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Pilot Study of Mitochondrial Bioenergetics in Subjects with Acute Porphyrias

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Abstract

[1] Background and Aims: The acute porphyrias are characterized by defects in heme synthesis, particularly in the liver. In some affected patients, there occurs a critical deficiency in a regulatory heme pool within hepatocytes that leads to up-regulation of 5-aminolevulinic acid [ALA] synthase-1, which is the first and normally rate-controlling enzyme in the pathway. In earlier work, we described defects in mitochondrial functions in cultured skin fibroblasts from patients with acute intermittent porphyria [AIP]. Others described defects in livers of murine models of AIP. Here, we explored mitochondrial energetics in peripheral blood mononuclear cells [PBMCs] and platelets in persons with AIP and hereditary coproporphyria [HCP]. Our hypotheses were that there are deficits in bioenergetic capacity in acute porphyrias and that subjects with more severe acute porphyria have more pronounced reductions in mitochondrial oxygen consumption rates [OCR].

Methods: We studied 17 subjects with acute hepatic porphyrias, 14 with classical AIP, one with severe AIP due to homozygous deficiency of hydroxymethylbilane synthase [HMBS], 2 with HCP, and 5 non-porphyric controls. We collected peripheral blood, isolated PBMCs, which we assayed either immediately or after frozen storage [−80 C] for up to 14 days. Using Seahorse XF-24-3, we measured OCR in the presence of glucose + pyruvate under basal condition, and after additions of oligomycin, carbonylcyanide p-trifluoromethoxyphenylhydrazone [FCCP], and antimycin +rotenone.

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[1]Abbreviations used in alphabetical order—AHP, acute hepatic porphyrias; AIP, acute intermittent porphyria; ALA 5-aminolevulinic acid; FCCP, -carbonilcyanide p-triflouromethoxyphenylhydrazone; HCP, hereditary coproporphyria; HMBS, hydroxymethybilane synthase; OCR, oxygen consumption rate; PBG, porphobilinogen; PBGD, PBG deaminase;

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Results: Most subjects [13/17, 76%] were female. Subjects with moderate/severe symptoms associated with acute porphyria had significantly lower basal and maximal-OCR than those with no/mild symptoms who were the same as controls. We observed significant inverse correlation between urinary porphobilinogen [PBG] excretion and OCR. The subject with homozygous AIP had a much lower-OCR than his asymptomatic parents.

Summary/Conclusions: Results support the hypothesis that active acute hepatic porphyria is characterized by a deficiency in mitochondrial function that is detectable in PBMCs, suggesting that limitations in electron transport and ATP production exist in such individuals.

Keywords

5-aminolevulinic acid; electron transport; heme; mitochondrial electron transport; oxygen consumption rate; porphobilinogen

Introduction:

1.1. The Porphyrias:

The porphyrias are a group of eight disorders, mainly due to inborn errors of metabolism, in which the primary defects are in normal heme synthesis. The principal sites of heme synthesis in vertebrates are the bone marrow (developing erythrocytes) and the liver (hepatocytes, which have high levels of cytochromes P-450), the principal users of hepatic heme. The human porphyrias generally are classified according to the principal site of overproduction of porphyrins or porphyrin precursors as being either hepatic or erythropoietic. The hepatic porphyrias are further classified as being “acute” or “inducible” or as being “chronic”. The acute designation is used for four disorders, all due to inherited defects in normal heme synthesis [acute intermittent porphyria, AIP; porphyria due to severe deficiency of ALA dehydratase; hereditary coproporphyria, HCP; and variegate porphyria]. In the USA, the most common and severe form is AIP, which is due to deficiency in activity of hydroxymethyl bilane synthase [HMBS, aka PBG deaminase], the third enzyme in the heme synthetic pathway. When AIP or other forms of acute porphyria are biochemically active, there is induction of 5-aminolevulinate [ALA] synthase-1, the first and normally rate-controlling enzyme of the heme biosynthetic pathway in the liver, leading to marked overproduction of ALA, and usually also of porphobilinogen [PBG]. [for reviews, see (1–3)].

1.1.1. Acute Porphyrias: The cardinal clinical features of symptomatic porphyrias are two-fold: (1) acute attacks of abdominal pain or pain in other parts of the body (chest, back, extremities), sometimes with the development of more diffuse neurological abnormalities, with weakness, loss of reflexes, cranial nerve involvement, delirium, and seizures; (2) cutaneous, with development of skin lesions on areas of skin exposed to sunlight.

Clinical manifestations of a porphyric attack are similar for each of the acute porphyrias (4), although usually more severe in AIP. Effects on the nervous system lead to most of the clinical features, including abdominal pain and neuro-visceral and circulatory disturbances. The extent of neurologic damage prior to correct diagnosis and therapy often determines the severity of the episode. Symptoms rarely occur prior to puberty and are more common and

more severe in women, with peak incidence in the third and fourth decades of life. Certain drugs, excess alcohol ingestion, periods of fasting, emotional or physical stressor exhaustion, and the luteal phase of the menstrual cycle, when levels of progesterone peak, may trigger acute episodes.

1.1.2. Pathogenesis of Acute Attacks: The primary metabolic defect in AIP is reduction in activity of hepatic HMBS of about 50% due to various gene mutations that have been identified. Whether residual enzyme activity is sufficient for normal heme synthesis and functions of hemoproteins, such as mitochondrial and microsomal cytochromes, remains unclear. The defect in HMBS predisposes affected subjects to the deleterious influences of factors that may trigger acute attacks, which may occur during and likely due to up-regulation of the hepatic ALA synthase-1 gene. Such up-regulation may occur due to a primary effect on gene transcription (e.g., by action of nuclear factors) and/or due to increased demand for hepatic heme or decreased regulatory heme and thus to derivative up-regulation of ALA synthase-1 levels.

1.1.2.1. Hypothesis of Disordered Mitochondrial Functions: A defect in mitochondrial function as a factor in pathogenesis of the acute porphyrias was first proposed by Bonkovsky etal. Studies in cultured skin fibroblasts from AIP subjects showed ~ 50% decrease in activity of HMBS and a defect in mitochondrial NADH oxidation (5). Recent studies performed in a murine model of AIP have shown that the mitochondrial respiratory chain [RC] and activity of the tricarboxylic acid [TCA] cycle were deficient in *Hmbs* −/− mice also treated with phenobarbital (6, 7). The authors proposed that marked induction of hepatic ALA synthase-1 led to shunting away of succinyl-CoA from the mitochondrial tricarboxylic acid [TCA] cycle into synthesis of ALA [cataplerosis]. There were major decreases in all respiratory complexes and enzymes of the mitochondria in livers, nerves, and muscles of the knockout mice. However, it is unclear to what extent studies in Hmbs −/− mice are a model relevant to human AIP: the mice require homozygous, rather than heterozygous deficiency of HMBS and they require further induction of ALA synthase-1 and cytochromes P-450 [Cyps] with phenobarbital.

1.2 Blood-based bioenergetic profiling:

Blood-based bioenergetic profiling has been successfully adopted as a reporter of systemic bioenergetic capacity in human studies (1–5). For example, Dr. Molina's team has reported that the respiratory capacity of peripheral blood mononuclear cells (PBMCs) is related to key measures of age-related physical function decline (4, 6). More recently, studies of non-human primates conducted in Molina lab provide striking evidence that blood cells are able to recapitulate the bioenergetic capacities of highly metabolically active tissues such as skeletal muscle, cardiac muscle, and brain (7, 8).

1.3. Aims of this Study:

Because of our involvement in the US Porphyrias Consortium, we have been evaluating and following a sizable number of subjects of diverse demographic and clinical features with well-characterized acute hepatic porphyrias. We have had the opportunity to perform studies of oxygen consumption rates [using the Seahorse Instrument] in some of these subjects and

to relate results to biochemical and genetic information that we collected and analyzed on the subjects. The aim of this study was to assess mitochondrial respiration in PBMCs of patients with acute hepatic porphyrias, compared to non-disease controls, and to assess whether defects in oxygen consumption rates correlate with clinical or biochemical features of disease. The main hypothesis that we tested is that subjects with biochemically active and clinically manifest AHP have significant defects in mitochondrial respiration that is detectable in PBMCs.

