REPORT

Exocrine Pancreatic Insufficiency, Dyserythropoeitic Anemia, and Calvarial Hyperostosis Are Caused by a Mutation in the *COX4I2* Gene

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Steatorrhea and malabsorption of lipid-soluble vitamins due to exocrine pancreatic insufficiency are common in patients with cystic fibrosis and are predominant in Shwachman-Bodian-Diamond, Pearson, and Johanson-Blizzard syndromes. In four patients who suffered from congenital exocrine pancreatic insufficiency, dyserythropoeitic anemia, and calvarial hyperostosis, we excluded these disorders and identified, by using homozygosity mapping, a mutation in the *COX4I2* gene. The COX4 protein is an essential structural subunit of cytochrome c oxidase complex and has two isoforms, encoded by two different genes. We show that the ratio of COX4I2 to COX4I1 mRNA is relatively high in human acinar cells. The mutation is associated with marked reduction of *COX4I2* expression and with striking attenuation of the physiologic COX4I2 response to hypoxia. Mutation analysis of *COX4I2* is warranted in patients with malabsorption due to exocrine pancreatic insufficiency and in patients with dyserythropoeitic anemia.

Exocrine pancreatic insufficiency (ePI) with failure to absorb fat is a life-threatening condition. Patients are not only depleted of fats but may also suffer from coagulopathy, rickets, anemia, and corneal disease as a result of deficiency of fat-soluble vitamins. ePI is a common feature of cystic fibrosis (CF, MIM 219700) and occurs in 95% of the patients.[1] The association of ePI and hematologic dysfunction is rarer and has been reported in Shwachman-Bodian-Diamond syndrome (SBDS, [MIM 260400]) and in Pearson syndrome (PS, [MIM 557000]). In CF and SBDS, the pancreatic acinar cells are replaced by fat, whereas in PS the pancreas is atrophic and fibrotic. Hematologic abnormalities are common in patients with SBDS and PS; in SBDS the bone marrow is hypoplastic and fatty, and patients suffer from intermittent deficiency of myeloid lineages and susceptibility to infections[2], whereas in PS there is typically refractory sideroblastic anemia, which is usually macrocytic, and the bone marrow has normal cellularity and distinctive vacuolization.[3] PS is caused by a deletion of mitochondrial DNA (mtDNA), and because of changing heteroplasmy, the condition may resolve spontaneously or progress to Kearns-Sayre syndrome (MIM 530000).[4,5]

We hereby report a new syndrome characterized by ePI, dyserythropoietic anemia, and calvarial hyperostosis in four patients originating from two families of Arab-Muslim origin. In family A, the parents were first cousins, and their three male offspring, patients 1991, 1990, and 1989, were affected (Figure 1). The patients were born at term, and their birth weights were normal (2800–3100 g). Soon after birth, they presented with steatorrhea, failure to thrive, and anemia. They had nearly no weight gain over their birth weight until 4–6 months of age, when supplementation with pancreatic enzymes began. Thereafter, the steatorrhea improved, but at 2 years of age their growth parameters were still at the third percentile for 6–12 months; catch-up with their chronological age percentiles occurred only at about 4 years of age. The hemoglobin levels were normal at birth but dropped to 5–9 g/100 ml at one month, and RBC transfusions were thereafter required at 6–8 week intervals. After 16–36 months, patients 1990 and 1991 maintained hemoglobin levels of 8–9 g/100 ml, whereas transfusion frequency increased in patient 1989 concomitantly with the development of massive splenomegaly. The parents noted several episodes of yellowish sclera associated with mild indirect hyperbilirubinemia. Transfusion frequency was not influenced by the administration of pancreatic enzymes. Additional physical findings included distended abdomen with hepatomegaly and progressive splenomegaly (Figure 2), inguinal and umbilical hernia, and generalized muscle hypotonia with delayed psychomotor development at around 2 years of age but near-normal development after 4 years of age. The three brothers each had a large, box-shaped skull with a bony groove between the frontal and occipital fontanelles, scaly skin rash over the perineum and 0.2–0.5 cm hyperpigmented lesions, bronchial asthma, maldentition, and severe dental carries.

