DIFFERENTIATION BETWEEN SPORADIC AND FAMILIAL PORPHYRIA CUTANEA TARDA

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The level of uroporphyrinogen decarboxylase (UROD) activity in erythrocytes has been used to distinguish familial (fPCT) and sporadic (sPCT) porphyria cutanea tarda, as fPCT is characterized by low UROD activity in all cells and sPCT only in liver cells. In this study the diagnostic value of UROD activity was assessed using sequencing of the UROD gene to identify the familiar PCT cases. So far 71 patients biochemically diagnosed with PCT at the Norwegian Porphyria Center, Haukeland University Hospital, Norway have been included prospectively in the study. Nine different mutations of the UROD gene have been identified in 43 patients, of these 38 had decreased UROD activity (<1.2 U/L erythrocytes). In 28 patients no mutation was identified, three of these had decreased UROD activity. Using UROD <1.2 U/L erythrocytes as a cut-off to identify the familial cases gives a positive predictive value of 93% and a negative predictive value of 83%. Two mutations count for 75% of the genetic findings. The prevalence of fPCT in Norwegian PCT patients (61%) is higher than reported in other populations.

WITHIN-SUBJECT BIOLOGICAL VARIATION OF THE URINARY EXCRETION OF ALA, PBG AND PORPHYRINS IN ACUTE INTERMITTENT PORPHYRIA

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Acute intermittent porphyria (AIP) is characterised by attacks of abdominal, neurological and mental symptoms. The diagnosis of an acute AIP attack is based on the assumption that it is accompanied by an increased excretion of urinary porphobilinogen (PBG). However, many AIP patients have a baseline excretion of PBG 5-10 folds the upper reference value even when in remission. To be able to distinguish an increase in u-PBG caused by a porphyria attack from the natural variation of PBG excretion, it is important to know the within-subject variation of porphyrins and porphyrin precursors in urine. Fifteen patients with latent AIP or AIP in remission without symptoms for the past two years, collected morning urine samples once a week for a period of ten weeks. The urine samples were stored in -80° C, and samples from each patient were analysed in duplicates in the same runs for the heme precursors PBG, δ-aminolevulinic acid (ALA), total porphyrins in addition to creatinine. The within-subject variation (CV) of PBG, ALA and porphyrins per creatinine are 18%, 20% and 28% respectively. This means that for an AIP patient with a u-PBG baseline excretion of 40.0 µmol/mmol creatinine, changes to a value between 19.6 - 60.4 PBG µmol/mmol creatinine can be explained by biological and analytical variation with a certainty of 95%.

TIN MESOPORPHYRIN POTENTIATION OF HEME THERAPY: A DOSE-RANGING STUDY IN ASYMPTOMATIC PORPHYRIA

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In acute intermittent porphyria (AIP) hepatic δ-aminolevulinic acid (ALA) synthase is induced and levels of the heme precursors ALA, porphobilinogen (PBG) and porphyrins are increased. Intravenous heme therapy represses hepatic ALA synthase and promptly reduces ALA, PBG and porphyrin excretion. Previous studies suggest that tin mesoporphyrin (SnMP), a potent inhibitor of hepatic heme oxygenase, may potentiate the effects of heme therapy during acute attacks by inhibiting its breakdown. Dose-ranging and pharmacokinetic observations are lacking, and are most feasible in asymptomatic patients. Asymptomatic patients with documented AIP and persistently increased levels of heme precursors, were enrolled in a dose-ranging study of single doses of intravenous heme (heme arginate or heme albumin, 1.0 or 3.0 mg/kg body weight) immediately preceded by SnMP, either 0, 0.5 or 1.0 µmol/kg. Serum PBG, urine ALA, PBG and porphyrins, and plasma heme and SnMP levels were measured up to 72 h. Following results were obtained: (1) Plasma levels of heme and SnMP were dose-dependent and followed first-order kinetics. (2) SnMP 1.0 µmol/kg prolonged the half-life ($t_{1/2}$) and decreased clearance (Cl) of heme given at the 1.0 mg/kg dose ($t_{1/2}$ 10.2±2.2 h vs. 13.8±2.8 h, p=0.035; Cl 202.9±13.4 vs. 135.6±30.4 mL/h, p=0.0014). Effects with heme 3 mg/kg, and effects of SnMP 0.5 µmol/kg with either dose of heme did not reach statistical significance. (3) Decreases in heme precursors induced by heme were strongly dose dependent. (4) An additive effect of both doses of SnMP was evident with heme 3 mg/kg, but not 1 mg/kg, achieving statistical significance on day 3. Change from baseline in plasma PBG 72 h after heme 3 mg/kg was –17.8±14.8, and after heme and SnMP, either 0.5 or 10 µmol/kg was –60.5±32.5 (p=0.05) or 61.9±15.8% (p=0.03), respectively. Corresponding changes in urinary PBG on day 3 were –20.8±46.6 mg/day with heme alone, and –75.3±12.6 (p=0.01) and –88.0±0.6 (p=0.001) mg/day with heme and SnMP 0.5 and 10 µmol/kg, respectively. Mild, dose-related photosensitivity was a common and expected side effect. In conclusion, SnMP follows first-order, dose-dependent kinetics in AIP, prolongs the metabolic clearance of intravenous heme and enhances its biochemical effects. SnMP may be beneficial as adjunctive therapy in AIP, especially for prolonging the effects of heme therapy. [G Drummond and A Kappas, Rockefeller University, New York, NY provided SnMP. Supported in part by grants from US FDA (FD-R-001459), American Porphyria Foundation and NCRR/NIH (MO1 RR-00073).]

MYELOPROLIFERATIVE DISEASE COMPLICATED BY LATE-ONSET ERYTHROPOIETIC PROTOPORPHYRIA AND LIVER DISEASE

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Erythropoietic protoporphyria (EPP) normally presents in childhood with acute photosensitivity. Onset after the age of 40 years is very rare. Most cases have been associated with myelodysplasia and may result from an acquired deletion of the ferrochelatase (*FECH*) gene at chromosome 18q21.3. Liver failure is a rare complication of the classical form of EPP but no type of liver disease has been reported in association with late-onset disease. We report a 66 year old man with longstanding polycythaemia rubra vera who presented with a short history of jaundice, intense burning and tingling of the hands and feet on exposure to sunlight and a haemorrhagic, blistering eruption on his right foot. Histology of this area was consistent with a severe photo-burn; no changes typical of EPP were seen. However, investigation confirmed that diagnosis; erythrocyte total porphyrin was 112 µmol/L (normal < 1.7) with > 95% free protoporphyrin and plasma porphyrin was markedly increased at 1673 nmol/L (normal <11) with a fluorescence emission peak at 635 nm. Serum bilirubin, alkaline phosphatase and aspartate aminotransferase were 188 µmol/L, 433 IU/L and 349 IU/L, respectively. Despite attempts at minimizing enterohepatic recirculation of protoporphyrin with cholestyramine and oral charcoal, his liver disease progressed rapidly. He died with an associated coagulopathy, a bleeding duodenal ulcer and a perforated abdominal viscus. Liver biopsy showed scattered porphyrin deposits, bile stasis without signs of biliary obstruction, feathery degeneration of hepatocytes and expansion of sinusoids by histiocytes, some containing porphyrin-like pigment. There was no fibrosis, cirrhosis or iron overload. A liver biopsy 2 years previously had shown only extramedullary erythropoiesis. The bone marrow was hypercellular with some erythroid dysplasia. The cytoplasm of erythroid cells showed intense red fluorescence indicating the presence of large amounts of porphyrin. Bone marrow cytogenetic analysis revealed an abnormal karyotype with partial deletion of chromosome 18. Fluorescent in situ hybridization and molecular analysis of the *FECH* gene in bone marrow DNA was consistent with predominance of a clone of erythroid cells lacking a *FECH* gene allelic to a low expression ferrochelatase allele.

No *FECH* mutation was detected by sequencing the gene in germ line DNA. Exogenous infusion of protoporphyrin is known to cause acute cholestasis in rats. We believe that this man's cholestatic liver disease was similarly caused by an acute hepatotoxic reaction to protoporphyrin released rapidly from erythroid cells that had developed an acquired ferrochelatase deficiency as his myeloproliferative disease deteriorated. This is the first report of this combination of events.

ACQUIRED PORPHYRIA CUTANEA TARDA PROBABLY RELATED TO ANTINEOPLASTIC THERAPY IN A PATIENT AFFECTED BY CHRONIC MYELOID LEUKAEMIA

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Porphyria cutanea tarda (PCT) is the most frequent cutaneous disorder of porphyrin metabolism and is due to an impaired activity of uroporphyrinogen decarboxylase enzyme. Three major forms of PCT can be distinguished: a sporadic form (acquired, type I) where the erythrocytes enzyme activity is normal but the liver enzyme activity is decreased; an hereditary form (type II) where both erythrocyte and liver enzyme activities are decreased; a familial form (type III) where erythrocyte enzyme activity is normal but the liver enzyme activity is decreased. Hepatitis C virus infection, alcohol and drugs, as estrogens, proved to be some of the most important triggering factors for acquired PCT. We report a case of a 64 years old female patient affected by chronic myeloid leukaemia since 1987. A few months after beginning therapy with antineoplastic agents (hydroxyurea and imatinib esylate) the patient showed skin hyperpigmentation and fragility, hypertrichosis of the temporal regions and blistering of the arms, hands and legs. A clinical suspicion of acquired PCT was made and confirmed by biochemical analysis. Dosage of antineoplastic agents was modified in relation to the course of chronic myeloid leukaemia. We checked porphyrins metabolism during different stages of therapy proving a direct correlation between dosage of antineoplastic drugs and total urine and serum porphyrins values. The authors show the possible porphyrinogenic effect of both the drugs.

MISCARRIAGE DUE TO RECURRENT ACUTE PORPHYRIC ATTACKS DURING PREGNANCY IN A PATIENT WITH ACUTE INTERMITTENT PORPHYRIA

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The porphyrias comprise a heterogeneous group of diseases resulting from an enzymatic dysfunction in heme biosynthesis. Acute intermittent porphyria (AIP; OMIM 176000) is an autosomal dominantly inherited disorder caused by a partial deficiency of the enzyme porphobilinogen deaminase (PBGD). Clinically, AIP is characterized by acute life-threatening neurovisceral attacks, which can lead to paralysis and death. These acute attacks can be precipitated by several triggering factors, e.g. porphyrinogenic drugs and hormones. However, little is known about the effects of pregnancy on the course of acute porphyrias and vice versa. Here, we present a 20-year-old woman previously diagnosed with AIP biochemically and by molecular genetic techniques. Due to recurrent porphyric attacks every three to four weeks frequent hospitalization including treatment in an intensive care unit was necessary. Therapy consisted of hemin arginate infusions, opiates and antiemetics. During one of these crisis, pregnancy was diagnosed. Although she continued to experience acute attacks, the initial course of pregnancy was uneventful and the fetus developed normally until week twenty. Subsequently, however, death of the fetus was diagnosed upon routine ultrasound check-up and curettage was performed in general anesthesia after cervical priming with prostaglandins. To date, only few reports about successful pregnancies in patients with acute porphyrias can be found. Additionally, there is little if any knowledge about the frequency of acute attacks and the appropriate treatment in such patients. Further, the rate of miscarriages in those patients is currently unknown. This case demonstrates that patients suffering from an acute porphyria like AIP confront gynecologists and general practitioners with challenging problems and emphasizes the need for close interdisciplinary collaboration in specialized centers.

FUNCTIONALIZED CALIX[4]PHYRINS: EFFICIENT ACCESS TO UNEXPECTED PORPHOMONO- AND PORPHODIMETHENES

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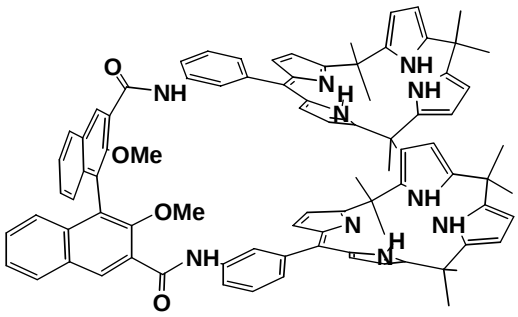

During the last decade, the development of novel specific receptors has been the main interest of supramolecular and analytical chemist. Chemical hybrids of porphyrins and calixpyrroles, called calix[n]phyrins, are oligopyrrolic macrocycles that contain at least one sp^3 hybridized meso-like bridging carbon atom. While porphyrins are well known cation coordinating ligands, the calixpyrroles have recently emerged as simple-to-make anion receptors. It is thus of interest to prepare and study calix[4]phyrins to ascertain whether they would react with cations, anions, or both. Here, we wish to report the unexpected reactivity of electron deficient aryl aldehydes with 5,5-dimethyldipyrromethane under acid catalysis in propionitrile that conveniently affords functionalized porphomethenes at the expense of the expected parent calix[4]phyrins in good yield. As part of our efforts on the development of easy-to-prepare oligopyrrolic macrocycles, we intended to design a new series of easily tunable calixphyrine-type systems. The functionalized calix[4]phyrins open the possibilities for constructing multifunctional receptors. This application will be demonstrated on the preparation of the first chiral calixphyrin dimer. Also, the preparation of water soluble calixphyrins considered for use in photodynamic therapy will be presented. The first electrochemical results will be reported. [Financial support from the Ministry of Education of the Czech Republic Grant No. MSM 223400008, the Grant EU QLRT-2000-02360, Grant Agency of the Czech Republic No. 309/02/1193 and from French government is gratefully acknowledged].

HOMOZYGOUS VARIEGATE PORPHYRIA. FIRST ITALIAN CASE - LONG TERM FOLLOW UP - IDENTIFICATION OF NOVEL MUTATIONS IN PPOX GENE

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Variegate Porphyria (VP) is a very rare autosomal dominant metabolic disorder of heme biosynthesis that results from partial deficiency of Protoporphyrinogen-oxidase enzyme activity caused by mutation in the PPOX gene. Homozygous VP (HVP) is a more than rare variant resulting from a mutation in both alleles of PPOX gene. Dermatological and neurological symptoms are severe and their age of onset is during first months of life. We describe a long term follow up of a HVP patient where severe photosensitivity leads to photomutilations; besides mental retardation aphasia and aggressivity developed in the course of years. Genetically he is a compoud heterozygotes: 1061C>T/397G>A

GENETIC SCREENING OF ACUTE INTERMITTENT PORPHYRIA IN HUNGARY: AN UPDATE

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Acute intermittent porphyria (AIP) has a large allelic heterogeneity of mutations. More than 200 different mutations of the hydroxymethylbilane synthase (HMBS) gene have been identified so far. High percent of mutations are located on exon 10, 12 and 14 (1), therefore we started our work with the screening of these exons. 26 Hungarian patients from unrelated AIP families were investigated. The diagnosis of AIP was based on clinical manifestations and biochemical studies. Mutation screening was performed using temporal temperature gradient electrophoresis (TTGE) of the PCR amplified exons. Automated DNA sequencing verified the presence of the mutations in the samples with altered TTGE profile. In 12 patients eight mutations were identified. Four novel mutations were found in addition to the four previously described (R167W, R173Q, R173W, gta->ata at position 825+1) mutations. One mutation causes a splicing defect (cag->ccg at position 652-2) resulting in the skipping of exon 12. Two deletion mutations (delCGCTGAAA in exon 12 at position 744-751 and delA in exon 14 at position 911) result in a frameshift and there was one nonsense (Q292X) mutation. In conclusion, screening only 3 exons of the HMBS gene we were able to identify mutations in 46% of the Hungarian AIP patients. These data provide efficient mutation screening strategy and support the findings of other authors. Four mutations out of eight were novel. This indicates that the mutations of the Hungarian population might be quite different from the mutations of other population and also adds novel mutations to those that have been previously reported. (1) H.Puy et al: Molecular epidemiology and diagnosis of PBG deaminase gene defects in acute intermittent porphyria. Am. J. Hum. Genet. 60: 1373-1383,1997. [This work was supported by the Hungarian Ministry of Welfare (ETT025/2000).]

