experiencing reflux and spasticity. Brain MRI at 6 years of age revealed abnormal intensities in the putamen, thalami, nucleus dentate, and to a lesser extent in the globus pallidus (Fig. 2B). At the age of 8, a percutaneous endoscopic gastrostomy was performed because of his feeding difficulties. A few years later, at the age of 11, he strongly deteriorated and could hardly make any contact with his environment. The following years he had recurrent respiratory tract infections. The performed muscle biopsy showed COX-negative fibres. Biochemical measurements of the oxidative phosphorylation complexes in Patient II:9 revealed no enzyme deficiency and the oxygen consumption measured with glutamate or succinate as substrate was normal. The many respiratory tract infections proved fatal and he died at the age of 15.

Control population

Blood samples were collected from umbilical cords of 230 unrelated newborns. They originated from different regions of Morocco and the Moroccan origin of their parents and grandparents was confirmed. Informed consent for DNA analysis was obtained from the parents. Genomic DNA was extracted from 3 ml blood using the salting-out method.

Homozygosity mapping

Homozygosity mapping was performed with the Affymetrix GeneChip Human Mapping 250 K NspI SNP array using peripheral blood DNA of Patients II:5 and II:6 from Family A. The DNA was processed and labelled according to the manufacturer's instructions. Genotypes were generated by the Affymetrix GeneChip® Genotyping Analysis Software (GTYPE). Homozygous regions were determined by the online tool 'HomozygosityMapper' (Seelow *et al.*, 2009).

Whole exome sequencing and analysis

The exome was captured for Patient II:6 of Family A according to the TruSeq Whole Exome Enrichment for Paired-end Sequencing Preparation Guide (Illumina). Briefly, 1 μg of genomic DNA was fragmented using a Covaris S2, with settings optimal for an average fragment peak size of 200–300 bp. Fragments were end-repaired, ligated to specific adapters with a single index after the addition of a 3'-A. Subsequently the fragments were size selected for ~400 bp on agarose gel and pre-PCR amplified (10 cycles) to yield a total amount of 500 ng for the hybridization step. The DNA library was mixed with the capture probes of the targeted exome regions. The hybridization steps were performed as follows 95°C for 10 min, 18 cycles of 93°C for 1 min, decreasing 2°C per cycle, and 58°C for 16–20 h. Streptavidin affinity was performed to capture targeted regions of interest. Additionally three washes were applied to remove non-specific binding from the beads. Next, the enriched library was eluted from the beads. A second round of hybridization and subsequent washing was done to increase enrichment of the targeted regions. A post-PCR (10 cycles) was performed to amplify the enriched DNA library. Quantification and quality determination of the library was done on a 2100 Bioanalyzer using the High Sensitivity DNA Kit (Agilent). Clusters were generated using the cluster generation kit V5.0 according to the manufacturers protocol (Illumina) and the paired-end library was sequenced using 2 × 150 cycles V5.0 paired-end by sequencing by synthesis (SBS) on a Genome Analyzer IIx system (Illumina).

Basecalling was done using Casava 1.6 (Illumina). Sequence reads were aligned against the human reference genome (UCSC hg19) using BWA 0.5.9. Single base variants were identified using samtools 0.1.16 and annotated using an in house developed annotation python/R script that matched variants to the RefGene and dbSNP132 hg19 tracks from the UCSC genome browser. The data obtained was filtered for genes containing at least two alleles with a non-synonymous variant or variant leading to premature stop codons, which had an allele frequency of <0.01 or were absent in dbSNP132 and the 1000genomes data (1000 Genome Project Consortium *et al.*, 2010) and present in the homozygous candidate regions.