The fourth patient (1964), a female, was the 11th child of another, allegedly unrelated, consanguineous family (family B, Figure 1) living in a neighboring village. One of her brothers died at 4 months with severe jaundice and hepatosplenomegaly, and a sister suffered from jaundice and anemia and received monthly transfusions until her death at 9 months. Patient 1964 was born at term, birth weight 3500 g. Since birth she failed to thrive and suffered

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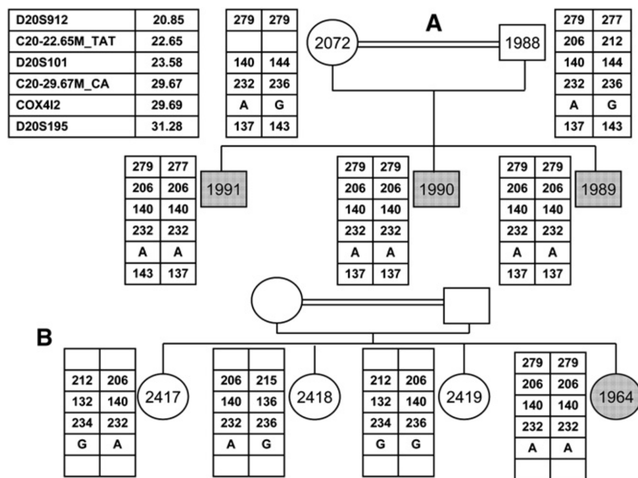


Figure 1. Pedigrees of Families A and B and the Haplotypes along the Critical Region on Chromosome 20

Patients' symbols are filled. Numbered symbols represent individuals whose DNA samples were available for analysis. The polymorphic microsatellite markers and their chromosomal locations (in Mb) are given in the upper left panel. C20-22.65M_TAT stands for hg18_chr20:22654664-22655109. C20-29.67M_CA stands for hg18_ chr20:29675861-29676305.

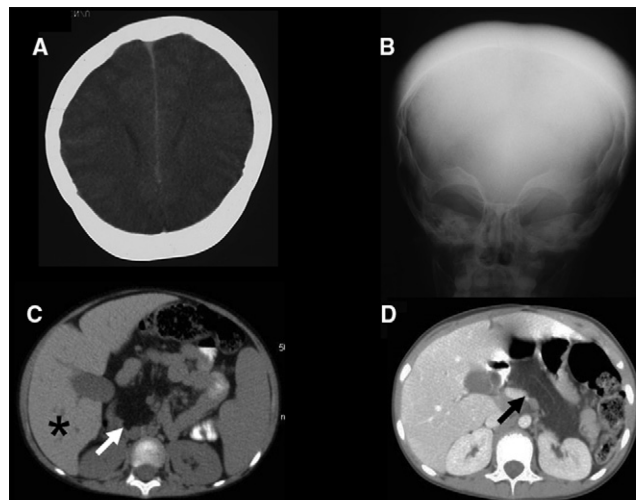


Figure 2. Skull and Abdominal Radiological Findings

(A and B) Patient 1989's thickened calvarium: (A) Brain CT scan, axial view; (B) skull x-ray.

(C and D) Abdominal CT scan of patients 1989 (C) and 3041 (D) disclosing pancreatic atrophy with massive fatty infiltration (arrows) and increased hepatic density (asterisk).

from watery diarrhea, which changed at 2 years to steatorrhea. Her development was delayed: she started walking at 30 months, and when we first saw her, at 3 years old, she had only 10 words. Her weight and height were at the third percentile for 12-month-old children. Supplementation with pancreatic enzymes, along with fat-soluble vitamins, was then added. Her stools normalized, and within four months she gained 2 Kg, added 6 cm to her height, turned more agile, and spoke in short sentences. Her disease course was otherwise similar to that of family A patients. Specifically, at one month her hemoglobin level was 8 g/100 ml, and she required monthly RBC transfusions at a hemoglobin level of 6–7.5 g/100 ml. Nonetheless, she had neither skull hyperostosis nor skin rash.