THE NORDIC DRUG DATABASE FOR ACUTE PORPHYRIA

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It is well known that certain drugs may precipitate attacks in acute porphyrias. Thus it is essential to identify those drugs which may precipitate the acute crisis and avoid prescribing them, except where no safer alternative exists and the indications outweighs the risks. A new and comprehensive drug database will be presented which is meant as an aid for doctors in the task of selecting the right drugs for these patients. The database takes into account the varying vulnerability to drugs among the gene carriers of acute porphyrias. This database is a collaborative work of the Norwegian and Swedish national porphyria centres. The porphyrinogenicity of the drugs are classified according to the newly revised Swedish drug list for acute porphyria. The Norwegian National Porphyria Centre has developed the database which has several advantages compared to other porphyria drug lists on the Internet.

• The database is not a list, but function as a search engine. It is user friendly since either the trade name or the generic name of the drug may be used.

•. The database takes into account that porphyric patients have a varying sensitivity to the same drug. The vulnerability of the porphyria gene carrier is estimated from age, gender, previous and current disease activity, and current exogenous or endogenous porphyric burden. In the database information about the vulnerability of the patient is combined with the porphyrinogenicity of the drug. A clear counsel about the use of the drug for that particular patient is then given by the database.

• The classification in the database is based on the pharmaceutical ATC system recommended by WHO. The database has the option "Show alternative drugs" which is a helpful tool in the search for a safer drug in the situation when your first drug of choice is classified as a porphyrogenic drug.

• The newly revised Swedish drug list for acute porphyria has been extended to cover all substances included in the Swedish Pharmacopeia 2003. It is now the most comprehensive of the different porphyria drug lists.

• For all drugs that are classified as more or less porphyrinogenic in the database, information is also given about the evidence behind its classification.

EFFECTS OF ISOFLURANE ON HEME SYNTHESIS IN A MOUSE MODEL FOR ERYTHROPOIETIC PORPHYRIA

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Isoflurane is a modern anaesthetic used in general anaesthesia. We have previously demonstrated that this xenobiotic produced in animals significant alterations in heme metabolism and its regulation and also when it was added to a B-lymphocyte cell line established from hepatoerythropoietic porphyria patients. The aim of this work was to investigate the effect of isoflurane in a genetic model for erythropoietic protoporphyria (EPP). The action of isoflurane administration (2 ml/kg, i.p.) to EPP mice (*+/Fech1pas*-, and *Fech1pas/Fech1pas*), was evaluated through the activities of δ-aminolevulinic acid synthetase (ALA-S), porphobilinogenase (PBGase), Heme oxygenase (HO) and P-4502E1; total P-450 (CYP) and GSH levels. When homozygous *Fech1pas/Fech1pas* mice received isoflurane, liver ALA-S activity was 100% induced. No significant changes in PBGase and HO activity were detected. In *Fech1pas/Fech1pas* mice, hepatic GSH was diminished when compared to wild-type animal, but the difference was only significant after isoflurane administration. Total CYP levels were unchanged, although CYP2E1 activity was induced. These results would indicate that administration of isoflurane to this genetic model of PPE would produce an increase in ALA levels as a consequence of ALA-S induction. Because ferrochelatase activity is drastically reduced in *Fech1pas/Fech1pas* mice, the excess of ALA formed could be accumulated leading to neurological consequences. In conclusion, findings here described confirm that isoflurane would be an unsafe anaesthetic for individuals with non acute porphyrias and could be considered as a porphyrinogenic drug because its administration greatly induced ALA-S activity.

PORPHYRINS STATUS IN SPONTANEOUSLY HYPERTENSIVE RATS

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Heme oxygenase (HO) is the rate-limiting enzyme in heme catabolism. HO and carbon monoxide (CO) participate in homeostatic control of cardiovascular functions, including the regulation of blood pressure (BP). Upregulation of HO has been shown to lower BP in young (8 weeks) but not in adult (20 weeks) spontaneously hypertensive rats (SHR), the animal model of human essential hypertension (HT). The aim of this study has been to examine the heme metabolism during the development of HT. Age-matched male SHR of the Okamoto-Aoki strain and normotensive WKY originally derived from Charles River Breeding Farm (Wilmington, Mass) were used. In young SHR rats (8 weeks), the level of δ-aminolevulinic synthetase (ALA-S) was 50% increased while HO was 45% decreased in liver. The blood activities of δ-aminolevulinate dehydratase (ALA-D) and deaminase were not affected, hepatic ALA-D was 30% decreased. In adults SHR rats (20 weeks) the blood and hepatic ALA-D activities were decreased 40% and 30%, respectively. Instead the levels of other enzymatic activities were within normal values. The plasma porphyrin index (PPI) was 200% enhanced in young SHR, but normal in SHR adults rats. The 24-h urinary porphyrin excretion was similar in WKY and SHR rats. However, the pattern gradually changed during the different stages of HT, showing an increase of uroporphyrin that correlated with BP

values. These heme metabolism disturbances occurring during HT might be attributed to the pathogenic mechanisms involved in the development of hypertension.

IN VITRO AND IN VIVO USE OF ALA DERIVATIVES TO OPTIMISE PHOTODYNAMIC THERAPY

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5-Aminolevulinic acid (ALA)-based photodynamic therapy (PDT) has been shown to be clinically beneficial for the treatment of certain tumours, including several skin cancers, however optimal tissue localisation is yet a problem. The use of more lipophilic ALA derivatives instead of ALA, to enhance protoporphyrin IX (PPIX) bioavailability is being examined. The aim of this work, has been therefore to test, using both *in vitro* and *in vivo* systems, the efficiency of different ALA derivatives: Hexyl-ALA (He-ALA), Undecanoyl-ALA and R,S-ALA-2-(Hydroxymethyl) tetrahydropyranyl-ALA (THP-ALA), compared with ALA. These compounds were assayed *in vitro*, employing a cell line derived from a murine mammary tumour and tumour explants, and *in vivo*, after injection of the cells into mice. In cells, PPIX synthesis from He-ALA was more efficient than from ALA. Instead, Undecanoyl-ALA and THP-ALA did not enhance ALA response. Kinetics of porphyrin synthesis from the different derivatives suggests different uptake mechanisms. I.p. injection of ALA derivatives to mice, resulted in a tumoural porphyrin concentration 4-times lower when compared with equimolar amounts of ALA. In tumour explants, porphyrin synthesis from He-ALA and ALA was similar, but it was 3.3-times lower from THP-ALA as compared to ALA. Undecanoyl-ALA produced nearly basal PPIX levels, these results were showing a good correlation between both *in vitro* models. ALA levels measured in the unperfused tumour, after ALA or ALA derivatives injection, did not correlate with porphyrin synthesis. Both *in vitro* and *in vivo* data, are suggesting that capillaries are playing an important role in the cellular uptake of ALA esters.

URINARY STEROID HORMONE METABOLITES IN PATIENTS WITH PORPHYRIAS

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Patients with acute intermittent porphyria (AIP) but not those with porphyria cutanea tarda (PCT) are reported to show predominance in urine of 5β-reduced androgen metabolites compared to the 5α-epimers. Steroids of 5β-androstane and pregnane types induce δ-aminolevulinic acid synthase in vitro, so altered 5-reduction may predispose to acute porphyric attacks. Urinary metabolites of both androgens and cortisol were quantified by gas-liquid chromatography and the urinary porphyrin and porphyrin precursor levels by spectrophotometry in 33 patients with AIP, 26 with PCT and in six with benign prostatic hyperplasia treated with the 5α-reductase inhibitor Finasteride. Ratios of 5β/5α reduced steroids for both androgen and cortisol metabolites showed increase vs. controls in both patient groups but that for androgens was only significant in males. In Finasteride-treated patients, a marked decrease of 5α-reduced metabolites was not accompanied by changes in porphyrin and porphyrin precursor levels. Apparent alterations in 11β-hydroxysteroid dehydrogenase activity were explained by the very diminished excretion of 5α-reduced metabolites. No changes in this activity were seen in acute porphyrics, but PCT patients had lower total cortisol metabolites. 5β-reduction predominates in both AIP and PCT. The 5β/5α ratio is more sensitively indicated by the cortisol than the androgen metabolite ratio.

ASSOCIATION BETWEEN THALASSEMIA MAJOR AND PORPHYRIA CUTANEA TARDA TYPE I

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Porphyria cutanea tarda (PCT) is a metabolic disorder due to an impaired activity of uroporphyrinogen decarboxylase, the fifth enzyme of the heme biosynthetic pathway. Three major forms of PCT can be distinguished: sporadic (acquired; type I); hereditary (type II) and familial (type III). Hepatitis C virus infection, alcohol and drugs, as well as estrogens, are proved to be the most important triggering factors for PCT. The authors describe the first case of association between Thalassemia Major and PCT type I. We report the clinical course of a patient affected by Thalassemia major and PCT. The course was complicated by hepatitis C virus infection, iron overload, anemia and estro-progestinic supplement. The authors discuss the weight that the copresence of the two different pathologies had in the choice of the therapeutic protocol. This is the first case of PCT associated with Thalassemia Major, where the use of low dosages of chloroquine resulted in improvement of clinical and biochemical picture of PCT.

GENETIC ANALYSIS OF VARIEGATE PORPHYRIA IN ITALY: IDENTIFICATION OF EIGHT NOVEL MUTATIONS IN THE PROTOPORPHYRINOGEN OXIDASE GENE

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Variegate Porphyria (VP) is one of the acute hepatic porphyrias, and is clinically characterised by skin lesions and acute neuropsychia-tric/visceral attacks that occur separately or together. The disorder is caused by a partial deficiency of protoporphyrinogen oxidase (PPOX), the penultimate enzyme in the heme biosynthetic pathway, and a number of mutations have been described for the corresponding gene PPOX. We report a genetic analysis of VP in Italy, and the identification of eight novel and three previously characterised mutations from thirteen affected individuals. Among those newly identified, two mutations were small deletions (c.418_419delAA; c.759delA), leading to the formation of premature stop codons, two were splicing defects (IVS10+2T>G; IVS12+1G>C), two were nonsense (c.384G>A, c.1013C>G) and three missense mutations (c.848T>A, 202>A, 694>C). This is the first molecular genetics VP study on patients of Italian origin. Finding eight identified novel mutations in thirteen patients confirms the genetic heterogeneity observed for this disorder.

EXAMINATION OF THE ACTIVE SITE OF HUMAN FERROCHELATASE

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Ferrochelatase catalyzes the terminal step in heme biosynthesis, the insertion of ferrous iron into protoporphyrin to form protoheme IX. In eukaryotes this enzyme is nuclear encoded, synthesized in the cytoplasm in a precursor form and translocated into the mitochondrion (and chloroplasts in plants). The mature enzyme is bound to the matrix side of the inner mitochondrial membrane. In animal cells ferrochelatase is a homodimer with monomer molecular weight of approximately 43,000. It possesses a [2Fe-2S] cluster that is coordinated by four cysteine residues. The crystal structure of human ferrochelatase has previously been determined at 2.0 A and clearly demonstrated the presence of the active site pocket that is present on a hydrophobic face of the molecule that is proposed to face the mitochondrial membrane. The active site pocket contains several highly conserved amino acid residues including R164, Y165, H263, F337, D340, H341, and Q343. Currently two models for catalysis of iron insertion exist. One, based upon the crystal structure of the monomeric, water soluble Bacillus subtilis ferrochelatase, proposes that deprotonation of the porphyrin

macrocycle and iron insertion are catalyzed by the conserved active site histidine (H263 in human). In this model both deprotonation and metal insertion occur from a single side. The second model is based upon the crystal structure of human ferrochelatase, a number of kinetic studies on specific mutants, and nonenzymatic, solution model studies. This second model proposes that deprotonation occurs via the histidine, but that metallation occurs from the opposite side of the active site pocket, possibly involving R164 and Y165. Both models suggest that the metallation reaction involves distortion of the porphyrin macrocycle. In the present work we have examined the crystal structures of three mutants of human ferrochelatase : H263C, H341C, and F337A. The mutant H263C has no enzyme activity while the other two mutants have significantly decreased activity. Mutation of H263 results in the reorientation of R164, H341, Q343 and F337 in the active site. Likewise the mutant H341C possesses the same reorientation of R164, Q343 and F337. No other residue side chain in the molecule appears to change orientation. However, in the mutant F337A the residues of H341, Q343 and R164 retain their wild-type positions. These data are explained by the fact that a hydrogen bond network exists among residues H263, H341 and Q343 which is disrupted by mutation of any of these residues. Normally F337 is restrained from movement by the presence of the side chain of Q343. However, disruption of the hydrogen bond network and the reorientation of Q343 allows F337 to swing into the active site pocket. Since F337A has no effect on the bond network, there is no movement of side chains in this mutation. We propose that the observed reorientation of this select set of residue side chains mimics what occurs during the catalytic cycle of the enzyme. We propose a model where deprotonation of the pyrrole proton of the porphyrin macrocycle via transfer to H263 causes a disruption of the hydrogen bond network with the resultant movement of F337. The phenyl ring of F337 then participates in the distortion of the macrocycle thereby facilitating iron insertion.

VISUALISATION OF PROTOPORPHYRINOGEN OXIDASE MITOCHONDRIAL TARGETING USING GREEN FLUORESCENT PROTEIN

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Variegate porphyria (VP), an autosomal dominant disorder of haeme metabolism, results from defects in the protoporphyrinogen oxidase (PPOX) gene. The disease is characterised by photosensitivity and a propensity to develop acute neurological crises. Protoporphyrinogen oxidase, an inner mitochondrial (mt) membrane protein, does not have a reported mt targeting pre-sequence. This is in contrast the other mt located haeme biosynthetic proteins, in which targeting pre-sequences have been reported. It is therefore, of great interest to establish how PPO is targeted as it is conceivable that a particular PPOX defect(s) may cause VP by disrupting the targeting and translocation of PPO to the mitochondria. The targeting signals of mitochondrial preproteins are typically 17-35aa's in length, and exhibit several common features: i) presequences are rich in positively charged residues (mainly arginines); ii) they generally lack acidic amino acid residues; iii) in most cases they have a high content of hydroxylated residues; and iv) many show the tendency to fold in an amphiphilic α-helix. The amphipathic signals function as matrix targeting signals that can direct import of preproteins to receptors on the mitochondrial surface and subsequently across outer and inner membranes. In addition, some preproteins contain sorting signals that are typically more hydrophobic and direct their specific sorting to intra-mitochondrial compartments.
Based on the (non)ability of clinically occurring mutant PPOs we propose that PPO has a recognisable mitochondrial presequence comprising the first 17aa at the N-terminus. We have further tested this hypothesis by creating different sized fragments of PPO-GFP fusion proteins and assessed mitochondrial targeting and translocation by transfection using a human hepatoma (HepG2) cell line followed by fluorescent microscopic analysis. By studying the mt (non)targeting of a number of VP-causing mutant PPOs we have shown that the N-terminal region is critical for efficient mt targeting. Results using the different sized N-terminus fragments further show that 17aa is the minimal length needed for efficient targeting and translocation of PPO to the mitochondria. The smaller fragments of 12, 14 and 15aa showed no targeting. Amino acid analyses and computer predictions of the 17aa pre-sequence show that it is a positively-charged fragment, and with intervening hydrophobic and neutral areas, has the ability to form an amphipathic α-helix.