SLC19A3 sequencing and mutation specific restriction assay

Specific intronic primers were designed for amplifying the exons and flanking introns of *SLC19A3* (Supplementary Table 1). PCR was performed in a 10 μl reaction volume using AmpliTaq® Gold Master Mix (Invitrogen) with 8% glycerol, 2 pmol of each primer and 5 ng DNA. The cycle conditions were: 96°C for 5 min, followed by 33 cycles of 94°C for 30 s, 60°C for 45 s, and 72°C for 45 s with a final elongation step of 72°C for 10 min. PCR products were directly sequenced with the PRISM® Ready Reaction Sequencing Kit (Perkin-Elmer Life Sciences) on an ABI3730 DNA Analyzer (Applied Biosystems). A mutation-specific restriction assay was developed to screen controls for the c.20A>G mutation. A 5 μl PCR product of exon 2 was digested with MseI for 2.5 h at 37°C. The mutation results in the gain of a restriction site presenting a fragment of 138 bp instead of 187 bp, which was visualized on an agarose gel.

Defining the *SLC193A* mutation haplotype

All patients and relatives and 230 Moroccan controls were genotyped for six polymorphic markers (D2S2354, D2S159, D2S401, D2S439, D2S362 and D2S2297) flanking the *SLC19A3* gene. Primers were fluorescently labelled. Amplicons were amplified in a 50 μl reaction volume using Bioline Taq polymerase. The cycle conditions were: 94°C for 5 min, followed by 33 cycles of 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min 30 s with a final elongation step of 72°C for 7 min. Subsequently the size of the amplicons was determined on an ABI3730 DNA Analyzer (Applied Biosystems) according to manufacturer's instructions. Analysis was conducted using the Peak Scanner™ Software 1.0 (Applied Biosystems). The following formula was used to assess the age of the c.20C>A mutation in generations (G): $G = \log \delta / \log (1-\theta)$, with $\delta = LD = (Pd-Pn)/(1-Pn)$. The measure δ refers to the linkage disequilibrium (LD) between the mutation and the nearest recombinant markers, in our case, D2S2354 and D2S439 (see Fig. 1). *Pd* is the frequency of the ancestral STR allele among chromosomes carrying the *SLC19A3* mutation and *Pn* is the frequency of the ancestral

Downloaded from https://academic.oup.com/brain/article/136/3/882/321679 by guest on 20 August 2022

STR allele among chromosomes not carrying the mutation. θ refers to the recombination fraction between marker and genes (Risch *et al.*, 1995).

Thiamine transport assay

An expression construct containing the *SLC19A3* open reading frame was obtained from Open Biosystems (*SLC19A3* in pCMV-SPORT6). The mutation was introduced using site-directed mutagenesis with forward primer (5′-GATTGTTACAGAACTTAACTAAGCAGTTCCT GG-3′ and reverse primer 5′-CCAGGAACTGCTTAGTTAAGTTCT GTAACAATCC-3′). The presence of the mutation was verified by sequencing. HEK293 cells were cultured in Dulbecco's modified eagle medium containing 10% foetal calf serum at 37°C. For over-expression experiments, cells were seeded in 6-well plates and transfected using Lipofectamine™ 2000 with expression constructs containing either wild-type or mutant *SLC19A3* or an empty vector. The transfection efficiency was checked by quantitative PCR of *SLC19A3* RNA and *SLC19A2* RNA of the wild-type and mutant transfected cell lines. Both the mutant and wild-type *SLC19A3* were highly expressed (data not shown). Transfected HEK293 cells were incubated with different concentrations of ^{3}H-thiamine in Krebs Ringer buffer (133 mM NaCl, 4.93 mM KCl, 1.23 mM $MgSO_4$, 0.85 mM $CaCl_2$, 5 mM glucose, 5 mM glutamine, 10 mM HEPES, 10 mM MES pH7.4) for 20 min at 37°C. Low concentration of radiolabelled thiamine (15 nM) was used to favour high-affinity, low capacity uptake, whereas a high concentration (10 μM) was used to favour low-affinity, high capacity uptake. The reaction was stopped with ice-cold Krebs Ringer buffer and samples were dissolved in 0.5 M NaOH and neutralized with 0.5 M HCl. A fraction of the sample was used for protein quantitation and the remainder was subjected to liquid-scintillation counting. Non-specific uptake was determined by repeating the experiment at 0°C and subtracted from the results. The results are calculated as pmol or fmol thiamine uptake/min mg protein.

