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The mitochondrial genome in Wolfram syndrome

EDITOR—Wolfram syndrome is the association of juvenile onset diabetes mellitus and optic atrophy,[1] also known as DIDMOAD (diabetes insipidus, diabetes mellitus, optic atrophy, and deafness). This is a progressive, neurodegenerative disorder, with diabetes mellitus and optic atrophy presenting in the first decade,[2] cranial diabetes insipidus, and sensorineural deafness in the second, and neuropathic bladder in the third, followed by neurological complications (cerebellar ataxia, myoclonus) and psychiatric illness in the fourth decade. The clinical phenotype is consistent with an ATP supply defect, suggesting a mitochondrial mediated disease.[3] Mitochondrial genome deletions and pathogenic point mutations[4 5] have been described in Wolfram patients. A nuclear gene *WFS1*, wolframin,[6 7] was recently identified, encoding a polypeptide of 890 amino acids. Wolfram syndrome thus appears to be genetically heterogeneous. Recently, a distinct "mitochondrial haplotype" was described.[8] Because recombination does not appear to be characteristic of mtDNA, the accumulation of polymorphisms can be used as a "genetic clock" to estimate diversity within and between populations.[9] A cluster of nucleotide exchanges at nucleotide positions 4216 and 11 251 roughly distinguished a series of 6/8 Wolfram patients from controls and patients with Leber hereditary optic neuropathy (LHON). The authors suggested that these mtDNA variants may predispose to Wolfram syndrome.[8] We investigated our cohort of 50 Wolfram syndrome patients[10] for evidence of a distinct mitochondrial haplotype and mitochondrial DNA rearrangements.

Patients for this study were included from a cohort recruited nationally in the UK.[10] Minimal ascertainment criteria were juvenile onset (less than 30 years of age) diabetes mellitus and optic atrophy. These were chosen as the only features consistently present and earliest to develop in 166/168 case reports.[11] Diabetes mellitus was defined as a fasting plasma glucose of more than 6.0 mmol/l (>3 SD above the mean of the normal population). All affected patients had been examined, with pupils dilated, by an experienced ophthalmologist. All patients were visited at home; blood samples were obtained from all available family members after informed consent was given. DNA was extracted from whole blood using Puregene DNA extraction kits (Gentra Systems), according to the manufacturers' instructions, and diluted to stock solutions of 500 ng/μl.

The mitochondrial haplotype of the Wolfram patients was investigated using PCR and direct sequencing of the region between 16 050 and 16 400 in the first hypervariable region of the large non-coding region as previously described.[12] Mitochondrial haplotype analysis was carried out on 32 Wolfram families of European origin. The presence of mtDNA variants at bp 4216, 11 251, and 15 257 were assessed using PCR and restriction digestion.[9 13] Mean pairwise differences[14] were estimated and compared with a control data set comprising 100[15] and 60 UK white subjects.[16] An unrooted tree was drawn based on previous studies of Europeans. A PCR *Apa*I restriction site assay was used to screen for the mitochondrial tRNA Leu (UUR) A to G (3243) mutation[17]; this has been associated with maternally transmitted diabetes and deafness.[18] The final cycle of PCR was labelled with ^{33}P-dCTP permitting detection of the mutation at levels of heteroplasmy below 1%. DNA from whole blood was also screened for the 11778A:T and 3460A:T mutations associated with Leber's hereditary optic neuropathy (LHON) using allele specific PCR.[19] These mutations were chosen as they account for 75% of all cases of LHON. The 14484 mutation was not investigated as it is phenotypically milder and has a better outcome.[20] Mitochondrial DNA was also analysed for the presence of major rearrangements by long range PCR[21]; PCRs were performed in a final volume of 50 μl with 0.25 μl each of primers L1 (nt2695-2720) and H3 (nt16459-16436), 2.5 mmol/l $MgCl_2$, 0.25 mmol/l of each dNTP, 1 × Bio-Optiform 111 buffer (Bioline), and 1.5 U Bio-X-ACT *Taq* polymerase(Bioline). PCRs were hot started at 80°C by the addition of 20-50 ng DNA and denatured at 95°C for 10 seconds and 68°C for 10 minutes plus 30 seconds for each subsequent cycle (25 cycles). PCR products were run on 0.7% Seakem agarose gels. The resolution for long range PCR is about 1 kb (sometimes 0.5 kb depending on the gel).