GENOTYPE-PHENOTYPE RELATIONSHIPS IN FAMILIES WITH ERYTHROPOIETIC PROTOPORPHYRIA

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Erythropoietic protoporphyria (EPP) is due to an inherited decreased activity of ferrochelatase (FECH). Inactivating mutations in the FECH gene are inherited in an autosomal dominant fashion, but only 8-10% of individuals with such a mutation are symptomatic. In symptomatic EPP individuals FECH activity has been shown to be < 30% of normal, in contrast to 50% in asymptomatic carriers. Recently an IVS3-48T>C transition in intron 3 was found to promote the use of an aberrant splice site and result in a decreased FECH mRNA level. To determine the importance of the splice site modulator IVS3-48C in trans to an inactivating mutation for the phenotype, we examined probands, parents and children of 45 families with EPP. Clinical symptoms were noted, protoporphyrin concentrations were measured in erythrocytes and FECH activity in lymphoblastoid cell lines. Sequencing analysis was used to detect mutations and examine polymorphic loci including the IVS3-48(T/C). In 26 families a previously reported or novel mutation was found, and haplotype analysis was possible. The combination of an inactivating mutation and IVS3-48C in trans was found in all families to be associated with raised erythrocyte protoporphyrin levels and a FECH activity of < 30%. IVS43-48C alone was associated with a slight decrease in FECH activity and IVS3-48T in trans with a mutation was associated with a 50% FECH activity, without symptoms or raised erythrocyte protoporphyrin levels. There was no clear relationship between the inactivating mutation and severity of symptoms or the level of biochemical abnormalities. In conclusion, DNA-analysis to detect inactivating mutations and examination of the IVS3-46(T/C) locus in trans explains the variance in phenotype in almost all families with EPP, and can be used in genetic counselling. Comparison of the IVS3-48(T/C) polymorphism results with erythrocyte PP and FECH activity has more clearly defined the "normal" values for these biochemical parameters. The combination of erythrocyte PP and FECH activity can now be used to predict carrier status and IVS3-48(T/C) status.

GENE TRANSFER INTO HUMAN HEMATOPOIETIC STEM CELLS. APPLICATION TO THE GENE THERAPY OF CONGENITAL ERYTHROPOIETIC PORPHYRIA

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Congenital erythropoietic porphyria (CEP) is an inherited disease due to a deficiency in the human uroporphyrinogen III synthase (UROS), the fourth enzyme of the heme pathway. It is characterized by accumulation of uroporphyrin I in the bone marrow, peripheral blood and other organs. The onset of most cases occurs in infancy and the main symptoms are cutaneous photosensitivity and hemolysis. For severe transfusion-dependent cases, when allogeneic cell transplantation cannot be performed, the autografting of genetically modified primitive/stem cells is the only alternative. In a previous study, the efficient mobilization of peripheral blood primitive CD34+ cells was performed on a young adult CEP patient. Retroviral transduction of this cell population with the therapeutic UROS cDNA resulted in both enzymatic and metabolic correction of CD34+ derived cells. To increase the efficiency of gene transfer in the most primitive cells, we investigated the use of HIV-1-based third generation lentiviral vectors. These SIN optimized vectors contained both cPPT and WPRE sequences. We chose the hEF1alpha promoter for constitutive expression in human hematopoietic cells. Lentiviral transduction of

porphyric cell lines and primary CD34+ cells with the therapeutic human UROS cDNA resulted in both enzymatic and metabolic correction, as demonstrated by the increase in UROS activity and the suppression of porphyrin accumulation in transduced cells. Very high gene transfer efficiency (up to 90%) was achieved in both cell lines and CD34+ cells without any selection. Expression of the transgene remained stable over long-term liquid culture. Furthermore, gene expression was maintained during in vitro erythroid differentiation of CD34+ cells. Therefore, the use of lentiviral vectors is promising for the future treatment of CEP patients by gene therapy. We are currently working on a selection system to overcome the need of a preconditioning regimen.

EUROPEAN PORPHYRIA INITIATIVE (EPI)

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The European Initiative (EPI) is a European network for acute hepatic porphyrias founded in 2000. The initiative has been funded by the INSERM (Institut National de la Santé et de la Recherche Médicale) and AFM (Association Française de Myopathie) as part of a project established to encourage research networks on rare disorders. Porphyrias are uncommon diseases for which diagnosis and treatment varies in individual countries. Clinical research in large patient numbers is complicated as patients are disseminated between many centres worldwide and most individual centres follow few patients. EPI was formed in order to compare experience between countries, attempt to develop a common approach to the management of these diseases and to facilitate international collaborative clinical research and development. The first phase of the project has been to set up a website; www.porphyria-europe.com presenting an up to date approach to the understanding of porphyria, focusing in particular on the prevention and treatment of acute attacks. It has been developed as a major information tool for the scientific community and the lay public.
The next phases of the project will address medical research questions including:

- setting up of a retrospective and prospective clinical survey of the acute attack
- establishing a multi-centre DNA databank from individuals with clinically overt and latent porphyria

TWO NOVEL MOLECULAR DEFECTS IN FERROCHELATASE GENE IN ITALIAN PATIENTS WITH ERYTHROPOIETIC PROTOPORPHYRIA

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Erythropoietic protoporphyria (EPP, MIM 177000) is an autosomal dominant disease with incomplete penetrance, due to reduced activity of ferrochelatase (FECH; EC 4.99.1.1), a mitochondrial enzyme located in the inner mitochondrial membrane that catalyzes the chelation of ferrous iron into protoporphyrin IX, the final step in the heme biosynthetic pathway. The clinical manifestations have a childhood onset, characterized by skin photosensitivity, mild anaemia and, in 5-10% of the cases, by progressive hepatic failure. Diagnosis of EPP can be supported by the presence of fluorescent erythrocytes in peripheral blood smears and increased protoporphyrin levels in erythrocytes, plasma and faeces. The human ferrochelatase gene (FECH) spans 45kb with a total of 11 exons and maps to chromosome 18 at region q21.3. The cDNA of FECH gene has an open reading frame of 1269bp, which encodes a precursor of 423 amino acid residues; the first 54 amino acids are the putative mitochondrial leader sequence. This precursor is processed to a mature protein of 369 amino acid residues. A single promoter directs both housekeeping and erythroid expression, but two polyadenylation site produce two mRNAs of different length. Phenotypic expression of EPP required coinheritance of a null FECH allele and a wild-type low expressed allele carrying IVS3-48 C polymorphism. So far molecular analysis of FECH gene has allowed the identification of more than 70 different mutations responsible for EPP, showing a high genetic heterogeneity. In this study, we applied a two-step screening strategy using denaturing gradient gel electrophoresis (DGGE) followed by direct sequencing, in order to rapidly identify FECH gene mutations in all 11 exons plus intron-exon boundaries in Italian patients affected by EPP. Two unrelated EPP patients and their relatives were investigated. The diagnosis of EPP was based on skin photosensitivity, presence of flurocytes and increased protoporphyrin concentrations in red blood cells and faeces. DGGE analysis of PCR fragments showed abnormal migration patterns compared to control and automated direct nucleotide sequencing identified two new molecular defects in FECH gene: one nonsense mutation (892 C>T) in exon 8 responsible for creation of a stop codon causing a protein truncation at amino acid 298 and a second nonsense mutation (930 G>A) in exon 9 responsible for creation of a stop codon at amino acid 310. First mutation was also identified in three symptomatic relatives. All patients displayed on the other allele the polymorphism IVS3-48C, responsible for allele low expression. Among probands' relatives we identified two asymptomatic carriers, confirming diagnostic power of gene analysis in genetic counselling for EPP.

COINHERITANCE OF A MUTATION IN PPOX GENE AND AN INTRONIC MUTATION IN HMBS GENE CAUSES A SEVERE PORPHYRIA'S PHENOTYPE

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Variegate porphyria (VP; MIM 176200) and Acute intermittent porphyria (AIP; MIM 176000) are autosomal dominant disorders caused by partial deficiency of protoporphyrinogen oxidase (PPOX; EC 1.3.3.4) and hydroxymethylbilane synthase (HMBS; EC 4.3.1.8) respectively, the 7th and the 3rd enzyme in the heme biosynthetic pathway. VP is clinically characterized by skin lesions and acute neurovisceral attacks that occur separately or together, similarly to other forms of chronic and acute porphyrias. Among the diagnostic criteria of VP, one of the most powerful is the detection of a plasma fluorescent peak at 630 nm, present in roughly 70% of the symptomatic VP patients. The clinical features of AIP instead are intermittent attacks of neurological dysfunction, including abdominal pain and neuropsychiatric symptoms. AIP is normally diagnosed on the basis of urinary overproduction of porphyrin precursors δ-aminolevulinic acid (ALA) and porphobilinogen (PBG). Diagnosis can be confirmed by measurement of erythrocyte HMBS activity. PPOX gene is situated on chromosome 1q22-23 and contains one non coding exon and 12 coding exons, that generate a protein of 477 aminoacids. The HMBS gene has been identified in the chromosomal region 11q24.1-11q24.2 and contains 15 exons and two distinct promoters, one active in all tissue and the other only in erythroid cells. So far, more than 100 mutations in PPOX gene and 210 mutations in the HMBS gene have been identified showing a high genetic heterogeneity. In this study, we investigated a patient aged 12 showing a severe porphyria's phenotype characterized by severe skin lesions since childhood, occasional abdominal pain and epilepsy. Using direct sequencing we identified a mutation missense 218 T>C (L73P) in exon 3 of PPOX gene and a deletion of 8 bp in intron 6 of HMBS gene. The proband's father carries PPOX mutation while mother carriers HMBS deletion. Parents are asymptomatic but the mother refers occasional mild abdominal pain. This study shows that the coinheritance of a single mutation in two different genes of the biosynthetic heme pathway can cause a severe porphyria characterized by early onset.

THE THIRD CASE OF DOSS PORPHYRIA IN GERMANY

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Delta-aminolevulinic acid (ALA) dehydratase (ALAD) deficiency porphyria (ADP), or Doss porphyria, has first been reported in Germany in 1979 (Klin. Wochenschr. 57: 1123; 1979). Two unrelated male adolescents suffered from severe, mainly neuropathic acute porphyria syndrome by nearly total ALAD deficiency. About seven cases of ADP have been reported to date, but only four cases have been confirmed by determination of the underlying ALAD mutation (Semin. Liver Dis. 18: 75; 1998). The molecular genetic studies confirmed that ADP is an autosomal recessive porphyria resulting from compound heterozygous mutations of the ALAD gene. A 17-year-old male suffered from colical abdominal pain and symptoms of severe polyneuropathy for two years. Due to an excessive porphyrinuria, porphyria was suspected and the patient was referred to our consultation. Urinary ALA was increased 33-fold, and coproporphyrin-III 79-fold. Porphobilinogen and uroporphyrin were only slightly increased. Fecal porphyrins were within the normal range. In erythrocytes zinc protoporphyrin was elevated 5-fold. ALAD activity in erythrocytes was decreased to 8% of normal controls and could not be activated by Zn and DTT, suggesting an ALAD protein deficiency. Blood lead levels were not elevated. ALAD activity was about 50% in both parents, whereas the patient's brother had normal activity. Urinary ALA and porphyrin excretion were within the normal range in both parents and the brother. Molecular genetic studies of the ALAD gene revealed two base changes in the family: ^{11}C to A in intron 3 in the mother and ^{11}C to T intron 3 in the father. No mutations were detected in his brother. Only the patient carried both mutations. These findings suggest that the observed compound heterozygosity of the ALAD gene may be responsible for Doss porphyria in the patient. The patient was treated several times by heme arginate. The clinical condition improved, and the excretion of ALA and coproporphyrin fell about 50% compared with the acute level. The heme therapy was continued intermittently for one year. Urinary parameters returned significantly to subclinical levels. The patient is currently in a good clinical condition. He is free of pain, and the polyneuropathy returned nearly to normal. [This study was supported by the German Research Association (GR 1363/2-3)].

RESIDUAL ACTIVITY OF HUMAN PORPHOBILINOGEN DEAMINASE WITH R167Q OR R167W MUTATIONS: AN EXPLANATION FOR SURVIVAL OF HOMOZYGOUS AND COMPOUND HETEROZYGOUS ACUTE INTERMITTENT PORPHYRICS

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To find an explanation for survival of homozygous or compound heterozygous variants of acute intermittent porphyria, we studied the three mutant forms of porphobilinogen deaminase (PBG-d) described in the four reported patients with homozygous acute intermittent porphyria. Wild-type human PBG-d and the PBG-d R167W, R167Q and R173Q mutants were expressed in Escherichia coli and the recombinant mutant human enzyme were examined for enzyme activity. Specific antibodies against human PBG-d detected the three human PBG-d mutants. All three had less than 2% of wild-type enzyme activity when examined under customary assay conditions (pH 8.0), but the R167W and R167Q mutants were found to have about 25% of normal activity when assayed at pH 7.0. This residual activity at a more physiological pH provides an explanation for survival when these mutations are inherited in a homozygous or compound heterozygous fashion.