Results

Homozygosity mapping was performed for Patients II:5 and II:6 (Family A) with the Affymetrix GeneChip Human Mapping 250 K NspI SNP array. Both patients were homozygous for an overlapping region on chromosome 2 (220–234 Mb) and chromosome 13 (80–101 Mb). In these regions a total of 258 genes were present, but no obvious candidate gene. As a next step for identifying the genetic defect, the whole exome of Patient II:6 was sequenced. An average coverage of 52× was established and in total 17 848 coding variants were detected. After filtering for non-synonymous variants and variants leading to premature stop codons, which had an allele frequency of <0.01 or were absent in SNP databases but present in the homozygous candidate regions, only *SLC19A3* contained two mutated alleles. The patient was homozygous for a substitution in *SLC19A3* (c.20C>A), which resulted in a premature stop codon (p.Ser7Ter). The presence of the mutation was confirmed by Sanger sequencing (Fig. 3). The patients' affected brother was homozygous for the mutation, whereas both parents and the healthy siblings II:2, II:3 and II:4 were heterozygous. The mutation was not present in 460 Moroccan control alleles. Measurement of the pyruvate dehydrogenase activity in muscle of Patient II:5 showed no activity in the absence of thiamine pyrophosphate as co-factor whereas the activity was normal in the presence of thiamine pyrophosphate supporting a thiamine deficiency (Table 1).

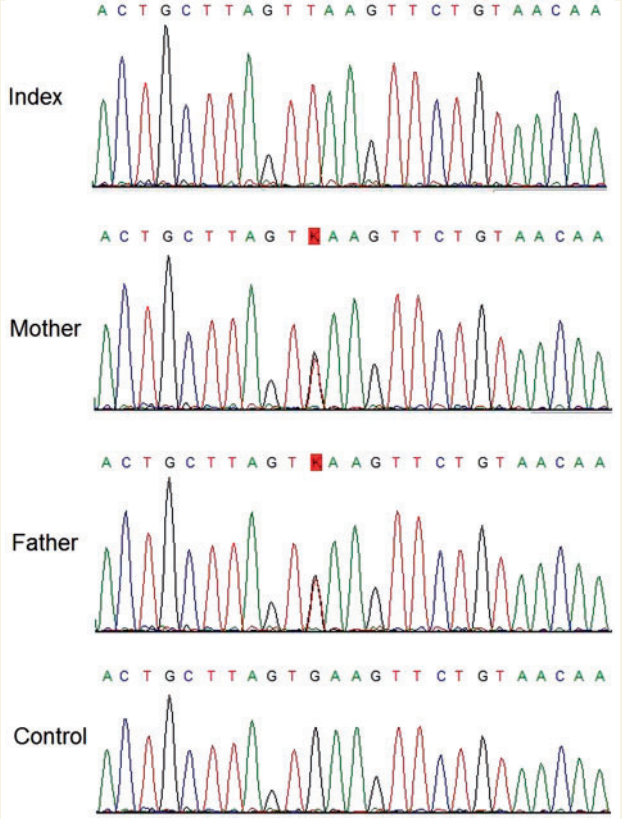

Figure 3 Mutation spectrum Family A. All are reverse sequences.

Table 1 Pyruvate dehydrogenase activity in muscle normalized to CS

	PDH complex activity -TPP	PDH complex activity +TPP
Control	2.8 ± 0.4	7.2 ± 2.2
Patient II:5, Family A	0	8.2

PDH = Pyruvate dehydrogenase; TPP = thiamine pyrophosphate.

