Stroke due to mitochondrial disorders in Saudi children

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ABSTRACT

Objective: To report on the clinical and biochemical features of patients who presented with stroke due to mitochondrial disorders amongst a prospective and retrospective cohort of Saudi children.

Methods: Children, who presented with stroke, were evaluated at the Division of Pediatric Neurology, or admitted to King Khalid University Hospital, College of Medicine, King Saud University, Riyadh, Kingdom of Saudi Arabia, during the periods July 1992 to February 2001 (retrospective study) and February 2001 to March 2003 (prospective study). Open muscle biopsies were obtained from patients suspected to have mitochondrial disorders, and examined using conventional histological and histochemical techniques. Biochemical, molecular pathological investigations, or both, of muscle could be arranged for only some of the patients.

Results: Mitochondrial disorders were the underlying risk factor for stroke in 4 (3.8%) of 104 children (aged one month to 12 years). Three patients (one male and 2 females) had Leigh syndrome (LS) and one had mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes (MELAS). At the time of stroke, the 3 children with LS were 11 months, 15 months, and 7 years old. They presented with psychomotor regression and seizures. Muscle histology and histochemistry showed mild non-specific changes but no ragged red fibers. Biochemical analysis of muscle (in one patient) revealed deficiency of pyruvate dehydrogenase complex. Analysis of mitochondrial DNA (mtDNA), [the other 2 patients] was negative for the 2 point mutations (T-G and T-C) at nucleotide position 8993, and for two T-C point mutations (at positions 8851 and 9176 of the ATPase 6 gene) that have been described in patients with LS. The girl with MELAS syndrome presented with a stroke-like episode at the age of 29 months and had focal brain lesions in the medial aspect of the left occipital and temporal lobes, and in the posteromedial aspect of the left thalamus, which resolved within 7 weeks. She had raised cerebrospinal fluid lactate but no ragged red fibers on muscle histochemistry. Biochemical assay of muscle homogenate showed reduction in respiratory chain complexes I, III and IV. Mutation screening of mtDNA at nucleotides 3243 (tRNA$^{Leu(UUR)}$) and 8344 (tRNA^{Lys}) was negative.

Conclusions: Mitochondrial disorders constitute a risk factor for stroke in Saudi children. However, demanding and highly specialized investigations are needed to confirm the diagnosis. These are better performed at supraregional centers where facilities for clinical, biochemical and molecular work-up are available.

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Mitochondria are involved in various pathways of metabolism, including the generation of cellular energy through the respiratory chain (RC), disposal of potentially toxic ammonia, removal and production of reactive oxygen species, and programmed cell death (apoptosis).[1-6] The most critical mitochondrial function

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is oxidative phosphorylation (OXPHOS), which results in the synthesis of adenosine triphosphate (ATP) from adenosine diphosphate (ADP) and inorganic phosphate. This entails transformation of energy derived from metabolism of nutrients to synthesis of ATP in the presence of oxygen. The system of OXPHOS includes 5 large multienzyme complexes, designated complexes I through IV and ATP synthase (complex V). These enzyme complexes are composed of many different proteins encoded by either nuclear or mitochondrial genes.[3] Mitochondrial encephalomyopathies constitute an important cause of stroke in children,[7] especially mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes (MELAS)[8] and Kearns-Sayre syndrome.[4,6] The latter disease also causes cardiac conduction disturbances, including complete heart block that may be a source of cerebral emboli. Implantation of a pacemaker may be needed to prevent such life-threatening complications. In MELAS, stroke-like episodes result in focal neurologic deficit due to metabolic decompensation of a brain region that can recover later. However, vascular insufficiency may also occur in mitochondrial diseases since endothelial mitochondria may be affected.[9] A heteroplasmic point mutation (A-G transition at position 3243) in the mitochondrial tRNA$^{Leu(UUR)}$ gene is the main cause of MELAS,[10] but many other point mutations are associated with MELAS. A third recognized cause of stroke due to mitochondrial dysfunction is Leigh syndrome (LS) (subacute necrotizing encephalomyelopathy, OMIM 256000).[11] As first described by Denis Leigh in 1951,[12] it has a characteristic neuropathology. This consists of focal, bilateral, and symmetric necrotic spongiform lesions associated with demyelination, gliosis and vascular proliferation in the brain stem, diencephalon, basal ganglia, cerebellum, and (occasionally) cerebral white matter. The symptoms typically begin in the first months of life and progress to death within 2 years although later onset or slower progression, or both, are known to occur.[13] Clinical signs include hypotonia, failure to thrive, psychomotor regression, brain stem, basal ganglia and cerebellar dysfunction, manifesting as ocular movement abnormalities, dystonia, ataxia, and swallowing and respiratory disturbances. Mutations in either nuclear DNA (nDNA) or mitochondrial DNA (mtDNA) can cause LS.[4,6] This condition has been associated with pyruvate dehydrogenase complex deficiency (mendelian inheritance), or with defects in any one of the five OXPHOS complexes (I to V) due to mtDNA or nDNA mutations.[4,6,13] Stroke-like episodes have also been described in overlap cases of Kearns-Sayre syndrome and myoclonic epilepsy and ragged red fiber (MERRF) syndrome.[9] It is noteworthy that several familial cases of mitochondrial diseases have been reported from Saudi Arabia.[14] During a retrospective and prospective study on childhood stroke, 104 Saudi children with suspected cerebrovascular disease were evaluated. Mitochondrial disorders accounted for 3.8% of identified stroke risk factors.[15] Herein, we report on the clinical and biochemical features of these patients.