A HIGHLY SENSITIVE AND SPECIFIC METHOD FOR THE MEASUREMENT OF THE I AND III ISOMERS OF PORPHYRINS IN PLASMA

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A highly sensitive and specific method for the measurement of the I and III isomers of porphyrins in plasma by a one-step liquid-liquid extraction followed by HPLC separation with fluorometric detection has been developed and validated. The analytical methodology has been validated in terms of specificity, extraction recovery, linearity, limit of quantitation, stability, between-run and within-run imprecision. Stock solutions of the I and III isomeres of uroporphyrin, hepta-carboxylporhyrin, hexacarboxylpor-phyrin, pentacarboxylporphyrin and coproporphyrin (Frontier Scientific Inc. Logan Utha, USA) were prepared by dissolving each compound in 3 mol/L HCl. To prepare calibrators the I and III isomere porphyrins were spiked in donor plasma from the local blood bank in concentrations from 1 to 1000 nmol/L. Quality controls of porphyrins were prepared by spiking the porphyrin acids chromatographic markers containing the I isomers of porphyrins (Frontier Scientific Inc. Logan Utha, USA) in plasma from the local blood bank in concentration of 10, 50 and 100 nmol/L. To 500 µL sample (calibrator, control or patient plasma) 250 µL dimethylsulfoxide and 250 µL 20% trichloroacetic acid were added in a glass tube. The mixture was vortexed for 1 min and then centrifuged at 3000 x g for 10 min. The clear supernatant was transferred to a brown vial. The Agilent 1100 series HPLC system (Agilent Technologies, Palo Alto, Ca, USA) consisted of a binary pump, a degasser, an autosampler operated at 5 °C, a column oven and a fluorescence detector connected to the Agilent ChemStation Software. Chromatographic separation of porphyrins was achieved by using a reversedphase LiChroCART® RP-18 (7µm) (250 x 4.0 mm) column connected to a LiChrospher® 100 RP-18 (5 µm) guard column (E. Merck, Darmstadt, Germany) operated at 30 °C. The mobile phase consisted of A: 1 mol/L ammonium acetate pH 5.16 and acetonitrile 90:10 (v/v) and B: methanol and acetonitrile 90:10 (v/v). Gradient elutions were performed using a flow rate of 1 mL/min. The initial concentrations were 90% A and 10 % B. During the following 15 min the concentrations were linearly changed to 1 % A and 99 % B. Porphyrins were detected with excitation and emission wavelengths set at 405 and 619 nm, respectively. The sample volume injected was 100 µL. Under these conditions complete separation was obtained for the I and III isomers of uroporphyrins, heptacarboxylporhyrins, hexacarboxylporphyrins, pentacarboxylporphyrins and coproporphyrins. Retention time and fluorescence emission spectra were used for the identification of each porphyrin isomer. The extraction recovery was 90-95%. The assay was linear from 0.5 - 500 nmol/L and the detection limit was < 0.2 nmol/L. Extracted porphyrins were stable for at least one week at 2-8°C. Within run and between run imprecision was < 4 % CV. In conclusion, the method developed and validated in this study is a sensitive, specific, fast and cheap method to measure the I and III isomers of porphyrins in plasma.

5-AMINOLEVULINATE SYNTHASE: MODULATION OF THE COFACTOR CHEMISTRY BY THE PROTEIN SCAFFOLD

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5-Aminolevulinate synthase, a pyridoxal 5'-phosphate-dependent enzyme of the α-oxoamine synthase family, catalyzes the first step of the heme biosynthetic pathway in mammalian cells. This reaction entails the condensation of glycine with succinyl-coenzyme A to yield 5-aminolevulinate, carbon dioxide and CoA. In the past few years, rapid scanning-stopped-flow spectroscopy and chemical quenched-flow studies of the ALAS reaction, under single- and multi-turnover conditions, have provided important results for the interpretation of the catalytic mechanism. In particular, the role of the protein scaffold in modulating the chemical reactivity of the pyridoxal 5'-phosphate cofactor and, thus, the catalytic pathway of ALAS has been investigated in our laboratory using a combination of circular permutation, transient kinetics and global analysis of the kinetic data.

NOVEL MUTATION IN PORPHOBILINOGEN DEAMINASE GENE IN A FAMILY WITH ACUTE INTERMITTENT PORPHYRIA FROM NEPAL

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Acute intermittent porphyria (AIP; OMIM 176000) is a low penetrant autosomal dominant disorder caused by reduced activity of porphobilinogen deaminase (PBGD; EC 4.3.1.8), the third enzyme of heme synthetic pathway. The human PBGD gene has been cloned and his organization characterized. Gene is localized in 11q23.3 region. It is split into 15 exons spread over 10 kb. AIP is the most frequent acute hepatic porphyria. The disease is characterized by intermittent acute porphyric attacks with abdominal pain, hypertension, tachycardia, neurologic and psychiatric manifestation. In difference to other hepatic porphyrias, skin photosensitivity is not present. Based on internet search, we were contacted by 50 year-old proband suffering from typical abdominal form of porphyria. He, not being a health professional, diagnosed himself as having acute porphyria attack, asked for heme-arginate (Normosang, Orphan Europe) treatment with excellent clinical effect, and arranged sending blood or genomic DNA from 15 members of his family to our laboratory. We, therefore, analyzed DNA from extensive family from Nepal. All 15 exons of PBGD gene with surrounding exon/intron boundaries were amplified by PCR. Consequently fragments of amplified DNA were analyzed by denaturing gradient gel electrophoresis. The abnormal pattern of the exon 15 was found. Subsequent sequencing analysis showed the insertion of one extra G in position 9205 on gDNA, resulting in shift of reading frame. In protein sequence, four amino acid after mutation are different and next the protein is prematurely truncated due to stop codon. The amino acid sequence of PBGD corresponding to proband was used to construct a homology model with *E. coli* PBGD as template. We also constructed a homology model with wild-type human PBGD. The resulting models were refined using CNS program to remove bad contacts and inspected using the program O. In the wild-type protein, the C-terminal helix protects the beta-strands from being exposed to solvent. In mutant protein, termination leads to the loss of this helix. This would be expected to expose the beta-strand core that could make the mutant protein prone to aggregation. [Supported by grant from MSMT - LN00A079]

INVENTORY OF MUTATIONS CAUSING PORPHYRIA IN SWEDEN

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The Swedish Porphyria Centre is a centralized national laboratory for porphyria diagnosis. Since several decades a genealogical data bank of porphyria kindreds has been organized at the centre. Porphyria diagnosis, principally based on clinical features and specific biochemical pattern, has during the last decade been reinforced by mutational analyses. The genetic picture has been shown to be heterogenetic. With the exception of one AIP mutation with "founder effect", each mutation affects only one or a few families. Many of the mutations have not been reported before and may thus be classified as novel. Mutational analyses have been of great use in early detection of silent carriers in families affected by acute porphyrias and has made possible early counselling with regard to life style and precipitating agents. In Sweden the dominating form of porphyria is AIP (10:100 000 inhabitants). So far we have found 38 different mutations in the HMBS gene. The prevalence of HCP and VP in Sweden is 0.5:100 000 and 1:100 000, respectively. Disease producing mutations affecting the CPO and PPOX genes have been identified in 5 and 13 families, respectively. In 9 EPP families six different mutations have been identified in the FECH gene. The splice site modulator IVS3-48C was found in trans to the mutated FECH allele in all cases of clinically overt EPP. The prevalence of EPP in Sweden is 0.5:100 000. The mutations underlying two cases of recessive porphyrias, ALAD-P and CEP, have been identified. In the case of CEP the identification of the mutation has been used in prenatal diagnosis of siblings to the proband. The mutations affecting the UROD gene have not yet been studied with exception of a compound heterozygous patient (HEP) in which case one of the mutations has been identified.

PORPHYRIA CUTANEA TARDA AND HAEMOCHROMATOSIS

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Anamnesis. The 67-year-old male patient was admitted to our Department with an acute erysipelas on his left leg. In his case history, diabetes mellitus, hypotension, AV-block I grade occurred. He had taken iron products for a long time, and half a year ago haemochromatosis developed, followed by hepatic fibrosis and portal hypotension.
Clinical symptoms. Periorbital hyperpigmentation, hypertrichosis, darkened skin suggested possible association of porphyria cutanea tarda (PCT).
Laboratory examinations revealed several abnormal values: glucose was: 12.9 mmol/L, liver function tests were moderately enhanced (total bilirubin: 9.2 μmol/L, GOT: 36.0 U/L, GPT: 67.0 U/L, γ-GT: 67 U/L, alkaline phosphatase: 197 U/L). Iron metabolism tests were markedly abnormal (serum iron: 41.8 μmol/L, TIBC: 43.0 μmol/L, ferritin: 1583.5 ng/ml, transferrin: 1.9 g/L). Homozygous mutation (C282Y) in the HFE gene was detected. Owing to the erysipelas, leucocytosis and accelerated sedimentation was seen. Urinary total porphyrin level was rather high: 7999 μg/day, with dominance of uro and heptacarboxyl porphyrins (56.6%, 29.1%, respectively). Levels of urinary δ-aminolevulinic acid, fecal porphyrin and plasma porphyrin were also increased. Catalytic activity of the erythrocyte uroporphyrinogen decarboxylase was 47% of control supporting the existence of type II (familial) PCT.
Therapy. Repeated phlebotomy (500 ml blood/every 2nd week) was applied; relative good response to therapy was observed.
Discussion. Although frequency of HFE gene mutations is known to be higher in PCT patients than that in normal population, however, association of type II PCT and heteditary haemochromatosis caused by a homozygous mutation in HFE gene at the position 282 is rare; this is the first case in Hungary.
[This work was supported by the Hungarian Ministry of Health (ETT 390/2003)].

SUCCESSFUL AND SAFE TREATMENT OF HYPERTRICHOSIS BY HIGH-INTENSITY PULSES OF NON-COHERENT LIGHT IN A PATIENT WITH HEPATOERYTHROPOIETIC PORPHYRIA

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Hypertrichosis is a common feature in cutaneous porphyrias which are characterized by the accumulation of excessive amounts of porphyrins. In porphyric patients, selective photothermolysis of hair follicles achieved by means of intense pulsed light may excite porphyrins and produce photocutaneous damage. Our aim was to design a safe protocol of photothermolysis to apply to a patient with hepatoerythropoietic porphyria. A non-coherent light from 755 nm to 1200 nm and an energy fluence of 42 J/cm^2 were applied on an albino porphyric mouse model and histological analysis revealed no skin lesions related to excitation of porphyrins. The same protocol was applied on a patient with hepatoerythropoietic porphyria. Hypertrichosis was almost completly removed after seven sessions without development of macroscopic skin lesions. Photothermolysis by means of intense pulsed light system was

found to be safe, and could be useful in clinical practice for the treatment of hypertrichosis in patients with cutaneous porphyrias.

EXTENSIVE ANALYSIS OF A FAMILY WITH CONGENITAL ERYTHROPOIETIC PORPHYRIA ABSENCE OF CLINICAL MANIFESTATIONS IN AN HOMOZYGOUS MUTANT SIBLING

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Congenital Erythropoietic Porphyria (CEP) is usually a severe form of erythropoietic porphyria, transmitted as an autosomal recessive trait and characterized by a profound deficiency in uroporphyrinogen III synthase (UROS). At least 40 different mutations have been described in the UROS gene, with one predominant missense mutation (C73R) associated with a severe phenotype. This report describes a large kindred from a Palestinian family, composed of 13 brothers and sisters born from healthy consanguineous parents. Seven members of the family were analysed, four presented with typical dermatological lesions and two were unaffected. In the four affected patients, typical profiles were observed : massive uroporphyrinuria and markedly deficient erythrocyte UROS activity. A new mutation of the UROS gene was evidenced by systematic sequencing of the ten exons of the gene: the substitution of Serine by Proline at the aminoacid residue 47 (S47P) encoded by the third exon of the gene was present at the homozygous state in the four patients. In two unaffected brothers, no accumulation of uroporphyrin was observed and UROS activity was either normal or decreased around 50 % of normal, consistent with genetic analysis showing either homozygous normal (Ser 47) or heterozygous mutant (Ser/Pro 47) profiles, respectively. Surprisingly, in one unaffected sister, UROS activity was markedly deficient and UROS gene analysis showed an homozygous mutant profile. The deleterious role of the mutant S47P protein on UROS activity was demonstrated by procaryotic expression. This observation is the first example of incomplete penetrance in the transmission of CEP. Some hypotheses are proposed for discussion.

FERROCHELATASE ALLELIC VARIANTS AND CLINICAL MANIFESTATION OF ERYTHROPOIETIC PROTOPORPHYRIA

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Genetic counseling in autosomal dominant disorders is often limited due to incomplete penetrance and variable clinical expressivity. It has been postulated that clinical phenotypes, even for simple Mendelian disorders, are complex traits under the control of modifier genes. Erythropoietic protoporphyria (EPP; MIM 177000) is an inherited disorder caused by a partial deficiency of ferrochelatase (FECH, protoheme ferrolyase, EC 4.99.1.1) which catalyses the chelation of iron into protoporphyrin to form heme. EPP is considered to be transmitted as an autosomal dominant disorder; related FECH gene defects are characterized by a high molecular heterogeneity and an incomplete penetrance. We previously reported that FECH enzyme activity was lower in overt disease than in latent EPP and that the wild-type gene expression level accounts for clinical expression of overt EPP; EPP phenotype requires a deleterious mutation in the FECH gene on one allele and a low-expression FECH allelic variant on the other. We have now identified and demonstrated the role of an intronic SNP, IVS3-48C/T, responsible for the low-expression mechanism using a haplotype segregation strategy. The polymorphism modulates the use of a constitutive aberrant acceptor splice site. Aberrantly spliced mRNA degraded by nonsense mediated decay mechanism leads to an additional FECH enzyme deficiency necessary for EPP phenotypic expression. Interestingly, the prevalence of EPP in different human populations differs widely and appears to be dependent upon the frequency of the SNP we report. Our results provide a dramatic improvement in risk prediction and patient management in EPP families, and shed new light on penetrance mechanism in dominantly inherited diseases.

ROLE OF THE WILD-TYPE ALLELE IN THE PENETRANCE OF DOMINANT ACUTE HEPATIC PORPHYRIAS

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In an autosomal dominant porphyria, erythropoietic protoporphyria (EPP), we have recently demonstrated that the coinheritance of a ferrochelatase (*FECH*) gene defect and a wild-type low-expressed *FECH* allele is generally involved in the clinical expression of EPP. This mechanism may represent a model for phenotype modulation by mild variation in expression of the wild-type allele in three autosomal dominant porphyrias with incomplete penetrance: acute intermittent porphyria (AIP), variegata porphyria (VP) and hereditary coproporphyria (HC), respectively due to a partial deficiency of hydroxymethyl bilane synthase (HMBS), protoporphyrinogen oxidase (PPOX) and coproporphyrinogen oxidase (CPO). To test this hypothesis, we first assess that the 132 deleterious mutations found in 200 overt porphyric subjects (55 EPP, 58 AIP, 56 VP; 31 HC), among them 20 are novel, are restricted to one allele, while the other is free of any mutations. We then analyze all the available non synonymous coding Single Nucleotide Polymorphisms (SNPs) presenting a high frequency in the general population spreading throughout the *FECH*, *HMBS*, *PPOX* and the *CPO* genes in 4 case-control association studies. Finally, we explored the functional consequences of polymorphisms on wild-type mRNAs abundance, and evaluated by relative quantifications of allelic mRNAs whether low-expressed *HMBS*, *PPOX* and the *CPO* alleles occurred in general population. We conclude that the wild-type low expressed allele phenomenon is not generally operative into the mechanism of variable penetrance in AIP and VP. For *CPO* gene, the mRNA quantifications strongly suggest the presence of normal *CPO* alleles with low-expression but it remains to be evaluated in HC families if this low-expression of the wild-type allele could modulate the penetrance of a *CPO* gene defect.

QUALITY OF LIFE IN CONGENITAL ERYTHROPOIETIC PORPHYRIA: LONG-TERM FOLLOW-UP OF THREE DIFFERENT CASES

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Congenital Erythropoietic Porphyria or Gunther's disease is one of the rarest porphyrias The enzyme defect concerns uroporphyrinogen III synthase, the fourth enzyme of the heme biosynthetic pathway that leads to overproduction of the isomer I porphyrinogens in bone-marrow erythrocyte precursor. Isomer I porphyrinogens cannot be decarboxylated by enzymes of the heme pathway further than to coproporphyrinogen I, and they accumulate as their corresponding oxidized photoactive porphyrin by-products. The accumulated uroporphyrin I and coproporphyrin I are released from the erythroid cells into plasma by hemolysis or diffusion, and they are excreted in urine and feces (chiefly coproporphyrin). The clinical severity is highly variable, ranging from hydrops fetalis for a severe hemolytic anaemia in uterus to milder, later onset forms, which have only cutaneous lesions in adult life. Some authors formed the hypothesis of a genotype/phenotype correlation predicting clinical severity of CEP. We describe three cases of CEP diagnosed at the Italian Porphyrias Center - S. Gallicano Institute. Different manifestations of this illness determined a different quality of life of these patients.