Sequence analysis of *SLC19A3* in 17 additional patients with Leigh syndrome revealed the same homozygous c.20C>A mutation in two patients of Moroccan origin, from different families (Fig. 1; Families B and C). One patient (Family B) showed the same MRI abnormalities as the patients of the first family and was born from consanguineous parents. The parents of the other patient (Family C) were not related. This family had previously been tested for linkage using the Human Mapping 10 K GeneChips for genotyping and Merlin for linkage analysis. Although not statistically significant, genomic regions on several chromosomes segregated with the disease [maximum log of odds (LOD) score 1.58] including a region from 222 to 231 Mb on chromosome 2 in which *SLC19A3* is located. Segregation analysis of the mutation in this family showed a perfect

Downloaded from https://academic.oup.com/brain/article/136/3/882/321679 by guest on 20 August 2022

genotype–phenotype association. Unfortunately no material was available for segregation analysis in Family B.

As all three families carried the same mutation and originated from Morocco, markers surrounding the mutation were genotyped to test for a common haplotype. The markers D2S2354, D2S159, D2S401, D2S439, D2S362 and D2S2297, located within 0.3–1.8 Mb of *SLC19A3*, were used to determine the haplotype of this region (Fig. 1). A shared genotype was detected for all patients for markers D2S159 and D2S401. Possible recombination events in the regions between markers D2S2354 and D2S159 and markers D2S401 and D2S439 resulted in a 0.74–1.35 Mb shared region between all affected individuals which includes *SLC19A3*. Additionally, 230 Moroccan controls were genotyped for these markers to determine the allele frequencies in the Moroccan population. These frequencies were used for the calculation of the linkage disequilibrium between the *SLC19A3* mutation and the recombinant markers D2S22354 and D2S439, which were $\delta = 0.542125$ and $\delta = 0.664317$, respectively. Taken into account the genetic distances between the mutation and these two markers, the c.20C>A mutation in *SLC19A3* was estimated to have occurred between 50 and 70 generations ago. Assuming 25 years per generation, this would mean that the mutation arose ~1250 to 1750 years ago.

To confirm a causal role for this mutation in the biochemical and clinical phenotype, we performed thiamine uptake assays in HEK293 cells overexpressing either wild-type or mutant SLC19A3. Low and high concentration of radiolabelled thiamine was used, testing both high-affinity, low capacity uptake and low-affinity, high capacity uptake. A clear increase (~4-fold) in thiamine uptake was observed in cells transfected with wild-type SLC19A3, confirming its function as functional thiamine transporter. In contrast, cells expressing mutant SLC19A3, do not show enhanced thiamine uptake when compared to mock-transfected cells, demonstrating that the mutation results in an absent or non-functional protein.

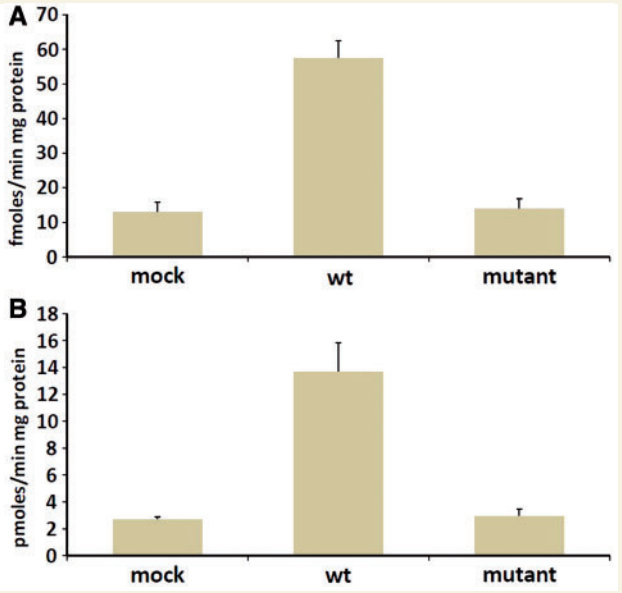

Figure 4 Thiamine transport assay. Thiamine uptake in HEK293 cells overexpressing empty vector (mock), wild-type (wt) or mutant (mutant) SLC19A3 in the presence of (**A**) a low concentration of radiolabelled thiamine (15 nM) or (**B**) a high concentration of radiolabelled thiamine (10 μM). Mock and mutant transfected cells take up only a marginal amount of thiamine whereas wild-type SLC19A3 overexpressing cells take up four times more thiamine. Error bars indicate the standard deviation.