Methods. The cohort included 104 Saudi children, who presented with stroke and were evaluated and followed-up at the Division of Pediatric Neurology (DPN), or were inpatients in the pediatric wards at King Khalid University Hospital (KKUH), Riyadh, Kingdom of Saudi Arabia. The duration of the prospective study was 2 years (February 2001 – March 2003), whereas the retrospective study extended for 8 years and 7 months (July 1992 – February 2001). The salient clinical, neuroimaging, neurophysiological and laboratory data were retrieved in a specially designed comprehensive protocol. Details of these are depicted elsewhere.[15] Open muscle biopsies were obtained from the vastus lateralis in each patient. These were frozen in isopentane cooled in liquid nitrogen and stored at -80⁰C. Conventional histological and histochemical techniques were performed on 10 μm thick transverse frozen cryostat sections.[16,17] Mitochondrial accumulation was explored with the Gomori trichrome stain in all samples. Histochemical enzyme reactions were confined to succinate dehydrogenase (SDH), which was performed in the samples of Patients 3 and 4. A portion of each biopsy was sent for biochemical and molecular analyses to the collaborating reference laboratories in dry ice. Due to logistic constraints, biochemical, molecular pathological investigations, or both, of muscle could be arranged for only some of the patients. Mitochondrial enzyme activities were measured in crude muscle extracts as described.[18-20] Total DNA extraction from muscle, preparation of mtDNA probes, and Southern blot analysis was also performed as previously described.[21]

Results. Four of 104 (3.8%) children (aged one month to 12 years) developed strokes in association with clinically identified mitochondrial disorders. They consisted of 3 patients with LS and one child with MELAS. The salient clinical characteristics of these patients are summarized in **Table 1**.

Patient one (**Table 1**) was a 5-year-old girl who was assessed because of epilepsy that had started 2 months earlier, and developmental regression first noted at the age of 8 months. Family history was negative for

Table 1 - Characteristics of patients with mitochondrial disorders.

Patient	Gender	Age at onset of initial stroke (years)	Age when evaluated at DPN (years)	Focal cranial/MRI lesions	Mitochondrial syndrome phenotype	Neurological features	Duration of follow-up (years)	Outcome
1	F	7	5	Bilateral thalamic. Central pontine	Leigh syndrome	Neurodevelopmental regression since age of 8 months. Epilepsy, severe mental retardation, ataxia, and spastic quadriplegia.	10	Alive
2	F	0.9	0.9	Bilateral symmetrical involving the temporal, parietal, occipital and thalamic regions	Leigh syndrome	Presented with intractable seizures and stupor. Condition resulted in spastic quadriplegic type of cerebral palsy and severe cognitive deficits.	7.8	Alive
3	M	1.3	1.3	Right frontal, parietal, and occipital regions. Left frontal lobe, both thalami, corpus callosum, right cerebral peduncle and mid-brain	Leigh syndrome	Neurodevelopmental regression since 7 months. Seizures and hemiparesis at 15 months.	0.3	Died at 19 months
4	F	2.4	2.4	Left occipital and temporal lobes, left thalamus, left hippocampus	MELAS	Presented with right focal seizures and hemiparesis at 29 months. Hemiparesis cleared within weeks. Had recurrence at 33 months.	0.3	Died at 33 months