*Patient 1 B*F: At birth of neonatal hyperbilirubinemia was diagnosed and the patient was exposed to UV ray and as a consequence a large bulla appeared on the side exposed. Splenectomy became necessary at the age of 2. At the age of 17 she underwent cholecystectomy and that occasion a micro- and macronodular cirrhosis was diagnosed with indication of transplant. In the meantime the need to submit to a BMT developed in the patient. The hematologist suggested that she undergo a

liver transplant first and then the BMT from the same donor. In February 2002 the patient underwent a living transplant from her mother. Post surgery course was good but when it seems possible to start thinking about the BMT a first set rejection takes place forcing the patient into a second transplant from a corpse. One year since this second transplant the patient intends to undergo a BMT.

Patient 2 AC: Photosentivity, blisters, erosions, scabs since early years. The patient was diagnosed as Porphyria Cutanea Tarda and treated with chloroquine and phlebotomy. Symptomatology remains serious but allows the patient to marry and father children having a normal working life. In 1995 he was diagnosed CEP at our Porphyrias Center. Therapy with chloroquine and phlebotomy were interrupted. Laboratory tests highlighted a well compensated cirrhosis. At present, after 8 years, the patient is affected by cirrhosis with abdominal dropsy.

Patient 3 SF: Diagnosis was made in the neonatal period, RBC transfusions were required every 4 weeks since birth, splenectomy became necessary at the age of 3. At the age of 12 he underwent BMT successfully, from an unrelated bone marrow donor. Urinary porphyrins excretion progressively decreased. Improvement of skin signs and he was able to tolerate sunlight exposure without scarring. The first patient is a L4F/V82F compound heterozygote, the second is C73T homozygote and we are investigating the third one.

ACUTE INTERMITTENT PORPHYRIA (A.I.P): A MULTIDISCIPLINARY APPROACH

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In February 1998 a 38-year-old female patient was admitted to Nephrology Department complaining recurrent abdominal colic pain, hyper-chromatic urine with transient urinary retention and bilirubin 2+ at urinary test. The clinical course was characterized by progressive asthenia affecting lower limbs till paralysis and psychiatric symptoms as irritability and aggressive behaviour in the familiar context.

Objectives:

1) prophylactic measures

2) strict clinical and laboratory surveillance

	Feb 1998	Sep 1998	Mar 1999	Feb 2000	Nov 2001	Dec 2001	Mar 2002	Apr 2002	Jun 2002	Jul 2002	Sep 2002	Nov 2002	Mar 2003
ALA	30.9 *0.1-4.5*	16.4 *0.1-4.5*		17.5 *0.1-4.5*	Normosange®	6.98 *0-6.0*	Normosange®	10.54 *0-6.0*		3.11 *0-6.0*	4.96 *0-6.0*	3.56 *0-6.0*	10.07 *0-6.0*
PBL	83.2 *0-2.0*	15.7 *0-2.0*		54.9 *0-2.0*		2.10 *0-2.0*		2.50 *0-2.0*		1.20 *0-2.0*	1.60 *0-2.0*	1.4 *0-2.0*	1.90 *0-2.0*
T.P.	1200 *0-150*	1151 *0-150*	339 *0-150*	1000 *0-150*				73 *50-200*	75 *50-200*	124 *50-200*	105 *50-200*	96 *50-200*	76 *50-200*

◄ DECAPEPTYL 3.75 ® ►

3) diet

overweight patient (real BW 62 kg vs ideal BW 55 kg)

normocaloric (30 Kcal/kg) and hyperglycidic diet (Kcal 1700): protein (gram 70: 16.6%) – glucose (gram 293: 64.7 %) - lipids (gram 35: 18.7%).

dietary supplements of malt dextrin (malt sugar) with the aim of integrate calories and glycide supplies.

4) menstrual cycle blockade

use of triptorelina (Decapeptyl 3.75 ®) for nine months (March - December 2002)

5) psychotherapy

since January 2002 the patient is following a program of Transactional Analysis Psychotherapy for the aggressive behaviour at home. Psychotherapy work enhanced the change of patient's feelings and her social interactions, and also the body manifestations versus the organic and molecular problems.

Results:

Clinical data in our patient show the psychobiologic unit of human being and confirm Damasio and Edelman's theories where body, mind, brain and environment are considered dynamic and interactive systems. The therapeutic relationship, activating chemical and neural response of the brain, can cause a deep change in the way human tissues or organs are operating. Energy bioavailability and metabolisms can be affected. The relationship between caring team and patient is essential element of the therapeutic work and founds his ground on the emotional communication that represents a powerful mean of the change.

CORRELATION BETWEEN PLASMA AND URINARY LEVELS OF PORPHOBILINOGEN (PBG) AND 5-AMINOLEVULINIC ACID (ALA) IN TEN ASYMPTOMATIC GENE CARRIERS OF ACUTE INTERMITTENT PORPHYRIA (AIP) WITH INCREASED PORPHYRIN PRECURSOR EXCRETION

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In acute intermittent porphyria (AIP) the heme precursors, porphobilinogen (PBG) and 5-aminolevulinic acid (ALA), accumulate during periods of overt porphyric crises. During remission, PBG and ALA in urine decrease to presymptomatic levels, but about 30 % of the gene carriers continue to exhibit high excretion also in the asymptomatic phase. In the present study plasma and urinary levels of PBG and ALA were studied in 10 asymptomatic excreters. Five healthy individuals were included as controls. Plasma levels were followed during 8 hours and urine concentrations during 8 and 24 hours. The following variables were evaluated: intraindividual variations in plasma and urine concentrations of PBG and ALA, the correlation between plasma and urine concentrations of PBG and ALA at different time intervals, and the 24-hour urinary excretion of PBG and ALA. Also the ratio between PBG and ALA in plasma and urine was calculated. The plasma levels of PBG and ALA were quantified by use of LC-MS technology (HemeBiotech A/S). Urine was analysed by ion exchange chromatography (Bio-Rad). The mean values for plasma levels of PBG and ALA in 10 asymptomatic AIP gene carriers were 3.1±1.0 µmol/L (range 1.7-5.1) and 1.7±0.7 µmol/L (range 0.9-3.6), respectively. In healthy controls the plasma PBG levels were under the detection limit for the method (<0.1 µmol/L), while ALA levels were low but detectable (0.4±0.1 µmol/L, range 0.2-0.5). The mean value for 8-hours urinary excretion of PBG and ALA for the 10 asymptomatic AIP gene carriers was 102±25 µmol/8-hour (range 68-147) and 56±17 µmol/8-hour (range 32-91), respectively. The corresponding values for healthy controls were 2.8±0.8 µmol/8-hour (range 1.7-4.1) and 9.2±1.2 µmol/8-hour (range 7.7-10.4), respectively. The correlations between PBG and ALA concentrations in plasma and urine of the asymptomatic gene carriers were 0.532 and 0.890, respectively. There was only slight intraindividual variation during the time of observation. The mean value for the ratio between PBG and ALA in plasma and urine was about 2.0 for the AIP gene carriers and 0.3 for the controls. This is to our knowledge the first biochemical report of plasma and urinary PBG and ALA concentrations and its relationship over a period of time.

ACUTE INTERMITTENT PORPHYRIA IN CATALONIA (SPAIN)

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Acute intermittent porphyria (AIP) is an autosomal dominant disorder with incomplete penetrance, caused by a deficiency of porphobilinogen deaminase (PBG-D) enzyme and characterised by acute neuropathic attacks. High prevalence is known in Sweden and in other areas of Europe. In Spain AIP occurs with very low prevalence but it may be underestimated. Material: Since 1992 fourteen patients with PAI attended the Hospital Clinic of Barcelona. We present the clinical,

biochemical, enzymatic and genetic analysis of 8 of these patients and 17 close relatives. Methods: We analysed (a): urinary excretion of porphobilinogen (PBG) and delta-aminolevulinic acid (ALA); (b) erythrocyte PBG-D activity; (c) DNA analysis through SSCP and sequencing the PBG-D gene. Results: Increased PBG and ALA urinary excretion and decreased erythrocyte PBG-D activity were observed in all the AIP patients. Moreover, seven cases of asymptomatic AIP were found in the relatives by means of these techniques. Five PBG-D gene mutations have been identified. Three of the mutations detected in four of the families have been previously reported in other countries (730delCT in exon 12, 340insT and G11R in exon 7). Two of the mutations identified correspond to novel mutations; one of them is present in 3 of the 8 families studied (37.5%). A strong correlation between the presence of any of the mutations and a low PBG-D activity has been observed. Conclusions: The prevalence of AIP in Spain may be much higher than previously estimated. Therefore porphyrin, enzymatic and genetic analysis must be performed in the relatives in order to detect asymptomatic carriers. AIP in Spain is also a heterogeneous disease and two novel mutations have been identified, one of them could be particularly prevalent in this area.

THE DIAGNOSTIC UTILITY OF PORPHYRIN ANALYSIS AND PLASMA FLUORESCENCE SCANNING IN VARIEGATE PORPHYRIA (VP): AN ACCURATE ASSESSMENT IN SUBJECTS IN WHOM THE PRESENCE OR ABSENCE OF A VP-ASSOCIATED MUTATION IS KNOWN

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Variegate porphyria (VP) is the autosomal dominant disorder associated with deficiency of the enzyme protoporphyrinogen oxidase. Clinical features include a photodermatitis and susceptibility to the acute attack; an acute neurovisceral crisis which may be fatal. Early detection is important since with education and avoidance of dangerous medication, the acute attack can be avoided. The standard method for diagnosis of VP is porphyrin analysis of urine and stool. A newer technique is plasma porphyrin fluorescence scanning. The sensitivity and specificity of neither test is accurately known since absolute determination of the genotype was until very recently not possible, and ;surrogate markers such as clinical symptoms were used. We have now undertaken a study to determine the utility of faecal porphyrin analysis and plasma fluorescence scanning in subjects in whom the absolute presence or absence of porphyria is reliably known through mutation detection. Subjects for the study were all those referred to our routine laboratory in whom both the genotype and the plasma fluorescence scanning result and/or faecal porphyrin chromatographic analysis result were available. The majority of subjects carried the common South African R59W mutation. Plasma porphyrin fluorescence scanning was conducted according to published methods. Biochemical porphyrin analysis was by thin layer chromatography. Stool porphyrin profiles were analysed in 400 adults of whom 172 adults carried a VP-associated mutation. Construction of receiver operating characteristics curves and discriminant analysis indicate that stool coproporphyrin is the most sensitive predictor of VP; stool protoporphyrin is less predictive. The sensitivity does not however exceed 0.76 for a specificity of 0.9. Plasma fluorescence scanning was conducted in 676 subjects of whom 205 were positive for a VP-associated mutation. In adults, sensitivity is 0.891 with a specificity of 0.996. Sensitivity is poor below the age of 20, and declines beyond the fourth decade of life. This is the first study to determine accurately the sensitivity and specificity of commonly used diagnostic tests for VP, using the genotype to determine the presence or absence of VP. Our results show that traditional stool porphyrin analysis is poorly sensitive in detecting gene carriers. Furthermore, in contrast to existing practice, the stool coproporphyrin is more predictive of VP than the stool protoporphyrin. Plasma fluorescence scanning is considerably more sensitive than stool porphyrin analysis in detecting carriers. Neither test is however useful in children, and mutation detection remains the most appropriate test for the detection of VP in children.

TWO COURSE ILLUMINATING SCHEME IMPROVES AMINOLEVULINIC ACID PHOTODYNAMIC THERAPY IN CELL CULTURES

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Photodynamic therapy with the pro-drug 5-aminolevulinic acid (ALA-PDT) is being used for the treatment of Barrett's oesophagus. We postulated that a first early course of ALA-PDT would increase protoporphyrin IX (PPIX) accumulation and thus the efficacy of a second course of ALA-PDT, by manipulating ferrochelatase (FC) and porphobilinogen deaminase (PBG-D) activity. Human EBV-transformed lymphoblastoid cells were used as a model of human tumour cells for the ability to form haem is present in all cells. After a single course of illumination (633 nm, 100 mW/cm^2) the FC activity decreased significantly whereas the PBG-D activity did not change. During continued incubation with ALA following the first illumination, cells accumulated up to four times more PPIX than non-illuminated controls (220±30% versus 55±5%; $p<0.001$). Two illuminations resulted in more cell death than one illumination (97±1% versus 80±2%; $p<0.001$). Since a second course of ALA-PDT within 3 hours after the first course resulted in a four fold increase in PPIX accumulation and significantly more cell death, we propose that a two course ALA-PDT scheme might improve the efficacy of this treatment for Barrett's oesophagus.

NON-REDUNDANT ROLES FOR HemZ AND HemF IN MAMMALIAN HEME BIOSYNTHESIS

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In humans the antepenultimate step in heme biosynthesis involves the conversion of coproporphyrinogen III to protoporphyrinogen IX by the oxygen-dependent coproporphyrinogen oxidase (HemF). Mutations in hemF lead to three clinically distinguishable pathologies: hereditary coproporphyria, its homozygous variant, and harderoporphyria. Although a small subset of bacteria have HemF as part of their heme biosynthetic machinery, most utilize HemN and HemZ, two closely related enzymes with no sequence similarity to HemF, to mediate oxygen-independent coproporphyrinogen oxidase activity. *Escherichia coli* HemN contains a motif, **CX_3CX_2CXC,** in which the first three cysteines are indispensable for coordinating an iron-sulfur cluster and the fourth is essential for catalytic activity (Layer, G., Verfürth, K., Mahlitz, E., and Jahn, D. *J. Biol. Chem*. 277: 34136-34142, 2002). Prokaryotic HemZ also contains this signature but lacks the fourth Cys and, yet, the *Bacillus subtilis* enzyme is functional. Both HemN and HemZ belong to the superfamily of '*Radical SAM*' enzymes. Here, we report the identification of a novel human HemZ homologue and demonstrate that it, like HemF, is a mitochondrial protein. We also show that hemZ and hemF are predominantly expressed in the adult heart, but only the former shows ubiquitous expression in fetal tissues. Whereas human hemZ is able to partially complement function in a heme-deficient (hemF$^-$, hemN$^-$) strain of *Salmonella typhimurium*, it lacks detectable levels of coproporphyrinogen oxidase activity when expressed in mammalian cells. Based on a genetic context analysis of the hemZ locus in enteric bacteria we propose that human HemZ participates in regulating porphyrin/heme utilization and/or iron sensing. Studies on human HemZ will contribute to our understanding of the regulation of tetrapyrrole biosynthesis and provide a model system to investigate how novel functions evolve from preexisting protein templates. This work is supported by a Pew Scholar Award (C.S.R.) and a grant from MSMT of the Czech Republic (LN 00A079 to P.M.)