Blood smears of all four patients showed moderate anisocytosis and poikilocytosis, mild polychromesia and basophilic stippling, and few normoblasts. There was no cellular debris, acanthocytes, target cells, sickling or spherocytes. The neutrophils had normal segmentation, and the lymphocytes were well shaped. Platelets had normal size and color. Ferritin levels were increased, but folic acid, iron, and TIBC were normal, as was hemoglobin electrophoresis. Bone marrow biopsy of patient 1989 at 7 years of age revealed hypercellular marrow with trilineage hematopoiesis. There was erythroid hyperplasia with megaloblastic change and bi- and multi-nucleated red cell precursors but no internuclear chromatin bridges (Figure 3). Iron-laden macrophages were seen, but there were no ring sideroblasts.

Serum transaminases, LDH, and bilirubin were slightly elevated, but gamma-GT, albumin, PT, PTT, cholesterol, triglycerides, HDL, and creatinine were normal. While patients were off pancreatic enzymes, vitamin E was low, and coagulation functions were impaired. Stool elastase was invariably low (20 μg/g of stool, normal 200–500 μg/g of stool). An oral glucose-tolerance test performed in patient 1991 at 6 years of age was normal. Sweat tests were repeatedly normal.

Plasma lactate levels were always normal, whereas plasma alanine was slightly increased (peak level 714 μmol/liter, n $<$ 547 umol/liter). Southern blot analysis of blood mtDNA disclosed a single, 16.5 Kb fragment. Urinary organic acid analysis and biotinidase activity in plasma were normal.

Skull x-rays of family A patients revealed abnormally thickened parietal bones with thinner bones along the sagital suture (Figures 2A and 2B). There were neither signs of ostepetrosis nor narrowing of the formina, and brain CT scan did not disclose signs of pressure over the brain. In a skeletal survey, delayed bone age and osteopenia were seen. The skull x-rays of the parents in family A disclosed normal shape and thickness of the calvarial bones.

In all four patients, abdominal ultrasound revealed enlarged homogeneous liver with normal bile ducts and portal flow, splenomegaly, and a thickened, granular pancreas. The abdominal CT scan disclosed pancreatic atrophy with massive fatty infiltration and increased hepatic density (Figure 2C).

The normal sweat test and the normal size of the mtDNA molecule excluded CF and PS, respectively. Despite the normal neutrophil count, the hypercellular marrow, and the lack of skeletal abnormalities, the diagnosis of SBSD could not be safely excluded at that stage. We therefore proceeded to homozygosity mapping by using Affymetrix Human Mapping 50K Array Xba240 as previously