DPN - Division of Pediatric Neurology, MELAS - mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes

neurodegenerative diseases. The parents were not consanguineous, but they originated from the same area of the Central Province. On examination, she was found to be mentally retarded, had truncal ataxia and spastic quadriplegia. Cranial MRI was normal apart from slightly widened lateral and third ventricles. Several other investigations were either negative or normal. These included arterial blood gases, ammonia, lactate, copper, ceruloplasmin, carnitine, amino acid profile (by tandem mass spectrometry), biotinidase, phytanic acid, brucella antibodies, α-fetoprotein, carcinoembryonic antigen, and vitamin E level. There were no acanthocytes on blood smear and urine examination by gas chromatography/mass spectrometry (GC/MS) revealed no abnormality in the organic acid spectrum. The EEG was diffusely slow, indicating generalized cortical involvement. Normal tests included nerve conduction studies (NCS), visual evoked potentials (VEP), electroretinogram (ERG) and brain auditory evoked potentials (BAEP). At the age of 7 years, she was admitted to a regional hospital with a history of fever and vomiting of one day's duration, followed by coma. Cranial non-enhanced CT scan showed evidence of brain atrophy and bilateral hypodense thalamic ischemic lesions. A week later, CT with contrast delineated the thalamic infarcts (**Figure 1**). She recovered from this episode but remained ataxic, spastic quadriplegic with generalized rigid tone. Three years later, cranial MRI (**Figure 1**) showed moderate brain atrophy mainly in the left parieto-occipital region, slight thinning of the left cerebellar peduncle and mild thinning of the body of the corpus callosum. A horizontally oriented, irregularly outlined lesion was seen in the central pons, together with small triangular lacunar infarcts in both thalami. Muscle histology and histochemistry showed neurogenic atrophy and no ragged red fibers. Biochemical analysis of muscle was not feasible. Analysis of DNA isolated from muscle was performed in July 1995 at the Laboratory of Neuromuscular Disease, College of Physicians and Surgeons of Columbia University, New York. It was negative for T-G and T-C point mutations in mitochondrial DNA at nucleotide position 8993 described in patients with neurogenic muscle weakness, ataxia and retinitis pigmentosa (NARP) and in children with maternally

inherited Leigh syndrome (MILS).[22-28] Analysis was also negative for two T-C point mutations (at positions 8851 and 9176 in ATPase 6 gene) described in children with LS.[29-31]

An 11-month-old girl (Patient 2, **Table 1**) presented 8 days after an episode of seizures followed by stupor. The seizures started focally in the left upper and lower limbs, and then became generalized. Following intensive care management, she recovered partially but had residual spastic quadriplegia, severe cognitive deficit and epilepsy. The epilepsy was controlled after 3 years of anti-convulsant therapy. Her parents were first-degree cousins. Hematologic and biochemical investigations, on admission, revealed normal results. These included complete blood count (CBC), blood glucose, blood lactate, ammonia and amino acids, blood culture and CSF cell count and culture. Urine GC/MS was negative for organic acids. Cranial CT scan (**Figure 2**) showed features of necrotizing encephalopathy in the form of multiple, bilateral, symmetrical, irregular hypodense areas involving the temporal, parietal, and, to a lesser extent, both thalamic and occipital regions. Brain SPECT showed perfusion defects in both right and left temporo-parietal regions. Abdominal ultrasound, followed by CT, showed left dysplastic cystic kidney and enlarged right kidney (compensatory hypertrophy) with normal excretion. Neurophysiological studies showed normal VEP and ERG, whereas BAEP was suggestive of bilateral brainstem involvement. Muscle histology and histochemistry showed mild non-specific changes and no ragged red fibers. Biochemical assay of muscle enzymes was performed in November 1994 at the Metabolic Division, Department of Pediatrics, University Hospital Nijmegen, The Netherlands. This revealed low pyruvate dehydrogenase complex of 1.3 mU/mg protein (control range = 2.7-8.2). Other mitochondrial enzymes were within the control range. These were cytochrome *c* oxidase (complex IV), NADH:Q1 oxidoreductase (complex I), succinate: cyt *c* oxidoreductase (complex II + coenzyme Q + complex III), and decylubiquinol:cyt *c* oxidoreductase (complex III). Analysis of DNA isolated from muscle was carried out at the Laboratory of Neuromuscular Disease, College of Physicians and Surgeons of Columbia University, New York. The results were negative for the two point mutations (T-G and T-C) in mitochondrial DNA at nucleotide position 8993 described in patients with LS and in patients with NARP.[22-28] Analysis was also negative for two T-C point mutations (at positions 8851 and 9176 in ATPase 6 gene) which have been described in patients with LS.[29-31]