2. Subjects Studied:

We studied a total of 17 subjects with AHP, 15 with AIP and 2 with HCP. Selected demographic and clinical features are summarized in Table 1. As is typical in AHP, the great majority [13/17] were women; most were European-Americans; however, the two with HCP [unrelated as far as we can ascertain] were African-Americans. They ranged in age from 10 months [a unique, homozygous deficient infant boy, described in greater detail below] to 84 years. Their clinical features were also highly variable, with one man [subject 15], having experienced repeated acute attacks that required monthly hospital admissions for IV heme therapy for many years; another younger woman [subject 3], having similarly experienced near-constant symptoms for several years, with nearly an inability to be able to be out of the hospital and who was cured virtually overnight after orthotopic liver transplantation; to a women [patient 7] with clearly chronically elevated urinary excretions of ALA and PBG, but who has never experienced an acute attack [she was evaluated and diagnosed only because her mother (patient 11) has symptoms that led to the diagnosis].

As summarized in Table 1, they harbor a variety of genetic mutations in the *HMBS* and *CPOX* genes. 6/15 of the subjects with AIP have the common R167Q mutation of *HMBS,* which is often associated with symptoms. However, as shown, subjects with the same mutation show rather wide variabilities in urinary excretions of ALA, PBG, and porphyrins, highlighting the importance of other factors in clinical penetrance of disease.

2.1 Clinical Summary of Infant with Compound Heterozygous, Severe Deficiency of HMBS

Patient # 13 [Table 1]-seen first at Wake Forest Baptist Health in September of 2017, was an 11-month old Caucasian male born to non-consanguineous parents at 37 weeks of gestational age by Cesarean section (breech presentation), birth weight of 3 600 grams with no perinatal complications except for mild jaundice without need for phototherapy. Around 4-6 weeks of age, he was first noted to have developmental delays, shortly after he developed apneic episodes, which worsened over time, occurring in clusters. By three months of age seizure activity was present (grunting, arms shaking with post ictal state, some episodes with associated oxygen desaturation). He had feeding difficulty with thickened and solid foods. His developmental milestones were delayed: he first rolled over at 5 months, babbled at 10 months. He was still unable to sit up without support or to perform pincer grasping at 11 months of age. For seizure control he had been receiving oral phenobarbital and levetiracetam with good reduction in seizure frequency.

On examination his weight and length were in the 56th and 60th percentile, respectively; no dysmorphic features were noted. He had an abnormal neurological exam with slight left lower facial droop, hypotonic throughout, absent reflexes in both lower extremities. Extensive evaluations were performed, including magnetic resonance spectroscopy of brain, which revealed an abnormal lactate peak in the cerebral white matter suggestive of a metabolic/mitochondrial disorder, and an abnormal spectroscopy peak at 2.4 ppm, which may be seen with abnormal glutamine, GABA or succinate metabolites. A comprehensive epilepsy panel (sequencing and deletion/duplication analysis of 87 genes associated with epilepsy) was negative with no pathogenic variant detected. (https://www.genedx.com/wp-content/uploads/crm_docs/info_sheet_epilepsy_comp.pdf)

Combined mitochondrial genome plus a mitochondrial nuclear gene panel (full sequence analysis and deletion testing of the mitochondrial genome plus sequence analysis and exon-level deletion/duplication testing of 319 nuclear genes) demonstrated single heterozygous variants of uncertain significance in autosomal recessive genes (https://www.genedx.com/wp-content/uploads/2014/01/info_sheet_Mito-Genome_Plus_Mito_Nuclear_Gene_Panel.pdf). Magnetic resonance imaging of brain showed enlarged bilateral fronto-parietal subarachnoid spaces (BESSI). Other relevant results included normal serum amino acids; normal chromosomal microarray; abnormal electroencephalogram with focal seizure activity. Whole Exome Sequencing (WES) of DNA from the infant demonstrated compound heterozygosity for the p. R167Q pathogenic variant and the p. T35M likely pathogenic variant in the *HMBS* gene, consistent with compound heterozygous severe deficiency of HMBS. The patient's mother [patient #2, Table 1] had not heretofore been diagnosed to have AIP. However, on detailed questioning, when she was around 20 years of age, she recalled having suffered from intermittent, diffuse abdominal pain episodes that were unexplained despite repeated medical evaluations. These pain episodes ultimately self-resolved. Biochemical porphyria testing of both parents demonstrated mildly increased urinary PBG and total porphyrins in the mother [patient #2, Table 1] and normal urinary analytes in the father [patient 14, Table 1], who denied any symptoms of AIP. Erythrocyte porphobilinogen deaminase activity in the infant was markedly decreased [6 nmol uroporphyrin/mL RBC/h], whereas both parents had decreased activity to approximately half normal [16 and 14 nmol uroporphyrin/mL RBC/h in the mother and father, respectively, ref range =20-50]. As expected, in view of the markedly increased production and urinary secretions of ALA and PBG, mRNA for ALA synthase 1 was increased in exosomes isolated from the serum of the infant [assay kindly performed by A Chen, Alnylam Pharma]. The mother of the infant was shown to carry a well-known disease-causing mutation in the *HMBS* [aka PBGD] gene, namely p.R167Q, which is common in the USA [Table 1]. The father was found to carry a previously undescribed, but likely disease-causing mutation, namely, p.T35M in the *HMBS* gene. Thus, both parents had findings consistent with biochemically inactive acute intermittent porphyria. After the diagnosis of severe deficiency of HMBS had been established for the infant, we recommended that his pediatric neurologist slowly decrease and discontinue phenobarbital, a well-known cause of exacerbations of AIP, as well as to avoid other cytochrome P-450 inducers, such as phenytoin, carbamazepine, or valproate.

In November, 2017, the infant received hemin intravenously via a central venous catheter at a dose of 3 mg/kg of body weight daily for five-consecutive days; the treatment was well tolerated with no side effects. Very significant reductions in ALA, PBG and total urine porphyrins were evident after the hemin course, although, as shown in Table 2, elevations persisted in this child with severe deficiency of HMBS.

2.2 Non-porphyric controls:

We obtained blood samples from 5 subjects without a personal or family history of porphyria, including 3 women, aged 34 to 49, and 2 men, aged 36 and 76 years. The controls consider themselves in generally good health and to be free of any genetic or metabolic disorders.

3. Methods:

We invited subjects with well-documented AHP to take part. The diagnosis had been established prior to the dates of study by the usual clinical, biochemical, and genetic criteria [8, 10]. We explained the procedures and the purposes of the study, answered all questions, and obtained written, informed consent from all adults. [For the infant, we obtained informed consent from both his parents.] We drew 10 mL of peripheral blood into Vacutainer tubes containing heparin as anti-coagulant. We placed the tubes on ice and processed them as described in ref 11. We found that 10 mL of peripheral blood was a sufficient volume for adequate numbers of PBMC and platelets for satisfactory and reproducible measurements of mitochondrial oxygen consumption rates.

3.1. Mitochondrial respirometry:

We isolated and assessed PBMCs as previously described (12). Detailed methods for respirometric analyses of platelets, monocytes, and lymphocytes have been published (11, 13–16). Briefly, we put 10 mL of blood through a Ficoll gradient to separate PBMCs and platelet rich plasma (PRP). We separated PRP and the buffy coat by centrifugation at 500 g for 10 min. We pelleted platelets by centrifugation of the PRP at 1500 g for 10 min and then washed with PBS containing prostaglandin I-2 (1 μg/mL). For some studies, we collected an aliquot of PBMCs for respirometric analysis and storage; and used the remainder for purification of monocytes and lymphocytes. We used CD14+ monocyte isolation kits (Miltenyi Biotech). We isolated lymphocytes by selectively depleting platelets and red blood cells using magnetic beads labeled with anti-CD65 antibody and anti-CD235a antibodies, respectively. We measured oxygen consumption rates (OCR) associated with various respiration states: basal respiration (Basal-OCR), maximal respiration (Max-OCR), spare respiratory capacity (SRC), ATP coupled respiration (ATP-OCR), proton leak (leak), and non-mitochondrial respiration (non-mito), as summarized in Figure 1 (14, 15, 17). We used a Seahorse XF-24-3 (Billerica, MA) to run samples in quadruplicate. We measured Basal-OCR in media containing pyruvate and glucose (A). We then added oligomycinto block ATP synthase. The resultant decrease in oxygen consumption reflects the amount of oxygen consumed for ATP synthesis, ATP-OCR (B). Next, we added FCCP to uncouple completely mitochondrial OCR from electron transport. The resultant increase in oxygen consumption represents Max-OCR. The difference in oxygen consumption rate between Max-OCR and

Basal-OCR represents the spare respiratory capacity [SRC] (C). Finally, we added antimycin-A and rotenone to block electron transport chain activity. Residual respiration is non-mitochondrial. Oxygen consumption not linked to ATP synthesis (leak) is measured by subtracting Non-mito OCR from the rate remaining after oligomycin treatment.