Needle muscle biopsies under local anaesthetic were undertaken on nine adult Wolfram patients. Informed, written consent was obtained, after a written and verbal explanation. Respiratory chain activity was analysed in fresh muscle biopsy samples from six patients by two separate methodologies. Flux experiments measuring rotenone sensitive cytochrome c reductase with glutamate, 2-oxoglutarate, and pyruvate + malate as substrates to assay complexes I and III, and antimycin A sensitive succinate cytochrome reductase for complexes II and III, were carried out.[22 23] Flux experiments on cytochrome oxidase for complex IV were also performed. Specific assays using n-decylubiquinone for complex I and the reduced form for complex III were also used.[24] The reference values were calculated from a control population of 100, which included normal muscle obtained from orthopaedic procedures, and muscle biopsy tissue from patients with neuromuscular disease including non-mitochondrial metabolic abnormalities, such as McArdle disease. Histochemistry results were available for three additional patients for cytochrome oxidase, succinate dehydrogenase, and NADH reductase. The study was approved by the ethics committee of South Birmingham district health authority.

The clinical features of our cohort of 45 patients have been described.[10] There were 29 index patients (14 male, 15 female) and 16 secondary patients (all sibs, seven male and nine female). Twenty seven of the 29 families were white UK patients. In addition, we included three affected sibs from Ireland and two affected sibs from New Zealand. The mitochondrial haplotypes of 32 white UK Wolfram patients are shown in the "unrooted tree" (fig 1). There was no evidence of clustering of the Wolfram haplotypes by eye or by calculating the mean number of differences between subjects in the Wolfram and control populations.[14] Only 4/32 (12%) fell into haplogroups 2A and 2B (equivalent to J and T of Torroni *et al*[25] and collectively to 4216+11251 of Hofmann *et al*[8]), which was less than the expected 6 based on prevalence of these lineages in our control population. This proportion was significantly different from the 7/8 (88% p=0.0001, Fisher's exact test) Wolfram patients found in this haplogroup by Hofmann *et al*.[8] Hence there was no evidence of a founder mitochondrial haplotype in our patients. The patients were then divided into those from families showing genetic linkage to the Wolfram locus on chromosome 4p and sporadic cases. Neither group showed evidence of haplotype clustering either on the unrooted tree or by comparison of the mean