Patient 3 (**Table 1**) was a 15-month-old boy who was admitted with left-sided focal seizures and hemiparesis associated with progressive deterioration in the level of consciousness. Past medical history revealed that he regressed in his milestones since the age of 7 months with progressive motor and cognitive deficits. Parents were first-degree cousins, and there was no family history of a similar illness. Examination at the Pediatric Intensive Care Unit (PICU) showed aphasia, left facial nerve palsy and left hemiparesis. Brain MRI (**Figure 3**) revealed extensive involvement of the right cerebral hemisphere with lesions, which were hypodense in T1-weighted and hyperintense in T2-weighted images. These lesions involved the right frontal, temporal, parietal and occipital regions, and the left frontal lobe. The right and left thalami showed ring-enhancing lesions, the right-sided ones being more prominent. The corpus callosum was also affected. The right cerebral peduncle and the mid-brain were involved, and there was expansion of these structures. The venous structures appeared normal. Several hematologic and biochemical investigations were negative or revealed normal results. These included CBC, ESR, prothrombin time (PT), activated partial thromboplastin time (APTT), protein C, protein S, serum brucella antibodies, anion gap estimation, lactate, ammonia and amino acid analysis by tandem mass spectrometry (MS). Examination of cerebrospinal fluid (CSF), revealed xanthochromia, white blood cells (WBC) 5 x 10^6/L (all lymphocytes), no red blood cells (RBC) or organisms, low glucose of 0.97 mmol/L (N = 2.5-4), raised proteins of 8.7 g/L (N = 0.15-0.45) and elevated lactate of 3 mmol/L (N = 0.5-2.2). Cultures were negative for acid-fast bacilli and viruses. Screening by PCR for herpes was also negative. Muscle histology and histochemistry showed no ragged red fibers but mild non-specific changes. There was slightly intensified staining with the oxidative enzyme succinate dehydrogenase (SDH), but no definite accumulation was noted. Staining for cytochrome *c* oxidase (COX) was not carried out. Further biochemical analysis of muscles and mitochondrial DNA screening was not feasible. The patient died at the age of 19 months despite supportive medications including biotin, carnitine and ascorbic acid.

A 29-month-old girl (Patient 4, **Table 1**) was admitted to KKUH with recurrent right-sided convulsions associated with right hemiparesis. Her first generalized tonic-clonic (GTC) convulsion occurred at the age of one year, and was associated with fever. She was diagnosed at another hospital as having encephalitis and was discharged after one weeks stay. Two weeks later, she had another GTC seizure without fever and was started on anticonvulsant therapy. At 18 months of age, she was
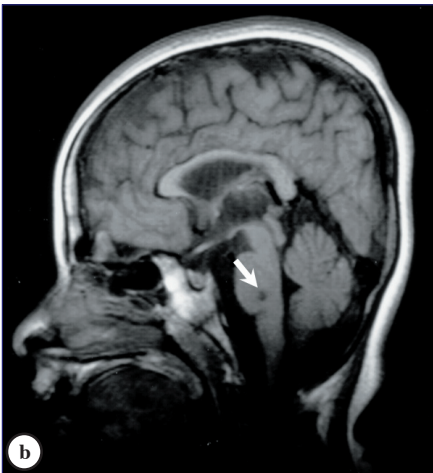
Figure 1 - **a)** Axial brain CT showing bilateral symmetric large low attenuation thalamic lesions due to infarction (arrows). **b & c)** Three years follow-up MRI showing regression of the thalamic lesions. Residual high signal intensity on coronal T2-weighted image (arrows in **c**) is seen in the thalamic region, bilaterally. Small central old pontine infarction appears as low signal on sagittal T1-weighted image (arrow in **b**) and high signal on coronal T2-weighted image (open arrow in **c**).
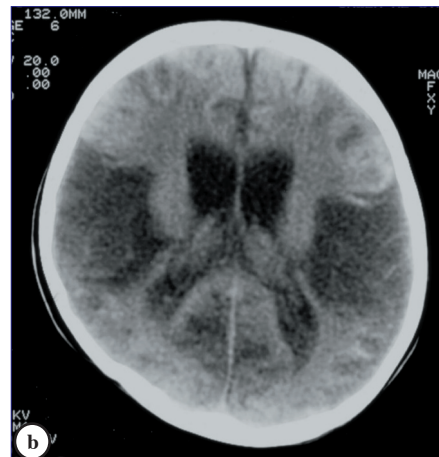