3.2 Measurement of porphyrins and porphyrin precursors:

Urines and blood samples were collected and kept refrigerated and protected from light. Portions were frozen at −85 C until they were shipped on dry ice by overnight air service to the reference laboratory of KE Anderson [UTMB, Galveston, TX]. Urinary 5-aminolevulinic acid (19) urinary and porphobilinogen (20) and total urinary porphyrins (21) were measured as previously described, except that porphyrins were determined by spectrofluorometry. Total plasma porphyrin concentration was measured by spectrofluorometry (22), and the neutral fluorescence emission spectrum of plasma diluted with phosphate buffer at neutral pH was recorded as described by Poh-Fitzpatrick (23, 24). Erythrocyte HMBS [also known as PBG deaminase] activities and protoporphyrin concentrations were measured as described by Granick, Sassa and coworkers (25, 26), with fractionation of metal-free and zinc protoporphyrin by ethanol extraction (27, 28).

4. Results:

A total of 17 subjects with acute porphyrias, aged 11.7 months to 84 years, were included in this study. 13/17 (76%) were female. Various genetic mutations of subjects with acute hepatic porphyria, including p.A331V, p.R167Q [the most frequent, being found in 6/15 subjects with AIP], p.T35M in the HMBS gene and p.H258R and p.R352C in the CPOX gene, amongst other mutations, are listed in Table 1. Urinary ALA, PBG, and total porphyrins are listed for each subject in Table 1 as well. Because AHPs are chiefly clinically and biochemically manifest in young women in their child-bearing years, we considered it of interest to assess whether, in our subjects with AHP, there was any influence of age on urinary excretions of ALA, PBG, or porphyrins. For all porphyric subjects, we failed to observe any correlation between age and urinary excretions of ALA, PBG, or total porphyrins (Figure 2).

Summary bioenergetic profiles of subjects with no/mild and moderate/severe symptoms and of the controls, respectively, are shown in Figure 3A. The basal-OCR and maximal-OCRs were highest in normal controls intermediate in subjects with no/mild symptoms and lowest in those with moderate/severe symptoms [Figs 3A & 3B].

Median values of PBMC basal OCR and FCCP OCR for subjects with none/mild and moderate/severe symptoms are shown in Figures 3B and 3C. The pattern of results for OCR in blood platelets, and in T vs. B cells, vs monocytes was generally similar [results not shown].

Bioenergetic profiles of one individual with very severe, compound heterozygous AIP (subject #13) and his two parents (subjects #2 and #14 - both heretofore unknown heterozygous for AIP) are depicted in Figure 4. The basal-OCR was 28.5 pmol/min for subject #13, 58.3 pmol/min for subject #2 (his mother), and 89.7 pmol/min for subject #14

(his father). The Max-OCR for subject #13 was 64.0 pmol/min, 161.4 for subject #2, and 282.2 for subject #14. Thus, there was markedly low maximal respiration in the severely affected subject #13 when compared to his asymptomatic parents who showed normal mitochondrial OCR [Figure 4].

5. Discussion:

The major findings of this pilot study are as follows:

1. It is possible to assess mitochondrial respiration in PBMC's and platelets derived from 10 ml_ volumes of peripheral blood; 2. Subjects with AHPs with no or mild symptoms and little or no increases in urinary ALA or PBG excretions have more nearly normal mitochondrial rates of oxygen consumption than subjects with AHPs with moderate or severe symptoms and with moderate to marked elevations in ALA and PBG excretions; 3. An infant with severe compound heterozygous deficiency of HMBS and severe clinical features, even at age ~ 1y, has a profound deficiency in mitochondrial OCR. Taken together, these pilot studies support our hypothesis that biochemically active acute porphyria is characterized by a deficiency in mitochondrial function. This was particularly evident in the basal and maximum oxygen consumption rates [after addition of FCCP]. These results suggest that limitations in bioenergetics capacity may exist in those individuals who are symptomatic with acute hepatic porphyria and who likely have partial deficiencies in at least some intracellular heme levels, as reflected in the overproduction of ALA and PBG, which imply up-regulation of hepatic ALA synthase-1. The results support and extend our earlier results obtained in cultured skin fibroblasts [5]. They are also generally consonant with recent findings of others in studies of murine models of AIP [6, 7]. Subjects with few or no symptoms have significantly higher maximum oxygen consumption rates than those with symptoms and biochemically active disease.

Other recent studies from our Center [Molina et al, unpublished] and elsewhere [34] have established the feasibility and accuracy of performing mitochondrial OCR studies with preparations that have been stored frozen. This is helpful from a practical standpoint of workflow and work schedules because, in such clinical studies, it is often not possible to plan ahead for the arrival of samples nor to obtain samples early in the work day.

The reasons and biochemical mechanisms for the decreases in mitochondrial OCR observed in subjects with AHP with moderate to severe clinical and biochemical activity remain to be unveiled. Among many possible hypotheses to account for this key finding are the following: a. There is a deficiency in heme supply, which leads to a deficiency in levels of one or more of the mitochondrial cytochromes or other hemoproteins that are important for the mitochondrial electron transport chain and/or oxidative phosphorylation; b. There is direct or indirect toxicity to mitochondrial structure or function produced by ALA, PBG, and/or porphyrins that are overproduced in AHP subjects with biochemical and clinical activities; c. There is a critical deficiency of endogenous substrates [cataplerosis] to support the mitochondrial electron transport system, due to the excess shunting of succinyl-CoA from the TCA cycle to the formation of excess ALA, PBG, and porphyrins, related to the uncontrolled up-regulation of ALA synthase-1, which is the biochemical hallmark of

biochemically active AHP, regardless of the primary distal defect in the heme synthetic pathway; d. There are deficient numbers of mitochondria in PBMCs, platelets, and other cells and tissues of subjects with active AHP. Other defects also are possible. Consonant with a deficiency in heme supply as being important are recent studies that showed association of heme deficiency particularly in components of the mitochondrial cytochrome oxidase complex [35]. In addition, both iron deficiency and iron excess [36] and biotin deficiency [37] have been reported to adversely affect heme and mitochondrial functions. At this time, we lack sufficient additional data to provide evdence for or against possible defects in these pathways and intermediates in AHP. Additional mechanistic studies are needed.

Strengths of this study include the careful clinical and biochemical phenotyping and the genotyping performed on all the subjects with AHP. We employed state-of-the-art methods and instruments with satisfactory reliability and reproducibility. In addition, for a few subjects, results obtained on samples obtained several months apart on the same subjects yielded closely similar results. [Subject #8 PBMCs were run nearly 1 year apart with similar results and subject #13 PBMCs were run nearly 2 weeks apart, also with similar results compared to the initial sample.]

Our study also has limitations, including limited numbers of study subjects and non-porphyric controls. A particular need is for studies and results in infants and children who heretofore have been studied little or not at all. In addition, we next need to perform additional studies in greater biochemical and molecular depth, in order to gain deeper insights into which complexes and components of mitochondrial electron transport are primarily and mainly affected in AHPs. Such studies can be performed by permeabilizing cells for respirometric assessments. In addition, possible beneficial effects of IV heme and/or glucose therapy and, perhaps, in future of Givosiran, an siRNA that down-regulates hepatic ALA synthase-1, and that shows promise for treatment of AHPs [38], seem worthy of further careful study. Of course, we would especially like to assess mitochondrial functions in hepatocytes, which are the main sites of ALA and PBG overproduction in biochemically active AHPs, but, until more sensitive assays in blood or urine exosomes from liver are perfected, we will need to continue to pursue most such studies in PBMCs or peripheral blood platelets.