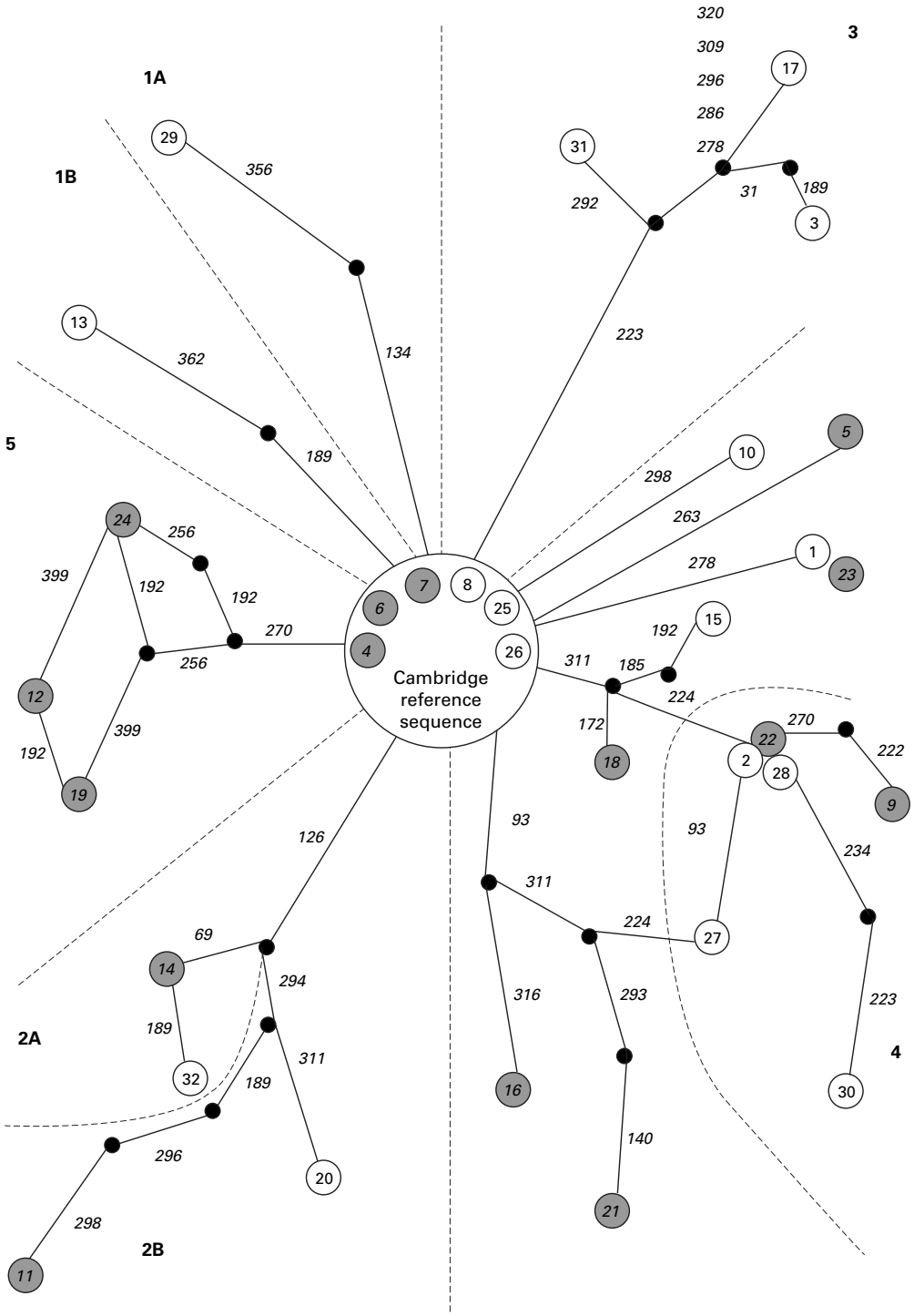

Figure 1 Unrooted mitochondrial phylogenetic tree of UK Wolfram patients. Each numbered open circle represents a UK Wolfram patient. Shaded circles represent patients in whom a (nuclear) WFS1 mutation has been identified. Each solid line represents a change in one base relative to the Cambridge reference sequence. Numbers in italics indicate the base change, for example, 298 represents a base change at nucleotide 16 298. Dashed lines represent divisions between Clades (for example, 1-5) and within Clades (for example, 1A, 1B).

pairwise differences. Mutations in *WFS1* have been identified in 15/16 patients investigated; there is no evidence of haplotype clustering of patients with *WFS1* mutations. Lymphocyte derived DNA was available from 40 patients in the cohort from 28 unrelated white families. There was no evidence for the mitochondrial tRNA Leu 3243G:C mutation or the 11778 A:T and 3460 A:T mutations associated with LHON. In addition, there was no evidence for major mitochondrial rearrangements. Nine Wolfram patients had muscle biopsies; of these, six showed normal respiratory chain complex activity and three normal histochemistry (table 1). The respiratory chain complex activity of four of these patients has been reported previously.[26] Sixteen of the 32 families were included in a mutation analysis of *WFS1*.[27] Loss of function mutations in *WFS1* were found in 15 of these families, including nonsense, missense, in frame deletions, in frame insertions, and frameshift mutations.