Figure 2 - **a & b)** Axial brain CT images showing bilateral symmetrical low attenuation areas in the temporal, parietal and thalamic regions due to necrotizing encephalopathy.
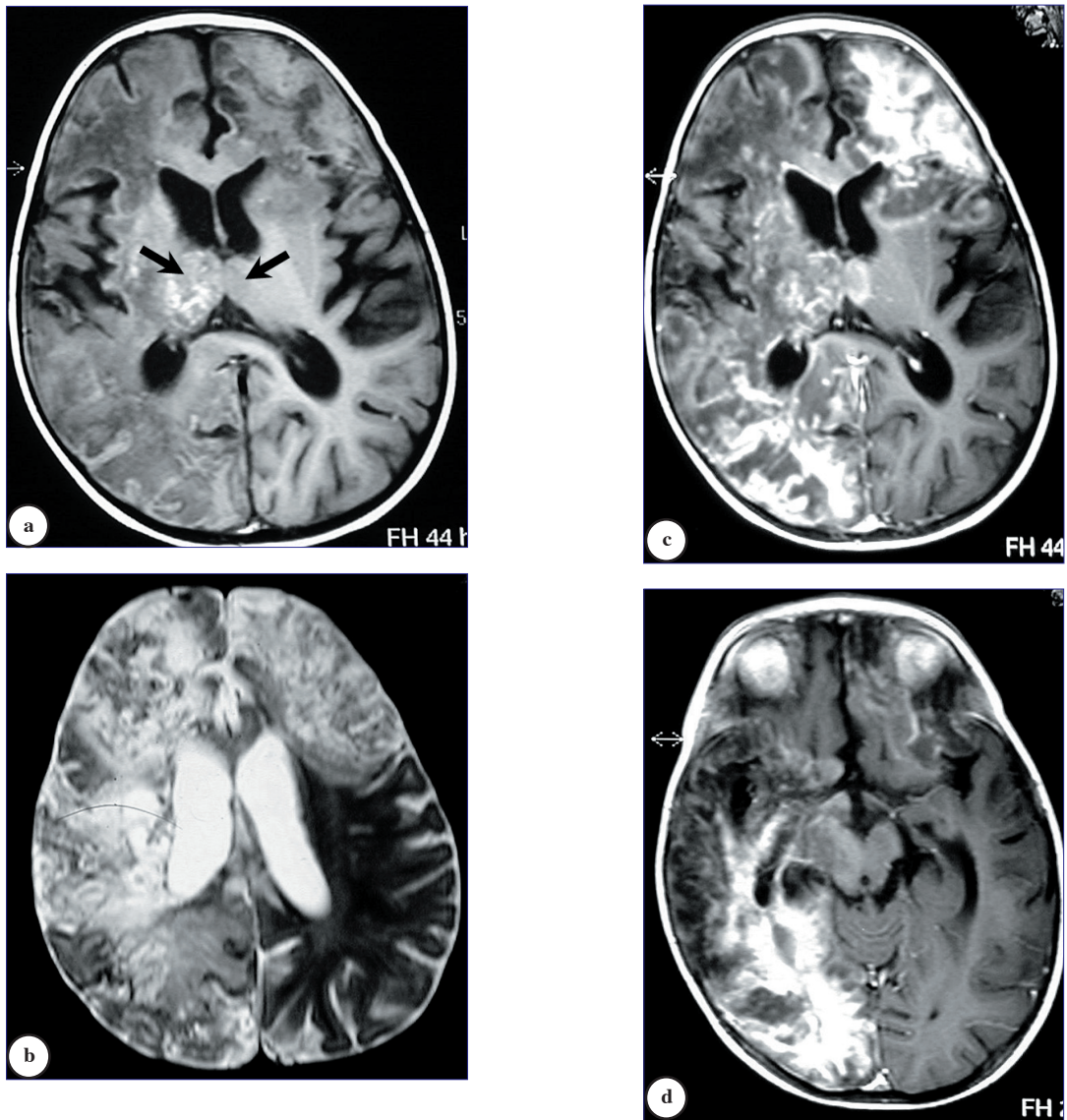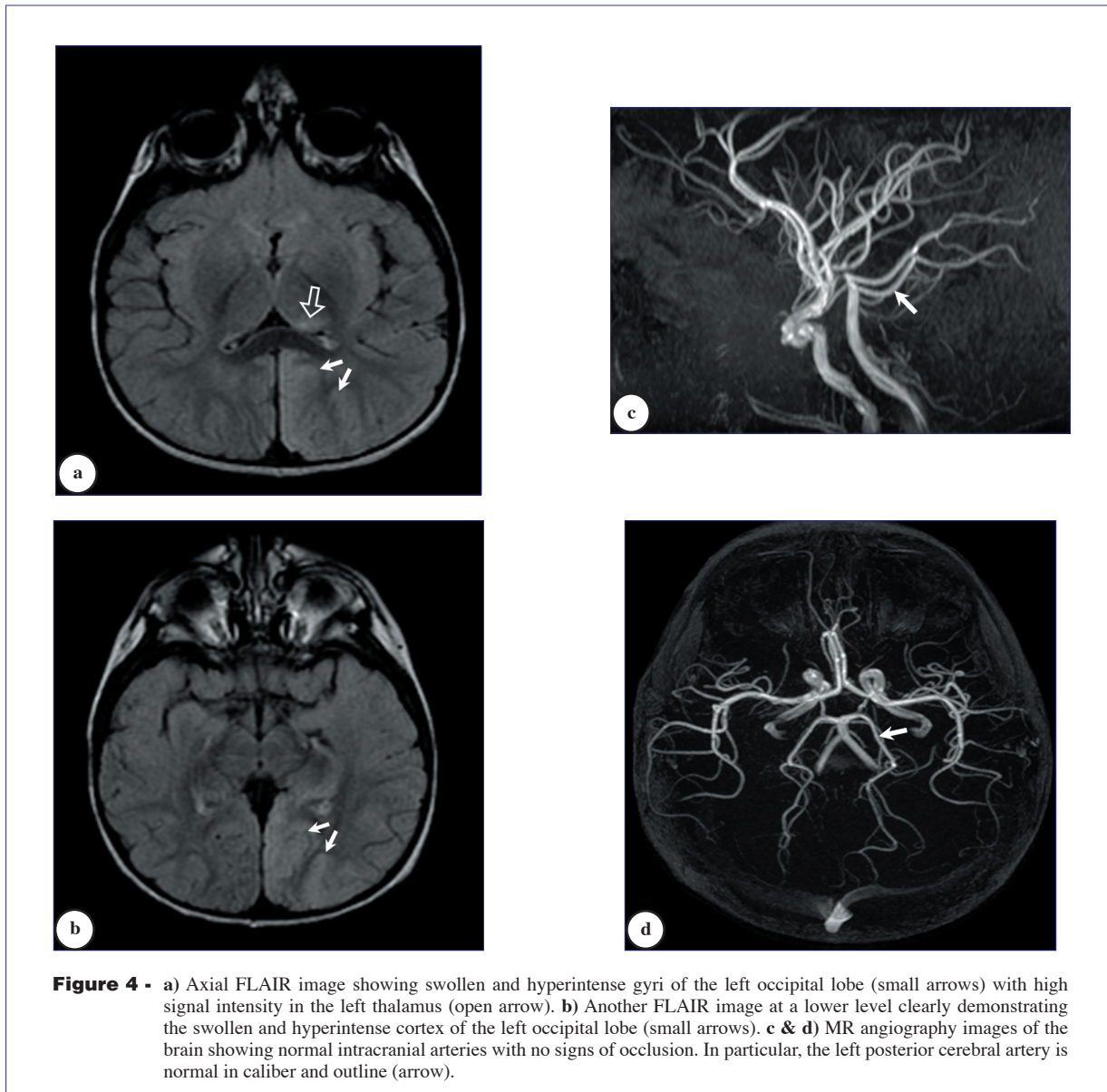
Figure 3 - a) Axial T1-weighted brain MR image showing large areas of low signal intensity due to extensive infarction involving the right frontal, temporal, parietal and occipital lobes, as well as the left frontal lobe and both thalami (arrows), more on the right. **b)** Axial T2-weighted brain MR image depicting the areas of infarctions as high signal intensity. **c & d)** Enhanced axial T1-weighted brain MR image showing intense enhancement of the infarctions due to luxury perfusion.