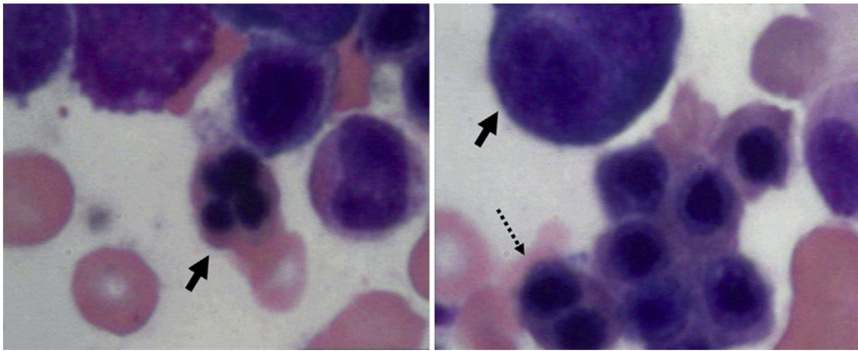


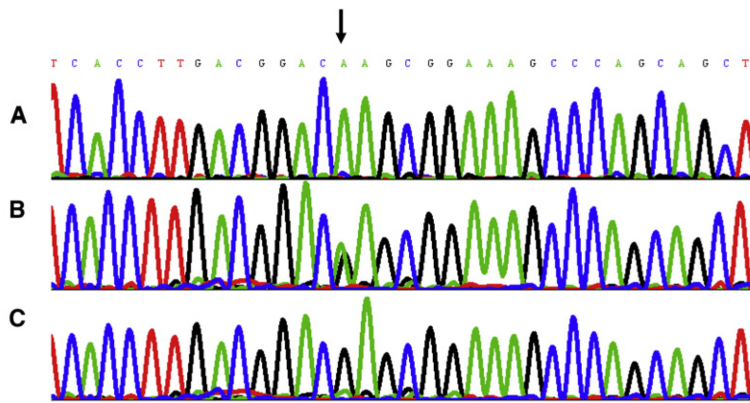
Figure 3. Bone marrow biopsy of patient 1989. Note multi-nucleated red cell precursors (solid arrows) and megaloblastic changes (dashed arrow)

described[6] and analyzed the DNA of the four patients. All experiments involving DNA of the patients, their relatives, healthy controls, and patients' cells were approved by the Hadassah Medical Center Ethical Review Committee. This analysis revealed two homozygous genomic regions, shared by the three brothers, on chromosome 20 at 17.12–33.19 Mb and on chromosome 17 at 22.43–28.94 Mb. Of note, the three brothers did not share any homozygous region on chromosomes 7, where the genes for CF and SBDS reside. Analysis of patient 1964's DNA sample revealed multiple genomic regions of homozygosity but only one region with a haplotype identical to that of the three brothers.

This region included 210 consecutive SNPs (rs956110-rs3746427) spanning the 16 Mb on chromosome 20. We further narrowed it down to 10.45 Mb (20.85–31.3 Mb) by using polymorphic microsatellite markers (Figure 1). The multipoint parametric linkage analysis, performed with SUPERLINK in the two families, gave an LOD score of ~2 in the region located between markers D20S101 and D20S195. No other genomic region gave positive LOD scores by linkage analysis, suggesting that chromosome 20p11.21-q11.21 contained the responsible gene. Within this centromeric region there were 117 open reading frames (ORFs). We have prioritized them according to their tissue expression (GEO profiles), their function (for example, by searching for proteins involved in RNA metabolism, as is the *SBDS* gene[7]), and the phenotypes of mutant mice. Thus, the sequence of the following genes was determined, but no mutation was identified: *FOXA2* (MIM 600288), *POFUT1* (MIM 607491), *ID1* (MIM 600349), *SSTR4* (MIM 182454), *NXT1* ([MIM 605811), *REM1* (MIM 610388)], *XRN2* (MIM 608851), *NKX2-2* (MIM 604612), *PAX1* (MIM 167411), *NXT1* (MIM 605811), and *PLAGL2* (MIM 604866). The sequence of the four coding exons of the cytochrome c oxidase subunit IV isoform 2 gene (*COX4I2* [MIM 607976]), which is also located within the defined region, was then determined because this gene was highly expressed in bone marrow (GEO profiles: GDS1096/212312_at /COX4I2). This resulted in the identification of a c.412G > A mutation, changing glutamic acid at codon 138 to lysine (E138K) (Figure 4). The four patients were homozygous for the mutation, and their unaffected relatives were heterozygotes or normal homozygotes (Figure 1). However, homozygosity for the mutation and the surrounding markers (up until the polymorphic microsatellite marker D20S101) was detected in an 8-year-old girl (individual 3041), the first cousin of the mother of patient 1964. This patient was born at term, and birth weight was 1900 g. At 3 weeks of age she presented with anemia and subsequently required blood transfusions monthly. Bone marrow aspiration at 7 months disclosed dyserythropoietic anemia. At 3 years hepatosplenomegaly became evident, and she underwent splenectomy at 5 years. Thereafter, she no longer required blood transfusions, and her hemoglobin level stabilized at 9–10 g/100 ml. Until she was 2 years of age, she failed to thrive, but her stools were always of normal consistency, teething and shedding occurred on time, and there were no signs of coagulopathy. Since early infancy she suffered from allergic rhinitis and severe asthma and was easily fatigued. At 8 years of age her weight and height were at the tenth and 30th percentile, respectively; she had a box-shaped head with a deep groove along the sagital suture, but her physical examination was otherwise normal. Her speech and comprehension were age appropriate, but she was doing poorly at school. A skull x-ray disclosed hyperostosis of the parietal bones, and an abdominal CT scan revealed pancreatic atrophy with massive fatty infiltration (Figure 2D).