We found no evidence supporting a role for mtDNA in Wolfram syndrome. Firstly, our data show no evidence for distinct mitochondrial haplotypes in Wolfram syndrome as previously described.[8] A cluster of nucleotide exchanges

Table 1 Mitochondrial respiratory chain activities

Assays	*Patients*						*Reference value*
	4	*14*	*30*	*32*	*23*	*34*	
Flux experiments							
Complexes I and III							
Pyruvate + malate cytochrome oxidase	0.245	0.327	0.426	0.164	0.418	0.139	0.1–1.0
Glutamate cytochrome reductase	0.009	0.019	0.098	0.147	0.262	0.025	0.1–1.0
Oxoglutarate cytochrome reductase	0.296	0.164	0.426	0.115	0.303	0.115	0.1–1.0
Complexes II and III							
Succinate cytochrome reductase	0.475	0.139	0.672	0.409	0.409	0.254	0.1–6.0
Complex IV							
Cytochrome oxidase	0.395	0.299	0.901	0.655	0.984	0.59	0.1–5.0
Specific assays							
Complex I			0.836	0.409	0.861	0.091	0.1–1.0
Complex II			4.918	9.835	10.359	1.967	3.0–15.0

Respiratory chain activities expressed as μmol/g/min (rate constant k/g/min for COX). Complexes I and III are specific assays. Patients 4, 14, 30, and 32 have been previously reported.[26]

were reported at nucleotide positions 4216 and 11 251 (designated haplogroup B by Hofmann *et al*,[8] haplogroup 2B by Richards *et al*,[28] and haplogroup T by Torroni *et al*[25]), which was found in 6/8 (75%) of Wolfram patients but in only 13/67 (19%) controls. Only 2/28 (7%) of our patients fell within this haplogroup and there was no evidence of polymorphism clustering in the remainder of our cohort of 40 patients. We conclude that because of the small size of the previous study, the results most probably occurred by chance.[8] On the other hand, it is clear that in LHON the 11 778 A:T mutation is associated with sequence changes at base pairs 4216 and 13 708 (which define haplogroup A of Hofmann *et al*,[8] 2A of Richards *et al*,[28] and J of Torroni *et al*[25]). This suggests that mtDNA sequence changes found in this haplogroup (not necessarily the 4216 and 13 708 variants) may play an aetiological role in LHON. Similarly, recent studies suggest that a common variant in the non-coding region of mtDNA at bp 16189 is associated with insulin resistance in adult life.[29] It remains possible that an unrecognised mitochondrial DNA variant may predispose to Wolfram syndrome.

Secondly, we found no abnormal mitochondrial function or mtDNA mutations in our series. Our search for mtDNA rearrangements only excluded them from one tissue (lymphocytes); one might expect patients to harbour mutations in other non-dividing tissues such as brain or pancreas. Wolfram syndrome is a neurodegenerative disease; it is known that mtDNA mutations normally accumulate with age in postmitotic tissues, and this may be increased in neurodegenerative disease.[30] We believe that the phenotypes of patients in whom mtDNA mutations have been reported are not typical of the majority of our cases and will not prove to have mutations in *WFS1*. The recent identification of a Wolfram syndrome gene (*WFS1*, wolframin), allowed us to screen our patients for mutations.[27] We found loss of function mutations in *WFS1* in 16 of the 17 families investigated. At present, the intracellular location of the WFS1 protein is not known. It is expressed in most organs, but exact localisation awaits the development of specific antibodies. The amino acid sequence does not have significant homology with other proteins in the databases and there is no evidence that the WFS1 protein has a mitochondrial targeting sequence.

Our study has implications for clinical practice; definition of inherited diabetes syndromes at a molecular level will help us to distinguish overlapping clinical phenotypes such as Wolfram syndrome and mitochondrial diabetes and deafness. In addition, exclusion of a role for mitochondrial DNA should simplify genetic counselling for families with Wolfram syndrome.