Figure 4 - **a)** Axial FLAIR image showing swollen and hyperintense gyri of the left occipital lobe (small arrows) with high signal intensity in the left thalamus (open arrow). **b)** Another FLAIR image at a lower level clearly demonstrating the swollen and hyperintense cortex of the left occipital lobe (small arrows). **c & d)** MR angiography images of the brain showing normal intracranial arteries with no signs of occlusion. In particular, the left posterior cerebral artery is normal in caliber and outline (arrow).

noted by the family to regress in her development and she gradually lost all her previously attained skills. The parents were first-degree cousins, and they had 2 other children, one of whom died at $1\frac{1}{2}$ years of age. He had been diagnosed (at another hospital) to have congenital toxoplasmosis and hydrocephalus. Three of the proband's maternal cousins (all males) have myoclonic epilepsy. Physical examination revealed, apart from right hemiparesis that weight, height and skull circumference were all below the 5th centile for age. She showed no dysmorphic features and had hypotonia of central origin. Brain MRI (**Figure 4**) showed high signal intensity on fluid-attenuated inversion recovery (FLAIR) sequence at the medial aspect of the left occipital and temporal lobes and posteromedial aspect of the left thalamus. A high signal intensity was also shown in the left hippocampus. Magnetic resonance angiography gave normal results. Electroencephalography showed features of encephalopathy. Brain auditory evoked potentials (BAEP), ERG and VEP were normal. Cerebrospinal fluid (CSF) examination showed normal cell counts and no bacterial growth, but a raised lactate level of 3.2 mmol/L (N = 1.0-2.2). Other normal hematologic, biochemical and serologic investigations included CBC, PT, APTT, protein C,

protein S, antithrombin III, Hb electrophoresis, liver function tests, blood lactate and pyruvate, blood amino acids by tandem MS, urine for amino acids and organic acids by GC/MS, blood culture, anti brucella antibodies, TORCH and hepatitis screening. Muscle histology and histochemistry showed non-specific changes and no ragged red fibers. Mitochondrial enzymes and DNA were assayed at the Division of Molecular Neurogenetics, National Neurological Institute "C. Besta", Milano, Italy. These showed reduction of activities of respiratory chain complex I (NADH : Co Q1 reductase) to 7.8 mmol/min mg (N = 13.6-27.7), complex III (DBH2: Cyt.*c* reductase) to 71.2 nmol/min mg (N = 75.7-140.7) and complex IV (cytochrome *c* oxidase) to 67.8 nmol/min mg (N = 85.6-195.2). The activities of complex II, complex V, succinate dehydrogenase and citrate synthase were normal. Analysis of mitochondrial DNA showed no mutations in tRNA$^{Leu(UUR)}$, frequently associated with MELAS.[10,32] It did not also show mutations in tRNA^{Lys}, which have been reported in myoclonic epilepsy with ragged red fibers (MERRF)/MELAS overlap syndrome.[33] Over the following 4 weeks, the hemiparesis cleared and a repeated brain MRI 3 weeks later, showed right cerebellar hemisphere focal hyperintensity on FLAIR images not appreciable on T1 or T2-weighted images. The cerebral cortex, white matter, and basal ganglia appeared normal. A third MRI, carried out 19 days later, was normal. A repeated EEG showed features of partial epilepsy with secondary generalization. The patient died at the age of 33 months.