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7. References Cited

1. Bonkovsky HL, Guo JT, Hou W, Li T, Narang T, Thapar M. Porphyrin and heme metabolism and the porphyrias. Compr Physiol 2013; 3:365–401. [PubMed: 23720291]
2. Besur S, Hou W, Schmeltzer P, Bonkovsky HL. Clinically important features of porphyrin and heme metabolism and the porphyrias. Metabolites 2014; 4:977–1006. [PubMed: 25372274]

3. Besur S, Schmeltzer P, Bonkovsky HL. Acute Porphyrias. J Emerg Med 2015; 49:305–312. [PubMed: 26159905]
4. Stein JA, Tschudy DP. Acute intermittent porphyria. A clinical and biochemical study of 46 patients. Medicine (Baltimore) 1970; 49:1–16. [PubMed: 4907358]
5. Bonkovsky HL, Tschudy DP, Weinbach EC, Ebert PS, Doherty JM. Porphyrin synthesis and mitochondrial respiration in acute intermittent porphyria: studies using cultured human fibroblasts. J Lab Clin Med 1975; 85:93–102. [PubMed: 1141734]
6. Homedan C, Laafi J, Schmitt C, Gueguen N, Lefebvre T, Karim Z, Desquiret-Dumas V, et al. Acute intermittent porphyria causes hepatic mitochondrial energetic failure in a mouse model. Int J Biochem Cell Biol 2014; 51: 93–101. [PubMed: 24727425]
7. Homedan C, Schmitt C, Laafi J, Gueguen N, Desquiret-Dumas V, Lenglet H, Karim Z, et al. Mitochondrial energetic defects in muscle and brain of a Hmbs−/− mouse model of acute intermittent porphyria. Hum Mol Genet 2015; 24:5015–5023. [PubMed: 26071363]
8. Bonkovsky HL, Maddukuri VC, Yazici C, Anderson KE, Bissell DM, Bloomer JR, Phillips JD, et al. Acute porphyrias in the USA: features of 108 subjects from porphyrias consortium. Am J Med 2014; 127:1233–1241. [PubMed: 25016127]
9. Tyrrell DJ, Bharadwaj MS, Van Horn CG, Marsh AP, Nicklas BJ, Molina AJ. Blood-cell bioenergetics are associated with physical function and inflammation in overweight/obese older adults. Exp Gerontol 2015; 70:84–91. [PubMed: 26226578]
10. Tyrrell DJ, Bharadwaj MS, Jorgenson MJ, Register TC, Molina AJA. Blood cell respirometry is associated with skeletal and cardiac muscle bioenergetics: implications for a minimally invasive biomarker of mitochondrial health. Redox Biol 2016; 10: 65–77 [PubMed: 27693859]
11. Anderson KE, Bloomer JR, Bonkovsky HL, Kushner JP, Pierach CA, Pimstone NR, Desnick RJ. Recommendations for the diagnosis and treatment of the acute porphyrias. Ann Intern Med 2005; 142:439–450. [PubMed: 15767622]
12. Kramer PA, Chacko BK, Ravi S, Johnson MS, Mitchell T, Darley-Usmar VM. Bioenergetics and the oxidative burst: protocols for the isolation and evaluation of human leukocytes and platelets. J Vis Exp 2014; 85: Mar 27. Doi: 10.3791/51301
13. Tyrrell DJ, Bharadwaj MS, Van Horn CG, Kritchevsky SB, Nicklas BJ, Molina AJ. Respirometric profiling of muscle mitochondria and blood cells are associated with differences in gait speed among community-dwelling older adults. J Gerontol A Biol Sci Med Sci 2015; 70:1394–1399. [PubMed: 25030980]
14. Chacko BK, Kramer PA, Ravi S, Johnson MS, Hardy RW, Ballinger SW, Darley-Usmar VM. Methods for defining distinct bioenergetic profiles in platelets, lymphocytes, monocytes, and neutrophils, and the oxidative burstfrom human blood. Lab Invest 2013; 93:690–700. [PubMed: 23528848]
15. Chance B, Williams GR. A method for the localization of sites for oxidative phosphorylation. Nature 1955; 176:250–254. [PubMed: 13244669]
16. Chance B, Williams GR. A simple and rapid assay of oxidative phosphorylation. Nature 1955; 175:1120–1121. [PubMed: 14394122]
17. Kramer PA, Chacko BK, George DJ, Zhi D, Wei CC, Dell'Italia LJ, Melby SJ, et al. Decreased bioenergetic health index in monocytes isolated from the pericardial fluid and blood of post-operative cardiac surgery patients. Biosci Rep 2015; 35 (4); e00237 (Jul 1). [PubMed: 26181371]
18. Brand MD, Nicholls DG. Assessing mitochondrial dysfunction in cells. Biochem J 2011; 435:297–312. [PubMed: 21726199]
19. Mauzerall D, Granick S. The occurrence and determination of δ-aminolevulinic acid and porphobilinogen in urine. J Biol Chem. 1956;219:435–46. [PubMed: 13295297]
20. Buttery JE, Stuart S. Measurement of porphobilinogen in urine by a simple resin method with use of a surrogate standard. Clin Chem. 1991 ;37(12):2133–6. [PubMed: 1764791]
21. Schwartz S, Edmondson P, Stephenson B, Sarkar D, Freyholtz H. Direct spectrofluorophotometric determination of porphyrin in diluted urine. Ann Clin Res. 1976;8:156–61. [PubMed: 1008486]
22. Egger NG, Pow-Sang M, Johnson SA, Motamedi M, Anderson KE, Orihuela E. Accumulation of porphyrins in plasma and tissues of dogs after ∂-aminolevulinic acid administration: implications for photodynamic therapy. Pharmacology. 1996;52:362–70. [PubMed: 8844786]