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1 Wolfram D, Wagener, H. Diabetes mellitus and simple optic atrophy among siblings: report of four cases. *Mayo Clin Proc* 1938;**13**:715-18.
2 Barrett TG, Bundey S. Wolfram (DIDMOAD) syndrome. *J Med Genet* 1997;**34**:838-41.
3 Bu X, Rotter, J. Hypothesis. Wolfram syndrome: a mitochondrial mediated disorder *Lancet* 1993;**342**:598-600.
4 Barrientos A, Volpini V, Casademont J, Genis D, Manzanares J, Ferrer I, Corral J, Cardellach F, Urbano-Marquez A, Estivill X, Nunes V. A nuclear defect in the 4p16 region predisposes to multiple mitochondrial DNA deletions in families with Wolfram syndrome. *J Clin Invest* 1996;**97**:1570-6.
5 Pilz D, Quarrell O, Jones. E. Mitochondrial mutation commonly associated with Leber's hereditary optic neuropathy observed in a patient with Wolfram syndrome (DIDMOAD). *J Med Genet* 1994;**31**:328-30.
6 Inoue H, Tanizawa Y, Wasson J, Behn P, Kalidas K, Bernal-Mizrachi E, Mueckler M, Marshall H, Donis-Keller H, Crock P, Rogers D, Mikuni M, Kumashiro H, Higashi K, Sobue G, Oka Y, Permutt A. A gene encoding a transmembrane protein is mutated in patients with diabetes mellitus and optic atrophy (Wolfram syndrome). *Nat Genet* 1998;**20**:143-8.
7 Strom TM, Hortnagel K, Hofmann S, Gekeler F, Scharfe C, Rabl W, Gerbitz KD, Meitinger T. Diabetes insipidus, diabetes mellitus, optic atrophy and deafness (DIDMOAD) caused by mutations in a novel gene (*wolframin*), coding for a predicted transmembrane protein. *Hum Mol Genet* 1998;7:2021-8.
8 Hofmann S, Bezold R, Jaksch M, Obermeier-Kusser B, Mertens S, Kaufhold P, Rable W, Hecker W, Gerbitz, K. Wolfram (DIDMOAD) syndrome and Leber hereditary optic neuropathy (LHON) are associated with distinct mitochondrial DNA haplotypes. *Genomics* 1997;**39**:8-18.
9 Hofmann S, Jaksch M, Bezold R, Mertens S, Aholt S, Paprotta A, Gerbitz K. Population genetics and disease susceptibility: characterisation of central European haplogroups by mtDNA gene mutations, correlation with D loop variants and association with disease. *Hum Mol Genet* 1997;**6**:1835-46.
10 Barrett T, Bundey S, Macleod A. Neurodegeneration and diabetes: UK nationwide study of Wolfram syndrome. *Lancet* 1995;**346**:1458-63.
11 Blasi C, Pierelli E, Rispoli E, Saponara M, Vingolo E, Andreani D. Wolfram's syndrome: a clinical, diagnostic and interpretive contribution. *Diabetes Care* 1986;**9**:521-8.
12 Morten K, Poulton J, Sykes B. Multiple independent occurrences of the 3243 mutation in mitochondrial $tRNA^{leuUUR}$ in patients with the MELAS phenotype. *Hum Mol Genet* 1995;**4**:1689-91.
13 Hofmann S, Bezold R, Jaksch M, Kaufhold P, Obermaier-Kusser B, Gerbitz K. Disease relevance of the so-called secondary Leber hereditary optic neuropathy mutations. *Am J Hum Genet* 1997;**60**:1539-42.
14 Macaulay V. *MULTIPAIR*. Department of Cellular Science, University of Oxford, 1996.
15 Piercy R, Sullivan K, Benson N, Gill P. The application of mitochondrial DNA typing to the study of white Caucasian genetic identification. *Int J Legal Med* 1993;**106**:85-90.
16 Poulton J, Marchington D, Brown M, Phillips D, Hagelberg E. Does a common mitochondrial DNA polymorphism underlie susceptibility to diabetes and the thrifty genotype? *Trends Genet* 1998;**14**:385-7.