Discussion. Three children (**Table 1**) had stroke with underlying LS-like features, which presented at ages ranging between 7-11 months, either acutely or with neurodevelopmental regression.[13,34,35] Neuroradiologic features (including both cranial CT and MRI) were also consistent with the diagnosis,[36,37] as cranial CT showed bilateral symmetric hypodensity involving the deep structures of the brain. On MRI (carried out for Patients 1 and 3, **Table 1**), the lesions showed low intensity on T1-weighted images and T2 hyperintensity, and involved the brainstem. In patient 3, where venous and CSF lactate were assayed simultaneously, CSF lactate was clearly raised whereas venous lactate was normal; emphasizing the importance of this clinical investigation in screening patients with suspected LS.[13,38] Cerebrospinal fluid proteins were also raised in this patient, as has been observed in similar cases.[34] In this patient also, MRI revealed extensive areas of cavitation and involvement of the corpus callosum similar to what has been observed in severe forms of LS.[36,39]

Histologic and histochemical analysis of muscle biopsies from the 3 patients with LS showed non-specific findings and were remarkable for the absence of ragged red fibers.[40] This is similar to the observation in other larger series.[41] However, specific histochemical stains for mitochondrial enzymes was confined to succinate dehydrogenase (SDH) in 2 patients (2 and 3) and the status of COX was not explored in any of the 3 affected children.[42] Deficiency of COX is frequently associated with LS and revealing it histochemically would help in exploring for mutations in COX assembly genes.[13,43]

Biochemical analysis of muscle was carried out in one patient (Patient 2, **Table 1**) and it showed a partial deficiency of pyruvate dehydrogenase complex (PDHC). Pyruvate dehydrogenase is a mitochondrial multienzyme complex that controls the entry of pyruvate, the glycolytic end-product, into mitochondria for oxidative metabolism. As such, it plays an important regulatory role in cellular energy metabolism in glycolytic-dependent organs such as the central nervous system. Brismar[36] reported 6 children from Saudi Arabia with LS due to defects of pyruvate utilization. Four patients had pyruvate dehydrogenase E1α subunit deficiency; one had E3 subunit deficiency; whereas the sixth had pyruvate carboxylase (PC) deficiency. Heterogenous biochemical defects have been detected in patients with LS. Apart from PDHC, these included PC, complex I, complex IV and combined defects in complexes I and III.[13,37,38,44] Other abnormalities such as complexes I, II, III, V and biotinidase deficiency have also been reported with features of LS.[4,6,45]

Two patients (Patients 1 and 2, **Table 1**) had their mtDNA screened for mutations known to be associated with LS. These revealed negative results. It is noteworthy that mutations of mtDNA are found in less than 10% of babies and children with mitochondrial disorders.[46] With the exception of MILS, associated with the T8993G mutation in the mtDNA ATPase 6 gene and the G13513A transition in the ND5 mtDNA gene, LS is usually a nuclear-related mitochondrial disease.[3]

Patient 4 was admitted with recurrent right-sided convulsions and hemiparesis (stroke-like episode) associated with large MRI signal alternations in the left hemisphere, not corresponding to well-defined vascular territories, similar to what is observed in MELAS patients.[37,47] Both the hemiparesis and the signal alterations disappeared over time. Other clinical features in this child, known to be associated with MELAS, included developmental regression and focal EEG discharges.[4,34] Lactate concentration was normal in blood and raised in CSF similar to the findings in other large series.[38] Muscle histology

showed mild non-specific changes. Histochemistry revealed no ragged red fibers, as has been observed in a few cases of MELAS syndrome.[48] Biochemical assay of muscle homogenate showed reduction in respiratory chain complexes I, III and IV. Combined defects of the respiratory chain complexes has been reported in patients with MELAS phenotype.[9] Because of the strong family history of myoclonic epilepsy, the mitochondrial DNA of this patient was screened for mutations at nucleotides 3243 (tRNA[Leu(UUR)]) associated with MELAS, as well as for mutations at nucleotides 8344 (tRNA[Lys]) known to be associated with the syndrome of MERRF.[49] However, these showed negative results, and the underlying molecular pathology in this patient, still awaits to be revealed.

Finally, and to the best of our knowledge, we are aware of only one case of stroke secondary to childhood MELAS being reported from the Arabian Peninsula.[50] This might reflect the demanding and highly specialized investigations needed to confirm the diagnosis in these cases. As has been suggested previously,[42] such studies are better performed in supraregional centers that can offer a complete diagnostic program where facilities for clinical, biochemical and molecular work-up are available.

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