Discussion

By combining whole exome sequencing with homozygosity mapping we identified a homozygous nonsense mutation in *SLC19A3* (c.20C>A; p.Ser7Ter) causing Leigh syndrome in a Moroccan consanguineous family. Because candidate regions after homozygosity mapping contained far too many genes for systematic sequence analysis and no obvious candidate gene, whole exome sequencing was performed yielding an average coverage of 52× and 17 848 coding variants. Variants were filtered in various steps to exclude those unlikely to be pathogenic. In the first step, non-synonymous variants and variants leading to premature stop codons were selected as these are most likely to affect protein function. In the next step, variants which are present in different SNP databases like dbSNP132 and 1000 genomes and have an allele frequency >0.01 were excluded. Subsequently only genes with homozygous variants were selected based on the autosomal recessive inheritance and consanguinity in the family. Finally, only the variants located within the homozygous candidate regions were considered candidates. This strategy revealed only one strong candidate, a homozygous nonsense mutation in *SLC19A3*, a gene previously reported to be involved in neurological disorders. The sequence of *SLC19A3* in 17 additional patients with Leigh syndrome was analysed and lead to the detection of the same homozygous c.20C>A in two unrelated patients of Moroccan origin. The brain MRI from Patient II:5 of Family B and Patient II:9 of Family C showed the same pattern of involvement of putamen, thalamus and cerebellar region as Patient II:5 of Family A suggesting the same disease. Marker analysis of the region surrounding the mutation in the three families showed a 0.74-1.35 Mb shared region between all affected individuals. This combined with the fact that the three families originated from the same province Al Hoceima in Northern Morocco supports the presence of a founder effect. Calculations to assess the age of the mutation revealed that the mutation occurred 1250–1750 years ago.

Metabolic investigations in all families indicated a mitochondrial defect due to possible deficiency of the respiratory chain, the pyruvate dehydrogenase, the 2 ketoglutaric acid dehydrogenase or the branched chain ketoacid dehydrogenase. A defect in the respiratory chain was excluded by the biochemical enzyme measurements. The normal oxygen consumption with glutamate and defective oxygen consumption with pyruvate indicated a defect in pyruvate dehydrogenase. However, the pyruvate dehydrogenase activity measured in the presence of thiamine pyrophosphate was found to be either normal or increased, indicating there is no genetic defect of the pyruvate dehydrogenase

Downloaded from https://academic.oup.com/brain/article/136/3/882/321679 by guest on 20 August 2022

complex but a defect of a co-factor. The co-factor which could explain the increase in the different metabolites such as lactate, pyruvate, 2-ketoglutaric acid, leucin and isoleucin is thiamine pyrophosphate, the active form of thiamine, indicating a possible defect in thiamine, thiamine transport or in the synthesis of thiamine pyrophosphate.

SLC19A3 is one of the two thiamine transporters present in the plasma membrane. In our patients, no functional SLC19A3 is predicted to be present, due to the premature stop codon, thereby preventing SLC19A3-mediated transport of thiamine in the cytoplasm. Mutations in *SLC19A3* have previously been associated with neurological disorders such as biotin responsive basal ganglia disease (BBGD) (Zeng *et al.*, 2005; Debs *et al.*, 2010), Wernicke's-like encephalopathy (Kono *et al.*, 2009) and epileptic spasm in early infancy, severe psychomotor retardation and progressive atrophy (Yamada *et al.*, 2010). Our results identified Leigh syndrome with specific MRI abnormalities as a new neurological disease phenotype associated with mutations in *SLC19A3*. It has been suggested that loss-of-function mutations are associated with the biotin responsive basal ganglia disease phenotype (Yamada *et al.*, 2010). However, our patients had Leigh syndrome caused by a homozygous nonsense mutation, which does not support this hypothesis. Moreover, the c.980-14A>G mutation described by Debs *et al.* (2010) in patients with biotin responsive basal ganglia disease does probably not result in a complete skipping of exon 4 as wild-type transcript was still detectable in patients which indicates there is a decreased amount of protein rather than a complete absence. Additionally, the biotin responsive basal ganglia disease families show typically striatal necrosis with caudate neurodegeneration which was not observed in any of our families, not even in the severely affected patients. Mutations in *SLC19A2*, the other thiamine transporter, have been described to cause megaloblastic anaemia (Labay *et al.*, 1999; Raz *et al.*, 2000; Scharfe *et al.*, 2000). The affected tissues in SLC19A2 or SLC19A3 deficiency correlate with the difference in expression of both thiamine transporters.