17 Goto Y, Nonaka I, Horai S. A mutation in the tRNA Leu (UUR) gene associated with the MELAS subgroup of mitochondrial encephalomyopathies. *Nature* 1990;**348**:651-3.
18 Van den Ouweland J, Lemkes H, Ruitenbeek W, Sandkuijl L, de Vijlder M, Struyvenburg P, van de Kamp J, Maasen J. Mutation in mitochondrial $tRNA^{Leu(UUR)}$ gene in a large pedigree with maternally transmitted type II diabetes mellitus and deafness. *Nat Genet* 1992;**1**:368-71.
19 Norby S. Screening for the two most frequent mutations in Leber's hereditary optic neuropathy by duplex PCR based on allele specific amplification. *Hum Mutat* 1993;**2**:309-13.
20 Brown MD, Torroni A, Lott MT, Wallace DC. Mitochondrial DNA haplotype and phylogenetic analysis of Caucasian Leber's hereditary optic neuropathy (LHON) patients indicates novel mutation associations and multiple independent occurrences of the common primary mutations. *Am J Hum Genet Suppl* 1994;**55**:A214.
21 Li Y, Hengstenberg C, Maisch B. Whole mitochondrial genome amplification reveals basal level multiple deletions in mtDNA of patients with dilated cardiomyopathy. *Biochem Biophys Res Commun* 1995;**210**:211-18.
22 Gohil K, Jones D, Edwards R. Analysis of mitochondrial function with techniques applicable to needle muscle biopsy samples. *Clin Physiol* 1981;**1**:195-207.
23 Scholte H, Busch H, Luyt-Houwen I, Vaandrager-Verduin M, Przyrembel H, Arts W. Defects in oxidative phosphorylation. Biochemical investigations in skeletal muscle and expression of lesions in other cells. *J Inherit Metab Dis* 1987;**10**(suppl 1):81-97.
24 Zheng X, Shoffner J, Voljavec A, Wallace D. Evaluation of procedures for assaying oxidative phosphorylation enzyme activities in mitochondrial myopathy muscle biopsies. *Biochim Biophys Acta* 1990;**1019**:1-10.
25 Torroni A, Huoponen K, Francalacci P, Petrozzi M, Morelli L, Scozzari R, Obinu D, Savontaus M, Wallace D. Classification of European mtDNAs from an analysis of three European populations. *Genetics* 1996;**144**:1835-50.
26 Barrett TG, Poulton K, Bundey S. DIDMOAD syndrome; further studies and muscle biochemistry. *J Inherit Metab Dis* 1995;**18** 218-20.
27 Hardy C, Khanim F, Torres R, Scott-Brown M, Seller A, Poulton J, Collier D, Kirk J, Polymeropoulos M, Latif F, Barrett T. Clinical and molecular genetic analysis of 19 Wolfram syndrome kindreds demonstrating a wide spectrum of mutations in WFS1. *Am J Hum Genet* 1999;**65**:1279-90.
28 Richards M, Corte-Real H, Forster P, Macaulay V, Wilkinson-Herbots H, Demaine A, Papiha S, Hedges R, Bandelt H, Sykes B. Paleolithic and neolithic lineages in the European mitochondrial gene pool. *Am J Hum Genet* 1996;**59**:185-203.
29 Poulton J, Scott-Brown M, Cooper A, Marchington D, Phillips D. A common mitochondrial DNA variant is associated with insulin resistance in adult life. *Diabetologia* 1998;**41**:54-8.
30 Wallace DC. 1994 William Allan Award Address. Mitochondrial DNA variation in human evolution, degenerative disease, and aging. *Am J Hum Genet* 1995;**57**:201-23.