23. Poh-Fitzpatrick MB. A plasma porphyrin fluorescence marker for variegate porphyria. Arch Dermatol. 1980;116:543–7. [PubMed: 7377785]
24. Poh-Fitzpatrick MB, Lamola AA. Direct spectrofluorometry of diluted erythrocytes and plasma: a rapid diagnostic method in primary and secondary porphyrinemias. J Lab Clin Med. 1976;87(2): 362–70. [PubMed: 1245797]
25. Granick S, Sassa S, Granick JL, Levere RD, Kappas A. Assays for porphyrins, d-aminolevulinic-acid dehydratase, and porphyrinogen synthetase in microliter samples of whole blood: applications to metabolic defects involving the heme pathway. Proc Natl Acad Sci USA. 1972;69:2381–5. [PubMed: 4506758]
26. Sassa S, Granick S, Bickers DR, Bradlow HL, Kappas A. A microassayfor uroporphyrinogen I synthase, one of three abnormal enzyme activities in acute intermittent porphyria, and its application to the study of the genetics of this disease. Proc Natl Acad Sci USA. 1974;71:732–6. [PubMed: 4522787]
27. Sato H, Ido KI, Kimura K. Simultaneous separation and quantification of free and metal-chelated protoporphyrins in blood by three-dimensional HPLC. Clin Chem. 1994;40(7): 1239–44. [PubMed: 8013093]
28. Gou EW, Balwani M, Bissell DM, Bloomer JR, Bonkovsky HL, Desnick RJ, et al. Pitfalls in erythrocyte protoporphyrin measurement for diagnosis and monitoring of protoporphyrias. Clin Chem. 2015;61(12):1453–6. [PubMed: 26482161]
29. Phillips NR, Sprouse ML, Roby RK. Simultaneous quantification of mitochondrial DNA copy number and deletion ratio: a multiplex real-time PCR assay. Sci Rep 2014; 4:3887. [PubMed: 24463429]
30. Barazzoni R, Short KR, Nair KS. Effects of aging on mitochondrial DNA copy number and cytochrome c oxidase gene expression in rat skeletal muscle, liver, and heart. J Biol Chem 2000; 275:3343–3347. [PubMed: 10652323]
31. Mengel-From J, Thinggaard M, Dalgard C, Kyvik KO, Christensen K, Christiansen L. Mitochondrial DNA copy number in peripheral blood cells declines with age and is associated with general health among elderly. Hum Genet 2014; 133:1149–1159. [PubMed: 24902542]
32. Rao M, Li L, Demello C, Guo D, Jaber BL, Pereira BJ, Balakrishnan VS. Mitochondrial DNA injury and mortality in hemodialysis patients. J Am Soc Nephrol 2009; 20:189–196. [PubMed: 18684894]
33. Short KR, Bigelow ML, Kahl J, Singh R, Coenen-Schimke J, Raghavakaimal S, Nair KS. Decline in skeletal muscle mitochondrial function with aging in humans. Proc Natl Acad Sci U S A 2005; 102:5618–5623. [PubMed: 15800038]
34. Karabatsiakis A, Böck C, Salinas-Manrique J, Kolassa S, Calzia E, Dietrich DE, Kolassa I-T. Mitochondrial respiration in peripheral blood mononuclear cells correlates with depressive subsymptoms and severity of major depression. Transl Psychiatry 2014; 4: e397; doi: 10.1038/tp.2014.44.
35. Atamna H, Killilea DW, Killilea AN, Ames BN. Heme deficiency may be a factor in the mitochondrial and neuronal decay of aging. Proc Natl Acad Sci USA 2002; 99: 14,807–12.
36. Walter PB, Knutson MD, Paler-Martinez A, Lee S, Xu Y, Viteri FE, Ames BN. Iron deficiency and iron excess damage mitochondria and mitochondrial DNA in rats. Proc Natl Acad Sci USA 2002; 99: 2264–69. [PubMed: 11854522]
37. Atamna H, Newberry J, Erlitzki R, Schultz CS, Ames BN. Biotin deficiency inhibits heme synthesis and impairs mitochondria in human lung fibroblasts. J Nutr 2007; 137: 25–30. [PubMed: 17182796]
38. Sardh E, Harper P, Balwani M, Stein P, Rees D, Bissell DM, Desnick R, et al. Phase 1 trial of an RNA interference therapy for acute intermittent porphyria. N Engl J Med 2019; 380: 549–558. [PubMed: 30726693]

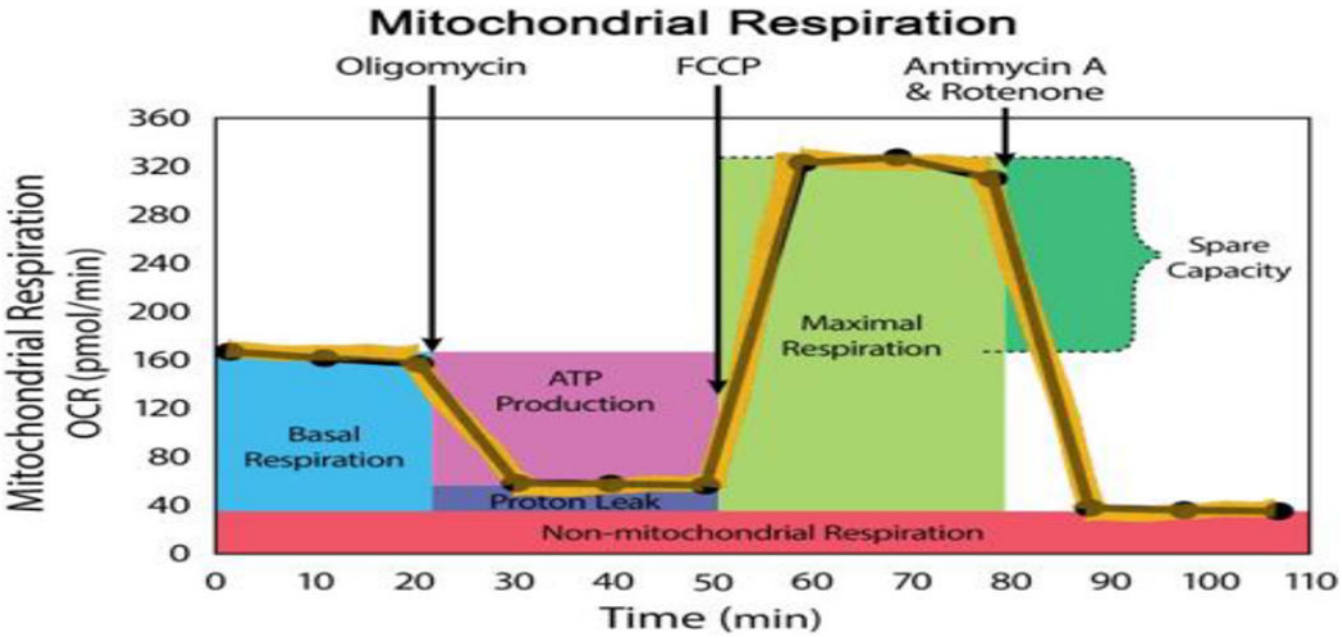
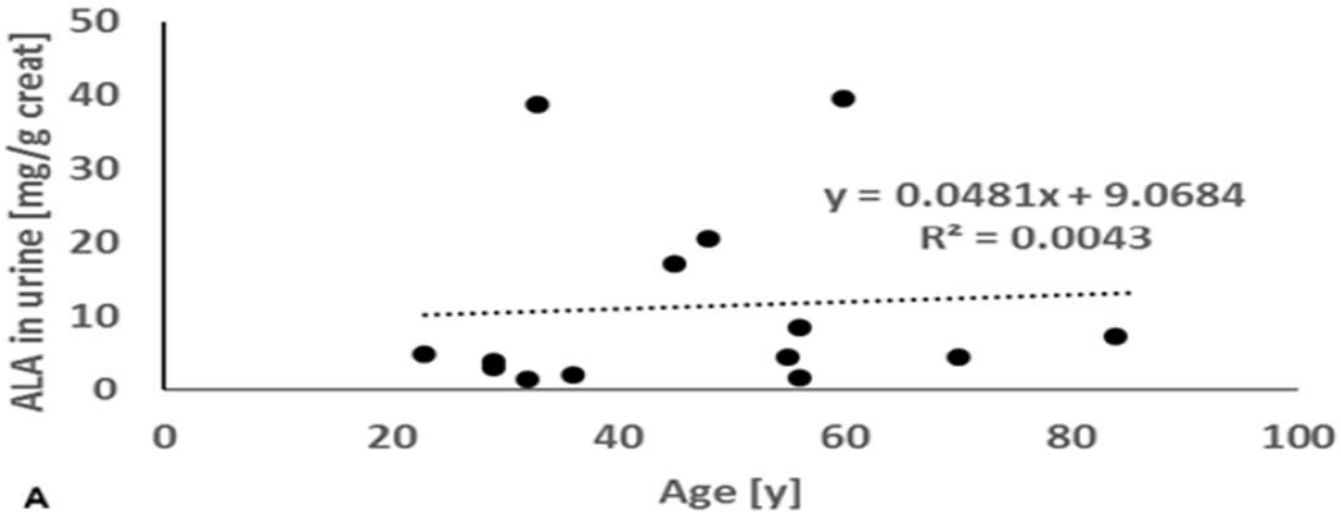
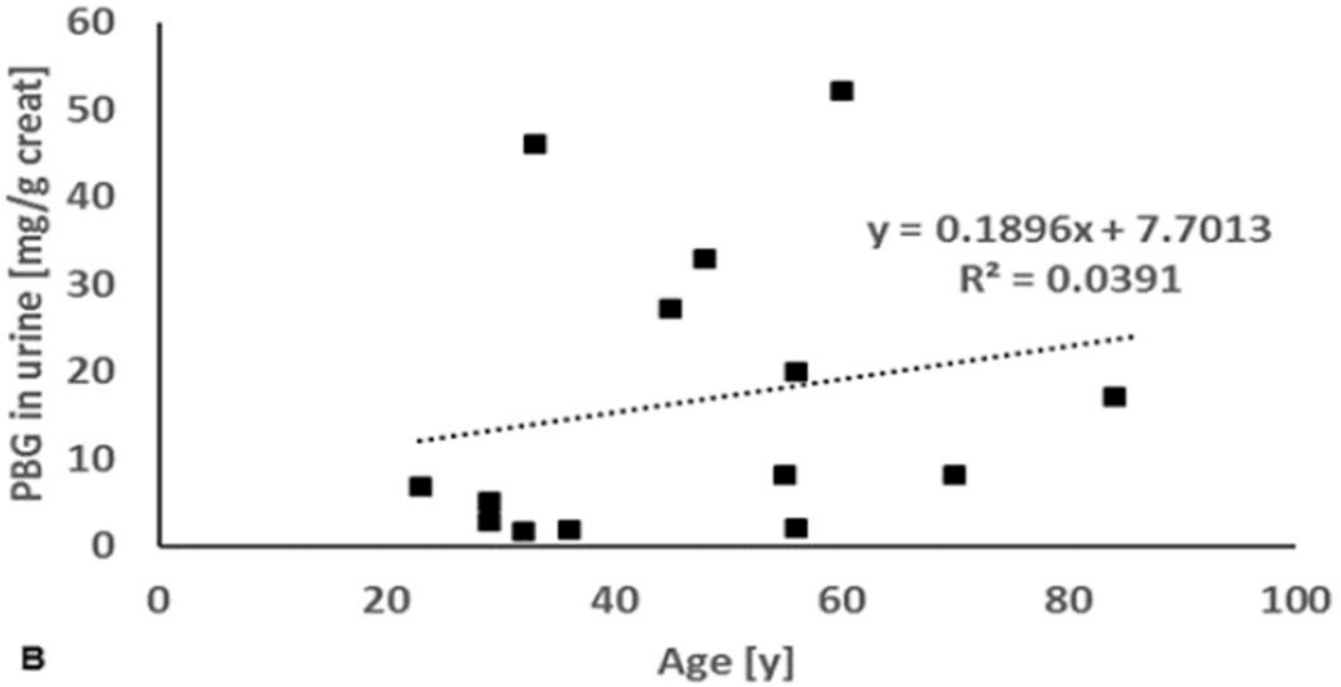