Thiamine is imported into the cytosol by either SLC19A2 or SLC19A3 and converted into the active form, thiamine pyrophosphate, by thiamine pyrophosphokinase 1 (TPK1). Thiamine pyrophosphate is an important co-factor in the cytosol for transketolase and in the mitochondria for pyruvate dehydrogenase, 2-ketoglutarate dehydrogenase and branched chain α-keto acid dehydrogenase. As SLC19A3 is the low affinity high capacity transporter, this will most likely lead to a deficiency of the co-factor thiamine pyrophosphate and of the thiamine pyrophosphate dependent processes. Whereas the other thiamine transporter, SLC19A2, is a high affinity low capacity transporter with a different tissue expression pattern. It is highly unlikely SLC19A2 can compensate the absence of a functional SLC19A3. Thiamine, or vitamin B_1, is not synthesized by humans or other animals and is required in the diet as the thiamine reserves only last for 20 days. Absence of thiamine in the diet or due to alcohol consumption leads to Beri-Beri or Wernicke-Korsakoff syndrome. Overlapping symptoms with Leigh syndrome are hypertonia, hypotonia, muscle weakness, dystonia, apneu, epilepsy, feeding problems, nystagmus and deafness.

A central role for thiamine deficiencies or thiamine transport defects in Leigh syndrome and related neurological disease can be further deduced from the clinical phenotypes associated with mutations in *TPK1*, the mitochondrial thiamine transporter (SLC25A19) and the thiamine pyrophosphate dependent processes. Whereas patients with mutations in *TPK1* presented with variable degrees of ataxia, psychomotor retardation, progressive dystonia and lactic acidosis (Mayr *et al.*, 2011), mutations in *SLC25A19* have been described in patients with bilateral striatal necrosis and chronic progressive polyneuropathy (Spiegel *et al.*, 2009). Mutations in *PDHA1* and *PDHB*, both encoding subunits of the E_1 component of the pyruvate dehydrogenase and sharing a thiamine pyrophosphate binding site, have been reported to cause Leigh syndrome (Schiff *et al.*, 2006; Quintana *et al.*, 2009). Finally, mutations have been identified in different subunits of the branched chain α-keto acid dehydrogenase in patients with maple syrup urine disease (Nobukuni *et al.*, 1993). Remarkably, all mutations in proteins involved in the thiamine metabolism, with the exception of SLC19A2, result in severe neurological symptoms demonstrating the importance of thiamine in brain development.