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Two further cases of Sener syndrome: frontonasal dysplasia and dilated Virchow-Robin spaces

EDITOR—Case 1, a male, was born at 38 weeks' gestation by normal delivery to unrelated parents. Mild bilateral renal pelvis dilatation was noted on prenatal scans as was a slight increase in liquor volume. An amniocentesis, which was performed for maternal age, showed a 46,XY karyotype. His birth weight was 2593 g (9th centile), length 47.5 cm (50th centile), and OFC 32.7 cm (50th centile). Features noted at birth included neck oedema, a large anterior fontanelle (6 cm × 7 cm), a short penis with a large scrotum, and an anteriorly placed anus. Echocardiography showed a patent ductus arteriosus with mild septal and right ventricular wall hypertrophy. His cranial ultrasound was reported to show partial agenesis of the corpus callosum. Postnatal renal ultrasound showed mild dilatation of the left pelvicalyceal system. At 7 months his growth parameters were weight 6.37 kg (0.4th centile), length 64.5 cm (2nd centile), and OFC 44 cm (25th centile).

Bilateral inguinal herniae were present from 3 months and repaired at 4 months of age. Ophthalmological examination showed a hypoplastic left disc and a small coloboma of the right disc. He also has hypermetropic astigmatism and bilateral entropion which required surgery. During his first year he developed eczema and persistent diarrhoea. He sat at 7 months, crawled at 1 year, and walked independently at 2 years. His linear growth progressed close to the 3rd centile and his OFC followed the 90th centile. The facial features of note are his wide mouth, long, smooth philtrum, and small posteriorly angulated ears (fig l). His hair grows well but is brittle and coarse. He has one extra tooth in the mandible and all his teeth are irregular and pointed. He was due to attend mainstream school. He had required speech therapy for delayed language. Urinary mucopolysaccharides, oligosaccharides, and white cell enzymes were normal. The triglycerides were at the upper range of normal at 1.4 mmol/l (range 0.5-1.8 mmol/1) and the cholesterol was at the lower range of normal (2.8 mmol/l, normal range approximately 1.7-5.2 mmol). A 7-dehydrocholesterol result was not available as the child left the country before this test became available. In addition, there were no records of the parental cholesterol levels. Thyroid function at 10 months showed T4 107 μmol/l and TSH 5.7 IU/l. Blood chromosomal analysis showed a normal 46,XY karyotype. Skeletal survey was normal.

Case 2, a male, was born at 32 weeks' gestation, weighing 2000 g, to unrelated parents, following spontaneous onset of labour and a normal delivery. There was a history of polyhydramnios during the pregnancy. He has one normal female sib. He was admitted to the special care baby unit for five weeks because of his prematurity. Dysmorphic features noted at birth included hypertelorism, a persistent large posterior fontanelle, a large anterior fontanelle, a narrow, high arched palate with midline cleft of the upper alveolar margin, two neonatal teeth, and a right inguinal hernia (fig 2).

Motor development was noted to be delayed; he sat at 10 months and walked at 18 months. He has had both inguinal and umbilical hernia repairs. He has required speech therapy and requires special help at school. At the age of 8 years, he is hyperactive and is said to have an

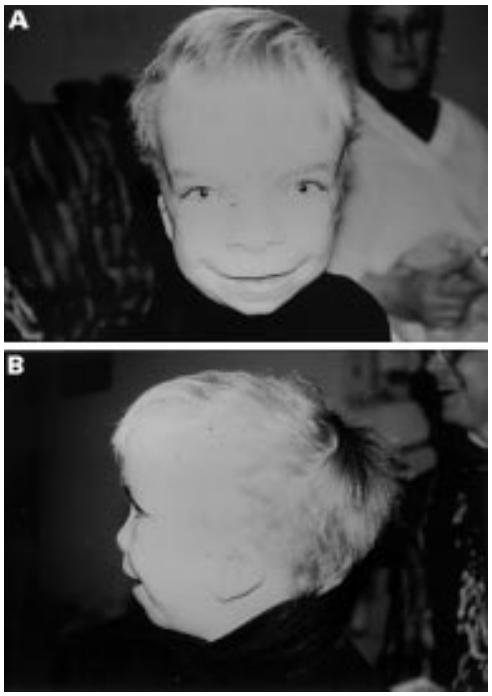

Figure 1 (A, B) Front and side view of case 1 showing hypertelorism, a wide mouth, and posteriorly rotated ears.