Fig 1.

Idealized tracing of mitochondria OCR analyzed by the Seahorse assay. OCR is measured before and after the addition of various inhibitors as described in text.



A



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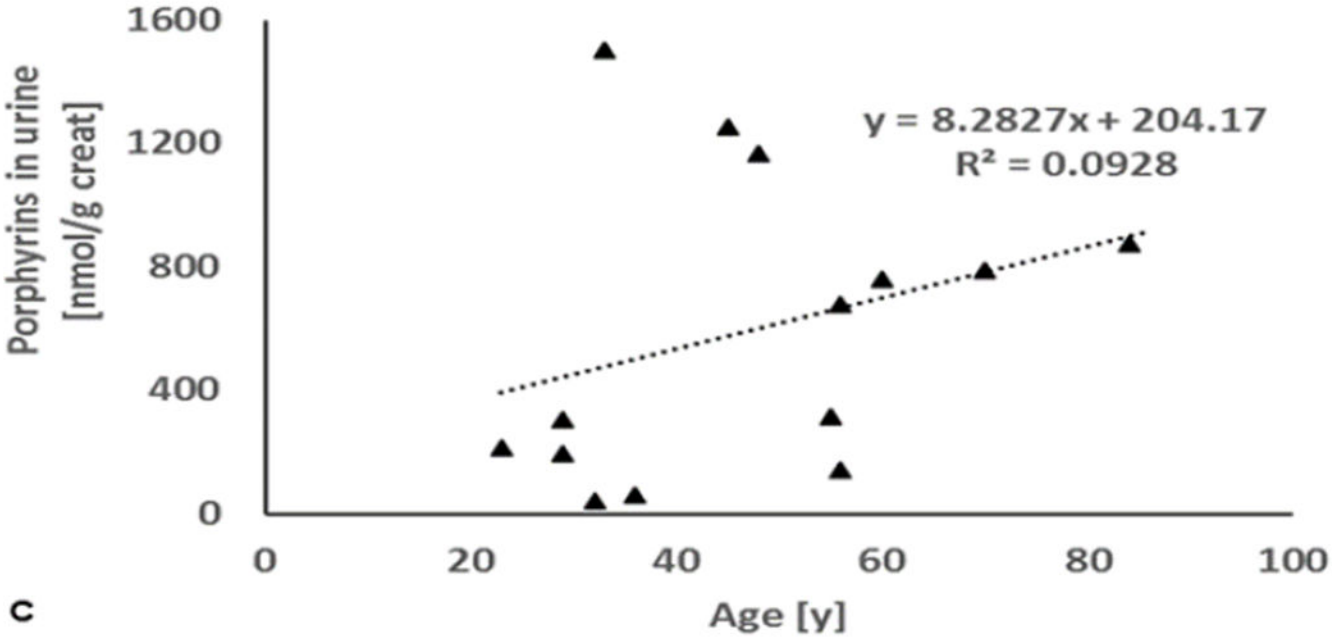
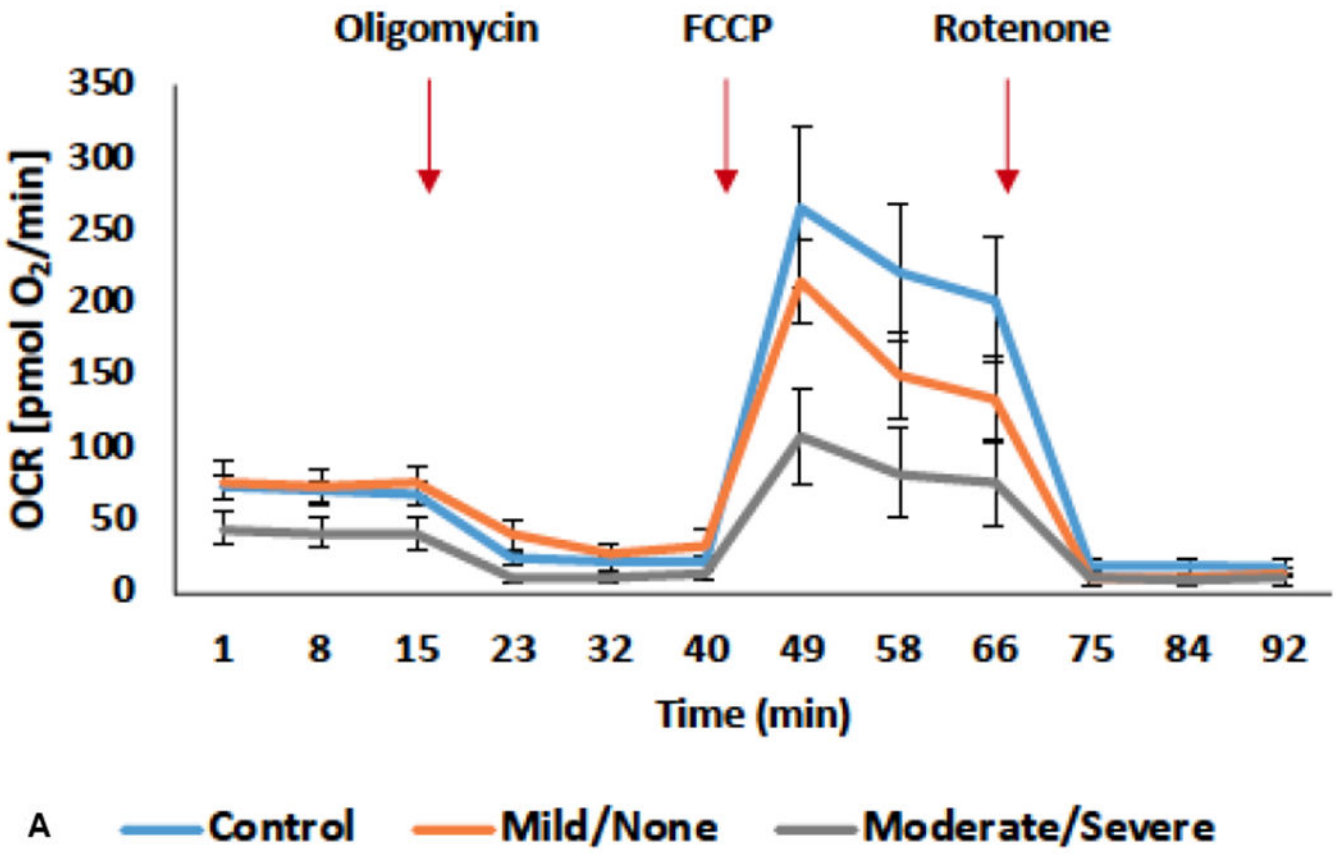
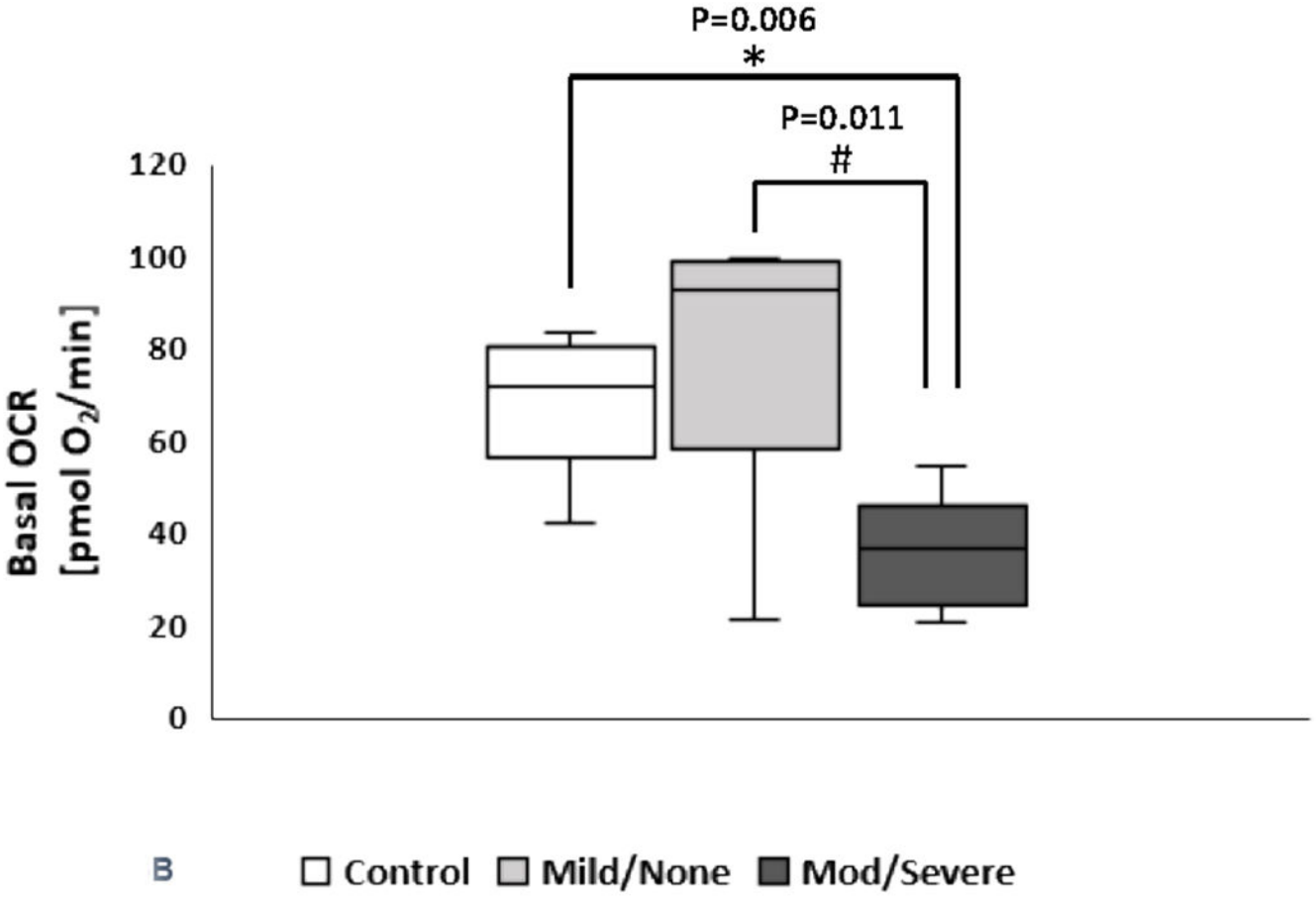