Previous studies have shown that SLC19A3 expression is dependent on biotin levels. The phenotype in patients with biotin responsive basal ganglia disease is likely the result of insufficient thiamine transport, which can partly be restored by overexpression of SLC19A3 through high doses of biotin. This supports our hypothesis that mutations in patients with biotin responsive basal ganglia disease lead to a decreased amount or activity of SLC19A3 instead of a completely non-functional protein. Besides biotin, also administration of high doses of thiamine has been reported to improve the clinical phenotype (Kono *et al.*, 2009). In this study it is suggested that the efficiency of high doses of thiamine indicates that dysfunction of SLC19A3 may induce expression of SLC19A2 increasing intracellular thiamine transport. The only patients in our families who survived childhood (Patients II:3 and II:9 of Family C) had been treated with a cocktail of vitamins, including thiamine and biotin. As it is highly unlikely that overexpression of the mutated *SLC19A3* gene will be beneficial, this treatment might have induced a partially compensating upregulation of SLC19A2, which could be sufficient for patients carrying this nonsense mutation to survive childhood. For patients with TPK mutations, thiamine pyrophosphate has been suggested as a therapeutic option (Mayr *et al.*, 2011). This might also be an option for patients with *SLC19A3* mutations. However, as stated by Mayr *et al.* (2011) the efficacy and safety of thiamine pyrophosphate as a drug still needs to be demonstrated and it is unclear whether thiamine pyrophosphate can cross the blood–brain barrier. In summary, we identified a nonsense mutation in *SLC19A3* in a consanguineous Moroccan family with Leigh syndrome, expanding the spectrum of neurological disorders associated with mutations in *SLC19A3* and pathways affected in Leigh syndrome. Our results further indicate that mutational screening of *SLC19A3* should be considered in patients with mitochondrial disorders and neurological symptoms, especially in specific parts of Morocco. Finally, these patients might benefit from high doses of biotin or, more likely, thiamine, which would make this a partly treatable form of Leigh syndrome.

Downloaded from https://academic.oup.com/brain/article/136/3/882/321679 by guest on 20 August 2022

Acknowledgements

We would like to warmly acknowledge the late neurologist Prof. dr. Herman F.M. Busch (Aug 21 1929 to Jan 21 2005; Erasmus MC Rotterdam, The Netherlands), who has been involved in the clinical investigations, and L.M. Hussaarts for excellent technical support.

Funding

The work is supported by the Alma in Silico project, which is financed by the Interreg IV European funds, The Walloon Region, The North Rhine Westphalia, The Flemish Community, The Belgian Province of Limburg and The Dutch Province of Limburg, as well as by the Universities of Maastricht and Liège, by the Prinses Beatrix Spierfonds (grant W.OR11-24) and the Stichting Metakids.

Supplementary material

Supplementary material is available at *Brain* online.

References

1000 Genome Project Consortium, Abecasis GR, Altshuler D, Auton A, Brooks LD, Durbin RM, et al. A map of human genome variation from population-scale sequencing. Nature 2010; 467: 1061–73.

Debs R, Depienne C, Rastetter A, Bellanger A, Degos B, Galanaud D, et al. Biotin-responsive basal ganglia disease in ethnic Europeans with novel SLC19A3 mutations. Arch Neurol 2010; 67: 126–30.

Grafakou O, Oexle K, van den Heuvel L, Smeets R, Trijbels F, Goebel HH, et al. Leigh syndrome due to compound heterozygosity of dihydrolipoamide dehydrogenase gene mutations. Description of the first E3 splice site mutation. Eur J Pediatr 2003; 162: 714–8.

Kono S, Miyajima H, Yoshida K, Togawa A, Shirakawa K, Suzuki H. Mutations in a thiamine-transporter gene and Wernicke's-like encephalopathy. N Engl J Med 2009; 360: 1792–4.

Ku CS, Naidoo N, Pawitan Y. Revisiting Mendelian disorders through exome sequencing. Hum Genet 2011; 129: 351–70.

Labay V, Raz T, Baron D, Mandel H, Williams H, Barrett T, et al. Mutations in SLC19A2 cause thiamine-responsive megaloblastic anaemia associated with diabetes mellitus and deafness. Nat Genet 1999; 22: 300–4.

Matthews PM, Marchington DR, Squier M, Land J, Brown RM, Brown GK. Molecular genetic characterization of an X-linked form of Leigh's syndrome. Ann Neurol 1993; 33: 652–5.

Mayr JA, Freisinger P, Schlachter K, Rolinski B, Zimmermann FA, Scheffner T, et al. Thiamine pyrophosphokinase deficiency in encephalopathic children with defects in the pyruvate oxidation pathway. Am J Hum Genet 2011; 89: 806–12.