REGULATION OF HEME OXYGENASE-1 GENE EXPRESSION VIA MAP KINASE SIGNALING PATHWAYS IN THE LIVER

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Cellular heme homeostasis is tightly controlled by a fine-tuned balance between enzymatic biosynthesis and degradation of heme. Heme oxygenase (HO)-1 is the inducible isoform of the first and rate-limiting enzyme of heme degradation and produces equimolar amounts of biliverdin, carbon monoxide and iron. HO-1 gene expression is not only induced by its substrate heme, but also by a large variety of oxidative stress stimuli. The stress-dependent HO-1 induction is of physiological significance because overexpression of HO-1 has been demonstrated to serve protective functions against the deleterious effects of various experimental injuries. Moreover, HO-1 knockout mice are highly susceptible to endotoxin-mediated liver damage which causes an increased mortality of these animals. Therefore, it is conceivable that activation of HO-1 gene expression may serve as a therapeutic target in hepatic injury (1). To investigate the stress-dependent molecular mechanisms and signal transduction pathways of HO-1 in the liver the regulation of HO-1 gene expression by the prototypical stress stimulus sodium arsenite has been examined in primary cultures of rat hepatocytes. In this cell culture model HO-1 gene expression is transcriptionally induced by sodium arsenite and is mediated via the c-Jun N-terminal kinase and p38 mitogen-activated protein kinase (MAPK) signaling pathways (2). The nuclear targets of these signaling cascades are the transcription factors AP-1 and Max that specifically interact with regulatory elements of the proximal rat HO-1 gene promoter region. Independently, others have demonstrated in a mouse hepatoma cell line that HO-1 gene induction by oxidative stress is regulated by the transcription factor NF E2 related factor-2 (Nrf2) that binds to the antioxidant response element in an upstream promoter region of the mouse HO-1 gene (3). Nrf2-dependent HO-1 gene activation is mediated by a posttranscriptional mechanism via the ubiquitin proteasome pathway (4). In conclusion, transcriptional activation of HO-1 gene expression by stress stimuli is regulated by cell- and species-specific signal transduction pathways in liver cells. References: (1) Immenschuh S et al. (2000) Biochem. Pharmacol. 60: 1121 (2) Kietzmann T et al. (2003) J. Biol. Chem. 278: 17927 (3) Alam J et al. (1999) J. Biol. Chem. 274: 26071 (4) Stewart et al. (2003) J. Biol. Chem. 278: 2396

CORRECTION OF THE BIOCHEMICAL DEFECT IN PORPHOBILINOGEN DEAMINASE DEFICIENT CELLS BY NON-VIRAL GENE DELIVERY

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Porphobilinogen deaminase (PBGD), the third enzyme in the biosynthesis of heme, is deficient in acute intermittent porphyria (AIP). AIP is a genetic disease characterized by neurovisceral and psychiatric disturbances. Despite a palliative treatment, it may still be lethal. An initial step towards gene therapy was recently taken by showing that PBGD could be expressed to correct the enzyme deficiency in AIP fibroblasts. The aim of the present study was to investigate whether the biochemical defect can be corrected by using non-viral gene delivery. The biochemical defect in human and mouse PBGD deficient fibroblasts was demonstrated by analyzing synthesis of the heme precursor, protoporphyrin (PP), after addition of 5-aminolevulinic acid (ALA). Human AIP fibroblasts synthesized 21 % and mouse PBGD deficient fibroblasts only 11 % of the PP amount synthesized in respective control cells. Gene delivery increased the PBGD activity 88-200 fold in human AIP fibroblasts and synthesis of PP was increased from 21 % to 152 % of normal after ALA incubation. Similar results were obtained in mouse PBGD deficient cells, although the PP levels were several-fold lower as compared to human cells. HPLC analysis confirmed that PP was the main porphyrin intermediate that was formed. Addition of porphobilinogen (PBG) resulted in 3-7 fold lower synthesis of PP as compared to ALA addition. These results show that non-viral gene delivery of plasmids encoding PBGD results in a high expression of functional PBGD shown by induced synthesis of PP in PBGD deficient cells after supplementation of ALA and PBG.

VALIDATION OF A DENATURING HPLC ASSAY FOR MUTATION ANALYSIS IN THE HUMAN PROTOPORPHYRINOGEN OXIDASE GENE

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A denaturing high pressure liquid chromatography (DHPLC) assay based on the formation of heteroduplexs, has been developed for mutation analysis of the PPOX gene. This method detects herozygotes for mutations in PPOX. Compared to other mutation analysis methods, the DHPLC method is much more automated and much faster to run. Exon 1 to exon 13 and the flanking intronic regions in the PPOX gene were blindly examined in DNA-samples from 14 patients with Variegate Porphyrias. The mutations in the 14 patients has previously been characterized in the laboratory of professor Jean-Charles Deybach by denaturing gradient gel electrophoresis (DGGE) and sequencing of abnormal band patterns. All the mutations previously found by DGGE were correctly identified using DHPLC. Futher information about the design of the assay and the results from comparison of the two methods will be presented.

A STUDY OF THE POSSIBLE ROLE OF LOW EXPRESSED FUNCTIONAL PPOX ALLELES IN THE VARIABLE PENETRANCE OF VARIEGATE PORPHYRIA

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The protoporphyrinogen oxidase (PPOX) gene is responsible for the penultimate step of the heme biosynthesis pathway where protoporphyrinogen-IX is oxidized to protoporphyrin. Mutations in the PPOX gene are associated with a diseased state known as variegate porphyria (VP). The term was chosen to describe the low penetrance and varied expression of the autosomal dominant inherited state, including the development of severe abdominal and neurological crises as well as cutaneous photosensitivity, with up to three-quarters of affected individuals remaining asymptomatic. South Africa has the highest incidence of VP in the world due to a founder effect. However, despite decreasing PPOX activity, the R59W founder mutation seems to play no role in the variable penetrance of the VP phenotype. It has recently been shown that where gene mutations are insufficient to convey a disease phenotype, symptoms may penetrate when the mutated allele is coinherited with a functional, yet low-expressed wild-type allele. This study investigates the possibility of a similar modulating mechanism for symptomatic penetrance in VP. Five conserved PPOX haplotypes have been identified based on four single nucleotide polymorphisms (SNPs) in the gene. Despite observed differences in haplotype frequencies between symptomatic, asymptomatic and control patients for at least two of the haplotypes, analysis of variance and association studies yielded negative results. However, due to the limited size of the sample population used the effects of varied expression of wild type haplotypes cannot be excluded. We are now focussing on a more causative approach to identify regulatory elements and possible low-expressed wild type alleles. The -1081G>A sequence variant in the promoter area of the PPOX gene has already been shown to reduce transcriptional activity relative to wild type alleles in in vitro expression assays, and allele specific quantification is now being used to confirm these results. Results from this study will additionally provide a better understanding of the mechanisms involved in PPOX gene regulation for future applications and possible therapies.

ON THE RELATIONSSHIP BETWEEN PORPHYRIA CUTANEA TARDA AND OTHER COMMON METABOLIC DISEASES (DIABETES, HYPERCHOLESTERINAEMIA)

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Porphyria cutanea tarda (PCT) is caused by a decrease of hepatic uroporphyrinogen decarboxylasis. By both endogenous and exogenous cofactors like HFE-mutations, hepatitis C and alcohol the biochemical defect turns into a clinical relevant disease. The relationship to other factor such as syndrome X as well as glucose- and cholesterol-metabolism are only scanty evaluated. In the present study we focused just on these parameters that were analysed before, during and after PCT-treatment. Forty-five patients (age 51±14, 11 women, 34 men) with PCT treated with chloroquine were enrolled in the study. Clinical examination, laboratory investigations including porphyrine metabolism, diabetes screening and serum lipids were performed on several occasions. Typical cutaneous signs were seen in all patients. At the first examination urinary porphyrine was elevated (3742±3161 µmol/d). After one year of treatment urinary porphyrine was 315±318 µmol/d ($p<0.00001$). The serum-glucose did not show any significant changes before and after treatment (4.05±1.48 mmol/l versus 4.35±1.32 mmol/l; $p=0.1547$). On the other hand a higher prevalence of diabetes mellitus type II (15.6 %) was seen in PCT compared to a control group (6.0 %), same age and sex ($p=0.0001$). In the case of serum cholesterol no differences were seen before and after successful treatment or in comparison with the control group. The insulin resistance and the associated hyperinsulinaemia might be a reason for the higher proportion of diabetes mellitus among PCT patients. It can be speculated that insulin resistance and PCT might share some genetic background.

PORPHYRIA CUTANEA TARDA (PCT) AND STEATOSIS HEPATIS

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PCT is the most common porphyria in Germany with particular interest for the dermatologist. The changes in liver morphology and metabolism are caused by the deposition of uroporphyrine and iron. In the present study we investigated weather there is a relationship of PCT and steatosis hepatis. The background for our study is the "syndrome X"-concept, which says that different ways of metabolism are connected with each other, and the clinical observation of an increased incidence of steatosis hepatis shown by liver biopsies among patients with PCT. We investigated semiquantitative the liver morphology by liver biopsies in 45 PCT-patients before chloroquine therapy. Follow-up biopsies were performed by Menghini technique. Statistics were performed by matching of pairs by two tailed sig-test. During chloroquine treatment a statistical significant decrease of the steatosis hepatis degree was seen (17.3 % versus 6.9 %, $p=0.001$, $n=27$). During the followup it was impossible to show a further improvement (5.2 % versus 5.0 %, $p=0.84$, $n=20$). Steatosis hepatis shows a wider range of causative factors. One major reason for steatosis hepatis in western population seems to be alcohol misuse or abuse. Our results are showing the reversibility of the steatosis hepatis by avoiding a reasonable inducer (porphyrine). One cofactor of steatosis hepatis in PCT is the iron overload of the liver. Chloroquine seems to support the excretion (or transport) of porphyrines from liver and has therefore a protective effect. Another important aspect is the higher rate of type II diabetes among PCT patients. It has to be investigated in further study which factors are more important. Nevertheless, chloroquine seems to be a reliable measure to lower the risk of hepatic damage in PCT patients.

REGULATION OF 5-AMINOLEVULINATE SYNTHASE-1 BY GLUCOSE AND HEME

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5-Aminolevulinic acid synthase-1 (ALAS-1) is the first and normally rate-controlling enzyme for hepatic heme biosynthesis. ALAS-1 is highly inducible, especially in liver, in response to changes in nutritional status, and to many drugs that induce cytochrome P-450 and/or affect heme synthesis. The critical biochemical abnormality of the acute porphyrias is an uncontrolled up-regulation of ALAS-1 in the liver. High intakes of glucose or other metabolizable sugars and intravenous heme are the cornerstones of therapy of acute attacks of these types of hepatic porphyrias. Both glucose and heme are known to repress the uncontrolled over-expression of hepatic ALAS-1, although their molecular mechanisms of action have not been characterized. In previous work, we and others showed that the LMH hepatoma cell line is extraordinarily useful for study of heme metabolism, including regulation of ALAS-1. In this work, we investigated the effects of glucose and heme in LMH cells transfected with ALAS-1 promoter-luciferase (Luc) reporter constructs. Treatment for 16 h with the barbiturate-like drug, glutethimide (Glut, 50 µM), and the inhibitor of heme synthesis, 4,6-dioxoheptanoic acid (DHA, 250 µM), produced a synergistic (5-fold) up-regulation of ALAS-1 promoter-reporter activity in LMH cells transiently transfected with a construct containing 9.1 Kb of the ALAS-1 5´flanking region and the ALAS-1 5´untranslated region (UTR) attached to the luciferase (pGL3 Basic) reporter gene (Fig 1). Addition of glucose (11 or 33 mM), in a dose-dependent manner, decreased the Glut+DHA up-regulation of pGcALAS9.1-Luc activity (Fig. 1). Exogenous heme (20 µM) repressed basal luciferase activity (4-fold) and had further and an additive effect on glucose repression and completely abrogated the induction by Glut and DHA (Fig. 2). The glucose analog 2-deoxyglucose, which cannot be metabolized to glucose, significantly augmented the induction by Glut and DHA and abrogated the glucose effect on ALAS-1, indicating that the glucose needs to be metabolized to exert its effect on ALAS-1. Metabolizable sugars such as fructose, galactose, glycerol and lactate, but not the non-metabolizable sugar sorbitol, also down-regulated ALAS-1 in LMH cells. In conclusion, these results establish that both heme and glucose or other metabolizable sugars lead to down-regulation of hepatic ALAS-1 by acting directly on hepatocytes through a mechanism that requires the first 9.1 Kb of the 5´- regulatory region of the ALAS-1 gene. These effects were observed in the absence of insulin, glucagon, other hormones or serum. Our focus is to perform fine mapping and detailed characterization of the 5´-flanking region to identify *cis*-acting elements that mediate these repressive effects on ALAS-1 gene expression.

Fig 1. Effect of Glucose on Basal and Induced pGcALAS9.1-Luc Activity in LMH cells

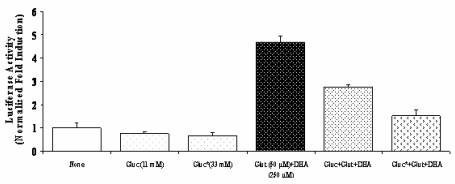
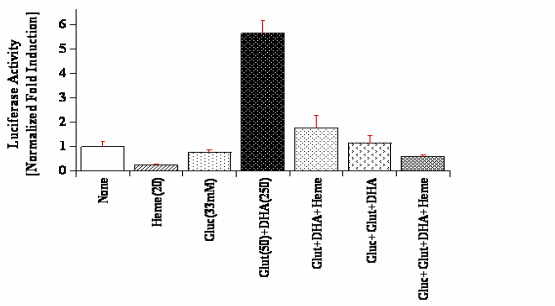


Fig 2: Effect of Glucose and Heme on Basal and Induced pGcALAS9.1-Luc Activity



THERAPEUTIC MONITORING OF PORPHYRIA CUTANEA TARDA (PCT) USING SERUM HAEMOPEXIN

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We investigated whether haemopexin (HPX) could be used in monitoring of chloroquine therapy (125-250 mg twice a week) in PCT. Ninety-eight patients with PCT were included: (1) patients with active disease n = 39, (2) patients after treatment n = 9, (3) patients in remission n = 36, (4) patients with relapses n =14. HPX levels were determined by nephelometry. Mean levels were 0.69±0.19 g/l (1) and 0.87±0.16 (3), $p<0.001$, 0.87±0.16 (3) and 0.65±0.13 (4), $p<0.05$. Intraindividual comparison after one year treatment (n = 34): 0.69±0.15 g/l and 0.84±0.11, $p<0.001$. Thus, during active PCT disease with high plasma levels of porphyrins HPX-porphyrin complexes are produced leading to a decrease of free HPX. Chloroquine therapy supports porphyrin exretion and should thereby increase free HPX. As far as we could demonstrate serum HPX seems to reflect the therapeutic effects. Serum HPX seems to be a reliable parameter for therapeutic monitoring.