Fig 2.

Lack of effect of age on biochemical activities. Urines from subjects with AHP were collected and levels of ALA (Fig 2A), PBG (Fig 2B), and total porphyrins (Fig 2C) measured as described in Methods. No correlation between age and excretions of porphyrins and porphyrin precursors was observed.





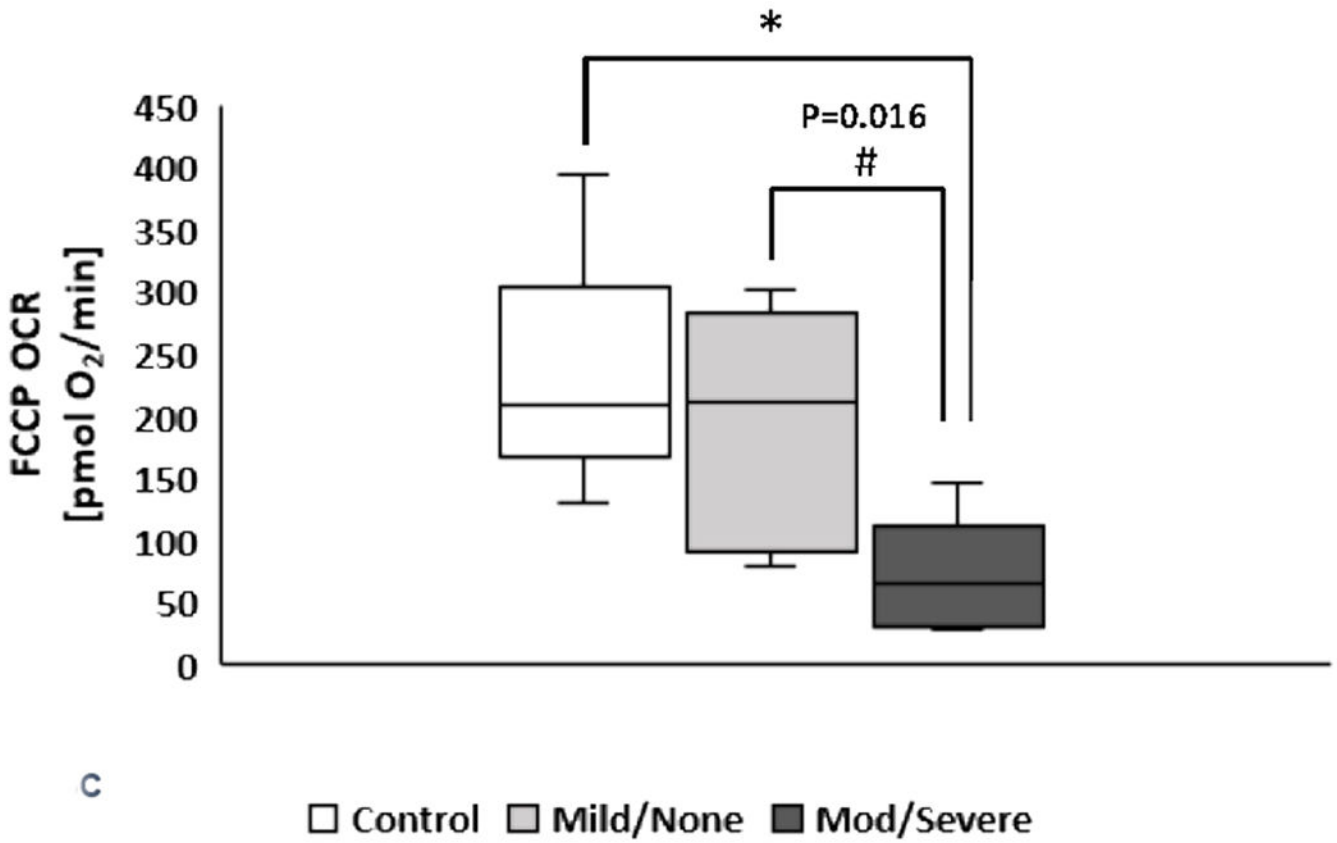


Fig 3.