Metzker ML. Sequencing technologies—the next generation. Nat Rev Genet 2010; 11: 31–46.

Mootha VK, Lepage P, Miller K, Bunkenborg J, Reich M, Hjerrild M, et al. Identification of a gene causing human cytochrome c oxidase deficiency by integrative genomics. Proc Natl Acad Sci USA 2003; 100: 605–10.

Nobukuni Y, Mitsubuchi H, Hayashida Y, Ohta K, Indo Y, Ichiba Y, et al. Heterogeneity of mutations in maple syrup urine disease (MSUD): screening and identification of affected E1 alpha and E1 beta subunits of the branched-chain alpha-keto-acid dehydrogenase multienzyme complex. Biochim Biophys Acta 1993; 1225: 64–70.

Pequignot MO, Dey R, Zeviani M, Tiranti V, Godinot C, Poyau A, et al. Mutations in the SURF1 gene associated with Leigh syndrome and cytochrome C oxidase deficiency. Hum Mutat 2001; 17: 374–81.

Quintana E, Mayr JA, Garcia Silva MT, Font A, Tortoledo MA, Moliner S, et al. PDH E(1)beta deficiency with novel mutations in two patients with Leigh syndrome. J Inherit Metab Dis 2009; 32: 339–343.

Raz T, Labay V, Baron D, Szargel R, Anbinder Y, Barrett T, et al. The spectrum of mutations, including four novel ones, in the thiamine-responsive megaloblastic anemia gene SLC19A2 of eight families. Hum Mutat 2000; 16: 37–42.

Risch N, de Leon D, Ozelius L, Kramer P, Almasy L, Singer B, et al. Genetic analysis of idiopathic torsion dystonia in Ashkenazi Jews and their recent descent from a small founder population. Nat Genet 1995; 9: 152–9.

Scharfe C, Hauschild M, Klopstock T, Janssen AJ, Heidemann PH, Meitinger T, et al. A novel mutation in the thiamine responsive megaloblastic anaemia gene SLC19A2 in a patient with deficiency of respiratory chain complex I. J Med Genet 2000; 37: 669–73.

Schiff M, Mine M, Brivet M, Marsac C, Elmaleh-Berges M, Evrard P, et al. Leigh's disease due to a new mutation in the PDHX gene. Ann Neurol 2006; 59: 709–14.

Scholte HR, Agsteribbe E, Busch HF, Hoogenraad TU, Jennekens FG, van Linge B, et al. Oxidative phosphorylation in human muscle in patients with ocular myopathy and after general anaesthesia. Biochim Biophys Acta 1990; 1018: 211–6.

Seelow D, Schuelke M, Hildebrandt F, Nurnberg P. HomozygosityMapper—an interactive approach to homozygosity mapping. Nucleic Acids Res 2009; 37: W593–9.

Spiegel R, Shaag A, Edvardson S, Mandel H, Stepensky P, Shalev SA, et al. SLC25A19 mutation as a cause of neuropathy and bilateral striatal necrosis. Ann Neurol 2009; 66: 419–24.

Tucker EJ, Compton AG, Thorburn DR. Recent advances in the genetics of mitochondrial encephalopathies. Curr Neurol Neurosci Rep 2010; 10: 277–85.

Yamada K, Miura K, Hara K, Suzuki M, Nakanishi K, Kumagai T, et al. A wide spectrum of clinical and brain MRI findings in patients with SLC19A3 mutations. BMC Med Genet 2010; 11: 171.

Zeng WQ, Al-Yamani E, Acierno JS Jr, Slaugenhaupt S, Gillis T, MacDonald ME, et al. Biotin-responsive basal ganglia disease maps to 2q36.3 and is due to mutations in SLC19A3. Am J Hum Genet 2005; 77: 16–26.

Downloaded from https://academic.oup.com/brain/article/136/3/882/321679 by guest on 20 August 2022