Finally, because the mutation abolished a cleavage site for the restriction enzyme BsrBI, we screened 90 anonymous ethnically matched individuals and 90 anonymous individuals of Ashkenazi-Jewish origin. None of them carried the mutation.

Cytochrome c oxidase (COX), the terminal enzyme in the mitochondrial respiratory chain, catalyzes the reduction of molecular oxygen by reduced cytochrome c.[8] It is embedded in the mitochondrial inner membrane and is composed of 13 subunits, ten of which are encoded by nuclear genes. Only the three mtDNA-encoded subunits (COI-COIII) form the catalytic core of the enzyme; the function of the remaining ten subunits is largely unknown.[9] The COX4 protein is a matrix-side polypeptide, located at the periphery of the complex. COX4 is essential for early stages of the COX complex assembly and becomes rate-limiting when expressed at less than 40% of its normal level.[10] Three of the ten nuclear-encoded polypeptides, subunits IV, VI, and VII, have tissue-specific isoforms, each encoded by a separate gene. *COX4I1* (MIM 123864) encodes 169 amino acids and is



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Human COX4I2	T	L	T	D	**E**	R	K	A	Q	Q	L	Q	R	M	L	D	M
Zebrafish COX4I2	T	L	S	Q	**E**	W	I	E	K	Q	T	Q	R	M	L	D	M
D. Melano. COX4I2	T	F	D	E	**E**	H	Q	K	A	Q	L	Q	R	I	I	D	L
Human COX4I1	S	F	D	K	**E**	W	V	A	K	Q	T	K	R	M	L	D	M

Figure 4. The *COX4I2* Mutation
(A and B) Genomic fragment of the *COX4I2* gene disclosing the c.412 G > A mutation (arrow) in a patient (A) and an obligate heterozygote (B).
(C) normal control.
(D) Conservation throughout evolution of the glutamic acid at codon 138 (bolded). D. Melano. stands for *Drosophila melanogaster*.

ubiquitously expressed, whereas *COX4I2* is mainly expressed in the lung and encodes 171 amino acids. The Glu138 residue of COX4I2 protein and the corresponding residue Glu136 at the COX4I1 protein are highly conserved at the subunit IV/VIIb interface (Figure 4). The study of the pathogenicity of this mutation was hampered by the normal COX activity in the patients' skin fibroblasts (data not shown) and by the lack of a COX4 ortholog in yeast. However, it has been suggested that the transcription of COX4 isoforms is regulated by oxygen concentration; a binding domain for the hypoxia-inducible transcription factor 1 (HIF-1) is found in *COX4I2*, and it was proposed that the expression of *COX4I2*, but not of *COX4I1*, is induced by hypoxia.[11] We have therefore determined the mRNA levels of the two COX4 isoforms by using real-time PCR in the patients' fibroblasts under physiologic and hypoxic conditions induced by incubation with 100 uM cobalt chloride for 48 hr. This analysis revealed that *COX4I2* expression in the patients' cells is reduced to 25% of the control in normoxic conditions (Table 1). When control fibroblasts were incubated with cobalt, *COX4I2* expression increased 25-fold, whereas in patients' cells COX4I2 mRNA level increased by less than 3-fold over the level in normoxic conditions. These findings suggest that the E138K mutation interferes with *COX4I2* expression and perturbs *COX4I2* response to hypoxia.