NOVEL OLIGOPYRROLIC MACROCYCLES AND PORPHYRIN DERIVATIVES: SYNTHESIS AND MEDICINAL APPLICATION

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The recent development in the area of oligopyrrolic mocrocycles will be reviewed. The novel methodology for porphyrin core modification is using Pd catalyzed C-C and C-N bond formation. Discovery of so called "confused or inverted porphyrins" will be described together with synthetic protocols for expanded porphyrins, as well as, for calix[n]phyrins. Porphyrins, long known for their versatile metal cation coordination chemistry, are macrocycles that contain only sp(2)-hybridized bridging meso carbon atoms within their framework. Calix[n]phyrins are macrocycles that contain both sp(2)- and sp(3)-hybridized meso carbon bridges and bear analogy to both porphyrins and porphyrinogens. This introduces novel structural features together with specific recognition properties. The novel water soluble calix[4]phrins and porphyrins with saccharide, ß-cyclodextrin and oligopeptide periphery were synthesized and tested for molecular recognition properties (cation, anion and oligosaccharide sensing) and PDT application. This work was supported by Grant Agency of Czech republic No. 301/01/0976 and 301/98/K042.

NOVEL PORPHYRIN AND EXPANDED PORPHYRIN DERIVATIVES WITH CAPACITY TO INDUCE APOPTOSIS IN VARIOUS TUMOR CELLS FOLLOWING PHOTODYNAMIC TREATMENT

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Synthesis and biolocalization properties of novel water-soluble porphyrin and sapphyrin derivatives will be presented. Novel fluorinated porphyrins with oligoethyleneglycol, oligopeptide, distamycin analogues, mono-, disaccharide, and b-cyclodextrin substitution showed significant photosensitizing potential in vitro and in vivo and displayed an increased selectivity for malignant tissue. The objective of this study was to explore their capacity to induce apoptosis. To study the mechanism of their action, we have investigated uptake, intracellular localization, cell phototoxicity and morphological and biochemical changes following photodynamic treatment in human leukemic cell line HL60 and also other tumor cells. Some of our novel PS exhibited a very effective induction of apoptosis as demonstrated by condensation of chromatin, DNA fragmentation, cytochrom c release, a loss of membrane phospholipid asymmetry (as evidenced by the externalization of phosphatidylserine), and an increase in caspase-3 protease activity. Moreover, polymethine and other polycationic porphyrin derivatives exhibit not only very specific tumor localization, but also into-cell antisense oligonucleotide transport properties as was demonstrated on the primary leukemic cells. [Financial support from the Czech Grant Agency No. 301/01/0976, 301/98/K042 and the Ministry of Education of the Czech Republic No. CEZ 2234000008 is gratefully acknowledged].

ALTERATIONS OF PORPHYRIN METABOLISM IN MICE BY GRISEOFULVIN

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Human erythropoietic protoporphyria (EPP) is an inherited disorder of porphyrin metabolism due to defective ferrochelatase. Griseofulvin (Gf) is believed to selectively inhibit this enzyme but is also known to precipitate acute attacks in patients with acute intermittent porphyria. Faecal coproporphyrin III (CP) and protoporphyrin (PP) in samples collected from mice treated for 1-7 days with oral 2.5% griseofulvin (Gf) in their diet were progressively increased compared with controls. A 2-fold and 4-fold increase in CP and PP, respectively, was seen after one day of feeding. After seven days the increases were 40-100-fold, respectively. Urinary ALA and PBG were, respectively, increased 20 and 2-fold. Mice fed with Gf for 3 days, enhanced the production of uroporphyrins I and III (10-fold increase), and heptacarboxylporphyrin. Mice fed Gf for 3 days and simultaneously injected with Haem-arginate (10 µg daily for 3 days), had normal coproporphyrin-lll, ALA and PBG-levels. The protoporphyrin-IX level was, however, only marginally reduced. The present study indicates that griseofulvin might be inhibiting protoporphyrinogen oxidase (PPOX) activity in addition to ferrochelatase (FC) simulating variegate porphyria (VP) and thus precipitating porphyric attacks in susceptible individuals.

FLUOROMETRIC MEASUREMENT OF 5-AMINOLEVULINIC ACID IN SERUM

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Measurement of 5-aminolevulinic acid (ALA) in serum is clinically important because it is increased in patients with inherited ALA dehydratase deficiency, acute intermittent porphyria, hereditary coproporphyria, variegate porphyria, lead toxicity, and tyrosinemia. Serum levels of ALA are about 100 times less than the urinary levels in healthy humans and a highly sensitive method is required. We describe a simple fluorometric method to determine serum ALA. ALA is separated from serum using a cation-exchange resin (DOWEX 50WX8-400), followed by addition of acetylacetone and formaldehyde to produce a fluorescent ALA derivative by the Hantzsch reaction. The intensity of fluorescence was measured by spectrofluorometer with an excitation wavelength of 370 nm. For comparison HPLC method (Tomokuni K et al. Clin Chem 1993;39:169) was performed with the same samples, isocratically on a C_{18} column with the mobile phase of methanol/water/acetic acid (50/50/1), a flow-rate of 0.7 ml/min, and a fluorescence detector. The calibration plot was linear for ALA concentration to 500 µg/L with a detection limit of 8.7 µg/L, which was equivalent to 3 S.D. above the zero in the plot. The emission of the fluorescent ALA derivative was peaked at 463 nm. The precision as measured by within-run variations (N=8) was 3.7% and 3.1% at 25 µg/L and 100 µg/L ALA, respectively, and day-to-day variations (N=10) for the same two levels were 7.2% and 6.1% respectively. The regression equation for this method in reference to the HPLC method was $Y = 0.99X + 10.34$ ($N=34$, $r = 0.98$, $S_{y/x} = 36.1$). The slope was near unity and the y-intercept was insignificant, although the results of fluorometry were slightly higher in general than those obtained from the HPLC method. A reference range of serum ALA was 0 - 79.4 µg/L (35.2 ± 22.1, mean±S.D.) as determined in normal subjects (N = 42). The distribution was skewed to the left because levels in some subjects (N =

8) were actually less than the sensitivity limit (< 9.0 μg/L). In conclusions, this fluorometric method for serum ALA correlated well with the results of HPLC method, although the values in the low range of less than 100 μg/L were somewhat higher with the fluorometric method. The method is simple and may be suitable especially when an HPLC instrument is not available.

IRON METABOLISM IN ERYTHROPOIETIC PROTOPORPHYRIC MOUSE MODEL

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Reduced activity of ferrochelatase in erythropoietic protoporphyria (EPP) results in protoporphyrin accumulation in erythrocytes and liver. However, iron, the other substrates of ferrochelatase is not obviously increased. Moreover, mild microcytic anemia is commonly observed in EPP patients. Controversial hypotheses have been reported: 1) a defective iron utilization in the bone marrow which leads to intramitochondrial iron deposits and sideroblasts, 2) iron deficiency with hematological sideropenic signs, 3) chronic hemolytic anemia by destruction of fragile porphyrin loaded red cells. We investigated the mechanisms underlying EPP-associated iron disorder in a mouse model of EPP. Liver, bone marrow, spleen, kidney, heart, duodenum histologies, blood hematological, plasma and intra-tissue parameters of iron metabolism are studied at both protein and mRNA levels in three different genetic backgrounds female mice homozygous for a point mutation in the ferrochelatase gene and in wild-type (Balbc, SJL/J, C57Black6). Microcytic anemia and low iron circulating parameters are observed in fech -/- mice. Microscopic examination shows a lower Perls staining coloration in ferrochelatase deficient mice without intramitochondrial iron deposits and sideroblasts in blood, bone marrow and spleen. Intracellular iron is around 50% decreased in liver, kidney and heart and normal in spleen compared with controls. Hepcidine, ferroportin, dcytb and others protein involved in iron metabolism analyses strongly suggest a dysregulation of iron absorption in the intestine in mice model of EPP.

ERYTHROPOIETIC PROTOPORPHYRIA: A RAPID METHOD TO DETECT ASYMPTOMATIC GENE CARRIERS

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As it always happens for different forms of porphyria, many individuals who inherit the gene mutation responsible for erythropoietic protoporphyria (EPP) remain asymptomatic (latent) throughout life. Nevertheless in some circumstances identification of asymptomatic EPP gene carriers could play an important role in the knowledge of this disease: through an extremely simple method - given the availability of a spectrofluorometer - it is possible to evaluate accurately the EPP prevalence in large populations. We studied: 14 EPP individuals (8 unrelated EPP patients and 3 couples of EPP siblings); 8 asymptomatic relatives from three unrelated families affected by EPP; a control group of 10 subjects. Fluorescence emission spectroscopy of red blood cell was carried out as described by Poh-Fitzpatrich with minor modifications. RBC from EDTA anticoagulated blood were scanned between 570 and 750 nm at an excitation wavelength of 405 in a Perkin-Elmer LS55 luminescence Spectrometer. Fourteen EPP patients were uniformly found, as already described by Poh-Fitzpatrich, to have a distinctive erythrocytes porphyrin fluorescence wavelength maximum at 626 (± 1) nm. Six out of the 8 asymptomatic relatives showed an evident peak at 626 nm even if the fluorescence intensity of this peak was at least 5 times minor then that identifiable in RBC of EPP patients. Scans from 10 normal subjects showed no peak or a very small peak with a maximum emission of 619 nm or less. All 8 asymptomatic relatives underwent genetic analyses to identify specific familial mutations. The genetic investigation confirmed biochemical data.

KINETIC AND PHYSICAL CHARACTERISATION OF RECOMBINANT WILD TYPE AND MUTANT HUMAN PROTOPORPHYRINOGEN OXIDASES

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Variegate porphyria (VP) is an autosomal dominant disorder of haem metabolism resulting from defects in the protoporphyrinogen oxidase (PPOX) gene. World wide heterogeneity for VP exists and over 100 mutations have been described to date. In this study, the effects of various PPOX mutations (R59W, H20P, R168C and Y348C) responsible for VP in South Africa, the roles of the arginine-59 residue and the glycines in the conserved flavin binding site, on catalysis and/or cofactor binding, were examined. Wild type recombinant human PPOX and a selection of mutants were generated, expressed and partially characterised. All mutants had reduced PPOX activity to varying degrees. However, the activity data did not correlate with the ability/inability to bind flavin. The positive charge at arginine-59 appears to be directly involved in catalysis and not in flavin-cofactor binding alone. The K_ms for the arginine-59 mutants suggested a substrate-binding problem. $T_{1/2}$ indicated that arginine 59 is required for the integrity of the active site. The dominant α helical content was decreased in the mutants. The degree of a helix did not correlate linearly with $T_{1/2}$ nor T_m values, supporting the suggestion that arginine 59 is important for catalysis at the active site. Examination of the conserved dinucleotide-binding sequence showed that substitution of glycine in codon 14 was less disruptive than substitutions in codons 9 and 11. Ultraviolet melting curves generally showed a two state transition suggesting formation of a multi-domain structure. Generally all mutants studied were more resistant to thermal denaturation compared to wild type, except for R168C. This work illustrates the use of studying expressed, purified mutant PPOXs in elucidating the importance of specific amino acid residues to fully understand the structure-function relationship underlying both normal and impaired PPOX activity.

IDENTIFICATION OF THE SPECIFIC MUTATIONS IN THE CPO GENE RESPONSIBLE FOR GENOTYPE-PHENOTYPE RELATIONSHIP BETWEEN HEREDITARY COPROPORPHYRIA AND HARDEROPORPHYRIA

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Hereditary coproporphyria (HCP), an autosomal dominant acute hepatic porphyria, results from mutations in the *CPO* gene that encodes the mitochondrial enzyme, coproporphyrinogen oxidase. A few patients have been reported who are homoallellic or heteroallelic for *CPO* mutations and are clinically distinct from those with HCP. In such patients the presence of a specific mutation (K404E) on one or both alleles produces an unifying syndrome in which hematologic disorders predominate: "harderoporphyria". Heterologous expression of another mutation (R401W) demonstrated functional properties to those of the K404E harderoporphyria mutation. Mutations on both alleles elsewhere in the gene give rise to the "homozygous" variant of HCP. The molecular relationship between the single *CPO* gene and these two different phenotypes (harderoporphyria and HCP) has not been defined and biochemical bases remained unexplained. We describe the molecular investigation and clinical features of harderoporphyric families reported so far, including a novel one. Investigations performed both in vivo and in vitro by heterologous expression studies after hydrophobic cluster analysis (HCA) of the secondary structure of CPO enzyme demonstrate that only few missense mutations restricted to 5 aminoacid encoded by exon 6 and localised in a hinge region between two b sheets may lead to harderoporphyrin accumulation and

subsequently harderoporphyric phenotype: D400, R401, G402, T403, K404. Moreover, all other type of mutations or missense mutations mapped elsewhere throughout the *CPO* gene lead to coproporphyrin accumulation and subsequently typical HC. Our findings add substantially to knowledge of molecular and biochemical bases responsible for the specific hematologic clinical manifestations of harderoporphyria and demonstrate that the type and location of the mutations in the *CPO* gene clearly modulate of phenotype.

CLINICAL AND NUTRITIONAL MANAGEMENT OF ACUTE PORPHYRIAS

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Porphyrias, either genetic or acquired, are metabolic diseases, produced by specific enzymatic deficiencies in the haem pathway. Each porphyria is biochemically characterized by an abnormal pattern of accumulation and excretion of the precursors aminolevulinic Acid (ALA) and porphobilinogen (PBG) and/or porphyrins. Independently on their hereditary or acquired origin, these diseases have a polymorphic clinical expression which might go from intense skin photosensitivity to an abdominal acute crisis, associated with severe hepatopathy and/or neuropathy, leading to death in 10% of the patients suffering the acute forms. Acute porphyrias are hepatic and genetic, depending on the enzymatic failure, they are identified as Acute Intermittent Porphyria (AIP), the most frequent of them all in Argentina, Variegate Porphyria (VP) and Hepatic Coproporphyria (HCP). They are most frequent in woman. Most of the carriers of the genetic deficiency might be asymptomatic throughout their life, however, around a third of them can develop an acute crisis due to the exposure to some triggering agents, such as several porphyrinogenic drugs, toxic substances, alcohol, stress or inadequate diet. The administration of relatively high amounts of carbohydrates, is the fundamental base of the nutritional treatment, both during the attack and their asymptomatic periods. Carbohydrates act through the mechanism known as "glucose effect", inhibiting the haem pathway by preventing the induction of the regulatory enzyme ALA-synthetase. An epidemiological retrospective clinical study was carried out in patients, both under admission and ambulatory, in the Department of Medical Clinics in the Ramos Mejía General Hospital of Buenos Aires, Argentina, during a period of 9 years (1994 to 2002). The following parameters were evaluated: type of porphyria, sex, age, patterns of excretion, nutritional state, number of admissions. All porphyric patients received the specific therapy for thier crisis during admission and for maintenance during remission. From a total of 152 patients, 78% were AIP and 23% mixed porphyrias (VP and HCP). The average age was 24 (13-44) for AIP and 23 (20-34) for mixed porphyrias. Admissions were 25 for AIP and 5 for mixed porphyrias. Of all admissions, 40% were due to an acute attack. In conclusion, the right clinical and very important, nutritional management of the porphyric patients assures both a more rapid recovery from the crisis and a much more extended remission period, often for the rest of their lives, meaning that they do not suffer another attack ever.