Summary of the key mitochondrial respirometry results in porphyric subjects and non-porphyriccontrols. (Fig 3A) Bioenergetic profiles from the control, mild/none, and mod/severe groups. Results are presented as mean values [+/− SE] for each cohort. (Fig 3B) Box and whisker plots of basal OCR for all subjects studied Results are presented as mean values [+/− SDs], (Fig 3C) Box and whisker plots of the maximal OCRs among the control, mild/none, and mod/severe groups. No significant differences were detected between the control and mild/none groups. However significant decreases were observed in the mod/severe group (control vs mod/severe P= 0.006, mild/none vs mod/severe P=0.011). Significantly lower FCCP-induced OCR was observed in mod/severe group (control vs mod/severe P=0.011 mild/none vs mod/severe P=0.016). Fisher's exact test was used for statistical comparisons.

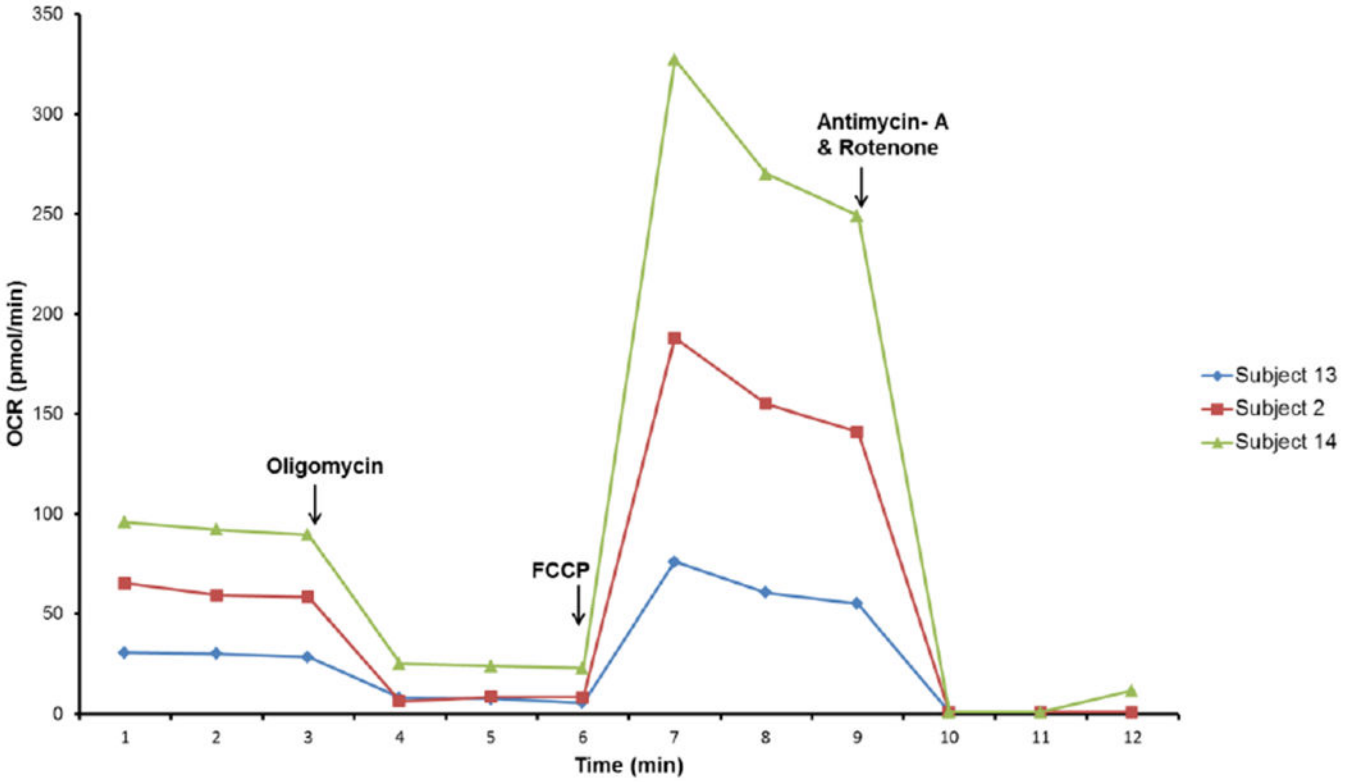


Fig 4.

Mitochondrial OCR in Kindred of Child with Severe Compound Heterozygous Deficiency of HMBS. The child's [Subject 13] basal and FCCP-induced maximal OCRs are both significantly lower compared to his parents' OCRs.

Table 1:

Acute Porphyrias Clinical Information and Demographics

Patient No.	Clinical Severity	Gender	Age [y]	ALA [mg/ g creat]	PBG [mg/ g creat]	Tot. Pns [nmol/ g creat]	HGB g/dl	WBC [# ×10-3/uL]	Genetic Mutation
AIP									
1	Mod/Severe	F	23	5	6.8	213	12.4	10.7	p.A331V(c. 992C>T)
2	Mild/None	F	29	3.9	5.2	306			p.R167Q
3	Mild/None	F	32	1.54	1.63	39	11.1	5.8	p.R173W
4	Mod/Severe	F	33	38.7	46.1	1504	10.9	4.4	p.R225X(c. 673C>T)
5	Mild/None	F	36	2.24	1.91	59	14.5	9.2	p. R167Q
6	Mod/Severe	F	45	17	27.3	1252	12.7	4.8	p. R167Q
7	Mild/None	F	48	20.4	33	1168			[c. 5′−155G>A]*
8	Mild/None	F	55	4.62	8.1	313	13.3	4.7	c.730-731delCT
9	Mild/None	F	56	1.82	2.08	143	15.1	7.2	c. IVS7-1G>A
10	Mod/Severe	F	56	8.59	19.9	680	12	10.3	p.R167Q(c. 500G>A)
11	Mod/Severe	F	70	4.6	8.1	788	14.6	4.8	c. 5′−155G>A
12	Mod/Severe	F	84	11	24.3	580	11.9	7.5	p. R167Q
13	Mod/Severe	M	1	105	289	4269	12.2	10.3	p.R167Q , p.T35M
14	Mild/None	M	29	3.2	2.9	195			p.T35M
15	Mod/Severe	M	60	39.5	52.1	758	13.8	21.9	p. L238P
HCP									
16	Mild/None	F	62	3	2.2	378	12.2	7.1	p. H258R
17	Mod/Severe	M	33	16.3	27.4	1048	11.6	4.9	p. R352C
Reference Range				0-7	0-4	30-300	12-16, F 12-18, M	4.5-11.5	

*Genetic defect in subject 7 is assumed from known defect in her mother, subject 11.

Subject 7 has not agreed to undergo genetic testing, due to expense

Abbreviations: ALA 5-Aminolevulinic acid; PBG, Porphobilinogen; Tot. Pns, Total porphyrins; Hgb, Hemoglobin; WBC, White blood cell count; NA, Not available.

Table 2:

Summary of Porphyrin and Porphyrin Precursor Profiles in an Infant with Severe Compound Heterozygous HMBS Deficiency and his Parents

	Infant		Parents		Ref Ranges
	Baseline	**Post Hemin**	**Mother**	**Father**	
Urine (Spot Urine)					
5-aminolevulinic acid					
mg/g creatinine	105.4	22.0	3.9	3.2	0-7
Porphobilinogen					
mg/g creatinine	289	68.0	5.2	2.9	0-4
Total Porphyrins					
nmol/g creatinine	4,269	2,000	306	195	0-300
Plasma					
Total porphyrins (mcg/dL) *Fluorescence emission peak=619 nm	1.3*				0.0-.9
Serum					
Porphobilinogen (mcg/mL)	0.4				0.0-0.6
Erythrocytes					
HMBS (nmol uroporphyrin/mL RBC/h)	6		16	14	20-50
Protoporphyrin (mcg/dL)	590				20-80
Zinc protoporphyrin (%)	81				<20
Metal-Free protoporphyrin (%)	19				>80