We next hypothesized that the selective nature of the disease is the result of the difference in the expression ratio of the two COX4 isoforms in various tissues. Given the normal glucose tolerance, a high COX4I1/COX4I2 mRNA ratio is expected in the pancreatic islet cells, and a low ratio is expected in the acinar cells. Because normal acinar cells were not available, we determined the mRNA levels of the COX4 isoforms in whole pancreatic tissue and in pancreatic islet cells. This analysis revealed that in the whole pancreas the COX4I1/COX4I2 mRNA ratio is >60-fold lower than the ratio in the pancreatic islets (Table 1). Because islet cells occupy only 2% of the pancreatic tissue but the acinar cells account for ~80% of the pancreas, the findings indicate that the acinar cells are the major source of COX4I2 mRNA in the pancreas, whereas COX4I1 is the predominant isoform in the islet cells. A defect in *COX4I2* would therefore affect the exocrine but not the endocrine function of the pancreas.

Isolated COX deficiency is manifested by various combinations of brain, myocard, kidney, liver, and skeletal muscle symptoms, and anemia is a relatively common finding.[8] More specifically, mutations in the assembly factor *COX10* (MIM 602125), which participates in the biosynthesis of COX prosthetic group heme A, were identified in patients with multi-system disease, which includes severe transfusion-dependent macrocytic anemia[12], and acquired sideroblastic anemia was reported in patients with heteroplasmic *COI* (MIM 516030) mutations.[13] The hematologic findings in our patients' bone marrow were consistent with dyserythropoietic anemia. Congenital dyserythropoietic anemias (CDAs) are characterized by markedly increased ineffective erythropoiesis and in many cases morphological abnormalities of erythroblasts.[14] In the most common form, CDA2 [MIM

Table 1. COX4 Isoform mRNA Quantification by Real-Time PCR

Sample	COX4I1/bACT	COX4I2/bACT	COX4I1/COX4I2
Control fibroblasts	1909.39	0.36	5303.86
Control fibroblasts in hypoxia	1821.23	8.82	206.49
Patient fibroblasts	1250.43	0.09	13893.67
Patient fibroblasts in hypoxia	1480.83	0.24	6170.12
Control lymphocytes	655.43	0.24	2730.96
Control pancreas	1245.76	119.41	10.43
Control pancreatic islets	738.89	1.08	684.16

Human pancreatic tissue was obtained from a pathologically proven tumor-free edge during subtotal pancreatectomy.

224100), the RBC has a shortened life span, about 10% of cases require regular transfusions during infancy and early childhood, and splenomegaly occurs in 65% of cases. Linkage analysis localized the CDA2 gene to chromosome 20q11.2[15], in close proximity to the *COX4I2* gene. Nonetheless, we did not detect a mutation in the coding sequence of *COX4I2* in a patient of Moroccan-Jewish origin with CDA2.

Although ePI and calvarial hyperostosis have not been reported in association with other COX-deficiency syndromes, our data suggest that both are caused by the *COX4I2* mutation. COX activity is important for the bone-resorbing activity of osteoclasts.[16] Because hypoxia is a major stimulator of osteoclast formation and bone resorption[17] and because calvarial osteoclasts are regulated in a distinctive manner from osteoclasts of long bones, it is tempting to speculate that the underlying mechanism of calvarial hyperostosis in our patients is due to COX4I2's being the predominant COX4 isoform in calvarial osteoclasts.

Heretofore isolated COX deficiency was associated with mutations in the genes encoding the three mtDNA subunits (COI-COIII), a single nuclear-encoded subunit[18], and several COX assembly factors (reviewed in[8,19]). The finding of COX4I2 dysfunction in patients with ePI, dyserythropoeitic anemia, and calvarial hyperostosis extends this list and should also be added to the differential diagnosis of ePI, currently including CF, SBDS, PS, and Johanson-Blizzard syndrome (MIM 243800).[20]

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Web Resources

The URLs for data presented herein are as follows:

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/sites/entrez
GEO profiles, http://www.ncbi.nlm.nih.gov/geo/
SUPERLINK, http://bioinfo.cs.technion.ac.il/superlink-online/

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