EFFECT OF POLYPHENOLS ANTIOXIDANTS ON A MOUSE MODEL OF PROTOPORPHYRIA

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Erythropoietic Protoporphyria (EPP) is an inherited disease biochemically characterized by protoporphyrin (PP) accumulation in bone marrow, erythrocytes, plasma, liver and faeces, due to a deficiency in ferrochelatase (FeChel). This mitochondrial enzyme is responsible for the insertion of ferrous iron in a molecule of PP to form haem. Hepatic clearance of PP through the biliary system can lead to precipitation of the highly hydrophobic porphyrin in biliary canaliculi, resulting in cholestasis, fibrosis and cirrhosis. Griseofulvin (Gris) is a widely used antimycotic. Besides its antifungal properties, it has many undesirable side effects. In laboratory animals, Gris can induce porphyrin accumulation and hepatocarcinogenesis. It has been shown that its porphyrinogenic action is due to the formation of N-methyl porphyrins, powerful inhibitors of FeChel. leading to the accumulation of the enzyme substrates, PP and Fe. The hepatic manifestations mimic those observed in EPP patients We have previously demonstrated the onset of oxidative stress in liver mice fed with variable concentrations of dietary Gris. Based in those observations, we have now studied the effect of several plant polyphenols on the oxidative stress induced by Gris in mice. Animals received ellagic acid (300 mg/l), quercetin (50 mg/l) or chlorogenic acid (50 mg/l) in drinking water, and simultaneously Gris (0.5%) in the diet. Control animals received standard diet for laboratory animals, and the corresponding controls for each polyphenol received standard diet plus the antioxidant in drinking water. ALA-S, GST and SOD activities, and liver porphyrins were elevated in all animals treated with Gris or Gris plus polyphenol (160%, 140%, 180% and 140%, respectively). Hepatic levels of GSH were only increased in mice treated with Gris or Gris plus quercetin (149% and 142%, respectively). Mitochondrial TBARS were slightly increased over the controls (129-133%) in animals receiving Gris plus antioxidant, while these levels were significantly increased (200%) in animals fed with Gris alone. Microsomal TBARS were increased only in animals receiving Gris alone (148%). Because FeChel is inhibited by Gris, hepatic porphyrin levels and ALA-S activity remain elevated. But polyphenols do protect the liver from lipid peroxidation induced by accumulated porphyrins. It is therefore expected that administration of biliary salts along with some of these polyphenols would increase clearance of hepatic porphyrins, so to improve the liver status of these animals.

TWO MUTATIONS IN UROPORPHYRINOGEN DECARBOXYLASE (UROD) GENE LEADING TO MILD PHENOTYPE OF HEPATOERYTHROPOIETIC PORPHYRIA (HEP) IN ITALY

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A deficiency in uroporphyrinogen decarboxylase (URO-D; EC 4.1.1.37) enzyme activity, the fifth enzyme of the heme biosynthetic pathway, is found in patients with sporadic porphyria cutanea tarda (s-PCT), familial porphyria cutanea tarda (f-PCT), and hepatoerythropoietic porphyria (HEP). Subnormal URO-D activity is due to mutations of the UROD gene in both f-PCT and HEP, but no mutations have been found in s-PCT. Genetic analysis has determined that f-PCT is transmitted as an autosomal dominant trait. In contrast, HEP, a severe form of cutaneous porphyria, is transmitted as an autosomal recessive trait. HEP is characterized by a profound deficiency of URO-D activity, and the disease is usually manifest in childhood, characterized by severe photosensitivity, skin fragility and hypertrichosis. Biochemical and genetic investigations suggested that HEP is the homozygous form of f-PCT. This hypothesis has been recently confirmed by the molecular analysis of the UROD gene. So far, 60 different mutations have been identified, 4 of whom (P62L, A80G, V134Q, G281E) detected either in HEP (in homozygosity or in compound heterozygosity) and in f-PCT, with 1 predominant mutation (G281E) detected in Spain. In this study we performed the molecular characterization in 4 subjects with coutaneous porphyiria at early onset. Patient 1 showed early onset, severe URO-D deficiency, marked hypertrichosis and ascertained familiarity; patients 2 and 4 both showed severe URO-D deficiency associated with a f-PCT-like phenotype. HEP in family 3 was suspected mainly by family history: the proband and her sister have a similar severe phenotype associated with early onset whereas the mother has a milder symptomatology. UROD gene mutations are detected by PCR-SSCP followed by nucleotide sequencing. Putative splicing mutations are analyzed by RT-PCR. The results obtained by molecular analysis are summarized in the following table.

Pt.	Sex	Mutation	Allelic state	Exon/ intron	Protein change	URO-D (% of N)	Diagnosis (years)	Reference
1	F	358 C>T 575 C>T	compound heterozygote	5 6	R120C L192P	24	7	Martinez 2002 Martinez 2002
2	F	IVS1+1 G>T 952 G>A	compound heterozygote	IVS1 10	Ex. 1 del. G318R	27	62	Martinez 2002 McManus 1996
3	F	756 C>G	homozygote	7	Ex. 7 del.	74	10	Martinez 2002
4	F	425 G>A	homozygote	5	R142Q	34	56	Cappellini 2001

The mutation screening has been extended, whenever possible, to other family members. In the case of pt. 1 we found the same two defects in a male twin with similar phenotype, R120C in the father and paternal aunt and L192P in the mother. In family 2, both the proband's daughters were carriers of the G318R mutation who are, at present, asymptomatic. The 756 C>G mutation (pt3), found in homozygosis in two sisters with the same severe phenotype, was detected in one allele of the mother. RT-PCR experiments showed that the correct splicing of exon 7 was not completely abolished by the mutation, possibly explaining the mild URO-D deficiency observed in RBCs. This study confirms, that HEP is the result of homozygosity or double heterozygosity for mutations occurring in f-PCT: among the 6 different mutations detected in this series, G318R has been previously detected in f-PCT heterozygotes in Northern Europe and R142Q was found by us in an unrelated patient of Italian ancestry.

MUTATIONAL ANALYSIS FOR ACUTE HEPATIC PORPHYRIAS IN THE UNITED KINGDOM

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The acute hepatic porphyrias comprise acute intermittent porphyria (AIP), variegate porphyria (VP) and hereditary coproporphyria (HCP). The diagnosis in the proband is made biochemically but mutational analysis is increasingly seen as an essential component in the management of families in order to identify affected family members and minimise their risk of a life-threatening acute neurovisceral attack. We report our experience of molecular investigation of a large number of families using direct automated sequencing of PCR-amplified genomic DNA. AIP: We have investigated 363 families, 203 by mutational analysis and identified a total of 102 mutations. Of these, 27 have not previously been described in the literature. In 7 families with an unequivocal biochemical diagnosis of AIP, we were unable to find a mutation in the HMBS gene and the sensitivity of mutation detection is therefore 96%. VP: 158 families have been investigated, 118 by mutational analysis and in all 118 families a mutation has been demonstrated. 56 different mutations were identified of which 23 appear to be novel. HCP: of 39 families, 27 have been investigated by mutational analysis. Since 2001, 13 of these families have been investigated by mutational analysis in Cardiff and 10 mutations have been found of which seven have not previously been reported. The sensitivity of mutation detection using the approach in Cardiff is thus 77%. These data demonstrate the high sensitivity of our method for the identification of mutations in the HMBS, CPO and PPOX genes. They confirm that the majority of families carry a unique mutation and that a sequencing based approach is therefore required for the management of this group of patients.

MOLECULAR GENETICS OF ERYTHROPOIETIC PORPHYRIA

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In most families, clinical expression of erythropoietic protoporphyria (EPP) appears to require inheritance of a low expression ferrochelatase (*FECH*) allele (IVS3-48C) trans to a severe *FECH* mutation [1]. Autosomal recessive (AR) inheritance has confirmed by molecular analysis in 2 families [2,3] but its prevalence remains uncertain. To determine the prevalence of these modes of inheritance in the United Kingdom, we investigated 111 consecutively referred, unrelated patients with clinically overt EPP. Patients and 100 control subjects were genotyped for the *FECH* IVS3-48C/T polymorphism [1]. The IVS3-48C allele was present in 108 (97%) of 111 patients compared with 13% of control subjects (chi^2 = 97; $p<0.001$). The 3 patients without an IVS3-48C allele were investigated by mutational analysis. For mutation detection, all exons with 30-250bp of flanking sequences and 250bp of the promoter region of the *FECH* gene were PCR-amplified from genomic DNA and sequenced using an ABI Prism 3100 Sequence Analyzer. One patient was homozygous for a missense mutation (416A>T; Q139L). Another was a compound heterozygote for the missense mutations 707G>A (C236Y) and 1137G>C (K379N). Expression of the C236Y and K379N mutants in E. coli showed that they decreased FECH activity to 12% and 38% of wild-type activity, respectively. In an asymptomatic parent, the C236Y mutation was *trans* to an IVS3-48C allele. No *FECH* mutation was found in the third patient. To search for AR EPP among patients with an IVS3-48C allele, the *FECH* gene was sequenced in 14 randomly selected patients. Mutations were identified in 11 patients (79%). One patient was an apparent compound heterozygote for the previously described missense mutations P334L and G55C, the latter having been reported only in AR EPP [2]. Our findings confirm the association between the IVS3-48C allele and overt EPP, suggest a minimum prevalence of 3% for AR EPP and have implications for genetic counselling of families with this disease. (1) Gouya L et al. Nature Genet 2002;30:27, (2) Lamoril J et al. Biochim Biophys Acta 1991;181:594, (3) Sarkany R et al. Lancet 1994;343:1394.

HO1 EXPRESSION AND IMMUNOHISTOCHEMICAL LOCALIZATION IN A MICE MODEL OF CHEMICALLY INDUCE HEPATOCARCINOGENESIS

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Heme oxygenase (HO) breaks down the pro-oxidant heme into carbon monoxide, iron, and the antioxidant biliverdin. The isoform HO1 plays an effective role to counteract oxidative damage and to control inflammation. Prolonged cellular damage due to chronic inflammation is one of the reasons leading to the development of tumors. We have previously demonstrated the role of oxidative stress in early stages of hepatocarcinogenesis (HCC) and the relevance of HO1 in this process. The aim of this work was to investigate HO1 expression and localization along the different stages of chemically induced HCC and the occurring morphological changes. To provoke sustained oxidative stress and chronic inflammation, *CF1* mice received dietary p-dimethylamino-azobenzene (DAB, 0.5%, w/w) during a whole period of 14 months. HO1 expression increased along the experimental trial in morphologically normal hepatocytes in DAB treated animals. HO1 expression diminished in altered hepatic foci (AHF) and oval cells, early preneoplastic lesions. Otherwise, marked HO1 overexpression was detected in Kupffer's cells and non fixed macrophagic cells surrounding necrotic and nodular areas. Adenomas were detected after 104 days of intoxication with decreased HO1 immunostaining. In hepatocellular carcinoma (at 10 months) an inverse relationship was found between the immunohistochemical expression of HO1 and tumor differentiation

degrees, which was negative in poorly differentiated tumors. These findings support the proposal of a protective role played by HO1 in oxidative stress and suggest that this protein expression would be critical in the inflammation process generated during carcinogenesis induced by chronic intoxication. Even more promising is the observation that in our experimental model of HCC down modulation of HO1 expression correlated with malignancy progression. Our data point out HO1 as a potential therapeutic target.

DUAL PORPHYRIA - PORPHYRIA CUTANEA TARDA AND VARIEGATE PORPHYRIA - IN ARGENTINA

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Porphyrias are hereditary and independent diseases due to a specific enzymatic failure in heme metabolism. Although the existence of two different porphyrias (Dual Porphyria) in one patient is unfrequent, cases of Dual Porphyria where Porphyria Cutanea Tarda (PCT) and Acute Intermitent Porphyria (AIP) and PCT and Variegate Porphyria (VP) coexisted, have been reported. Superimposition of both urinary and faecal porphyrin excretory patterns and clinical symptoms are observed in these patients. We report here a case of Dual Porphyria in a patient having both PCT and VP. In Argentine PCT and AIP are the most frequent porphyrias. We have already diagnosed a big family carrying PCT/AIP Dual Porphyria. Because VP is not very frequent in our country, its association with other porphyria is yet more rare. We present the case of two sisters showing cutaneous signs typical of PCT: blisters, photosensitivity, hyperpigmentation, hypertrichosis, cutaneous fragility, and no acute symptomatology. GW (age 28 years): urinary porphyrins (UP) 1975 µg/ 24 h exhibiting characteristic PCT pattern but with high coproporphyrin content (Uro: 36%, Hepta: 25%, Hexa: 4%, Penta: 10%, Copro: 25%). MW (age 32 years) UP: 256 µg/24 h showing abnormal but not PCT pattern (Uro: 4%, Hepta: not detectable (nd), Hexa: nd, Penta: 16%, Copro: 80%). In both patients urinary δ-aminolevulinic acid (ALA) (GW: 1.0 mg/24 h; MW: 1.5 mg/24 h) was normal and porphobilinogen (PBG) was only slightly increased (GW: 3.2 mg/24 h; MW: 2.8 mg/24 h. Faecal porphyrins were elevated: GW: 270 µg/dry weight (Uro: 2%, Hepta: 1%, Hexa: 1%, Penta: 10%; Copro: 35%, Isocopro: 10%, Proto: 36%), MW: 896 µg/dry weight (Uro: 2%, Hepta: 1%, Hexa: 1%, Penta: 2 %; Copro: 35%, Isocopro: 10%, Proto: 49%). In this patient faecal porphyrin content was rather high for a PCT however characteristic for a VP pattern with predominance of Proto. Plasma porphyrin index (PPI) was above normal (<1.30) in both patients: GW: PPI was 4.33 with a wavelength (λ) at 618 nm characteristic for PCT, MW: PPI was 6,20 with λ at 628 nm characteristic for VP. According to these results patient GW presents a Dual Porphyria PCT/PV while her sister, up to date, has only developed VP. Genetic studies are been carrying out in the uroporphyrinogen decarboxylase and protoporphyrinogen oxydase.

COPROPORPHYRINOGEN OXIDASE: STRATEGIES FOR UNRAVELING STRUCTURE-FUNCTION RELATIONSHIPS IN ENZYME DEFICIENT IN HEREDITARY COPROPORPHYRIA

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Heme, iron(II)-protoporphyrin, is indispensable for life. The biosynthetic pathway for heme is conserved across all three phyla - bacteria, archaea, and eukarya. Heme acts as a prosthetic group for numerous essential hemoproteins. Genetic defects in the heme biosynthetic pathway results in disorders known as porphyrias. Coproporphyrinogen oxidase (CPO) catalyzes the antepenultimate step in heme biosynthesis. CPO deficiency causes hereditary coproporphyria (HC) (1), inherited in an autosomally dominant fashion. Although a decade has passed since the cloning of human CPO, structural insights into this very important enzyme has not been forthcoming due to difficulties with crystallization. To overcome these hurdles we have resorted to two surefire strategies: (a) utilizing disease-causing CPO variants and (b) cloning of CPO from a number of mesophilic and thermophilic bacteria. The basic idea behind this strategy is that it utilizes naturally occurring variants to increase the chances of obtaining diffraction quality crystals. Two naturally occurring CPO mutants (R331W and Δ390Gly) and CPO from *Chloroflexus aurantiacus* and *Thermosynechococcus elongatus* were chosen as lead candidates for our trials. Cloning and overexpression of these proteins in *E. coli* has allowed us to successfully narrow down crystallization conditions for one or more of these proteins. In addition, we have also cloned CPO from the sea slug *Aplysia californica* and will present its expression profile in this well-studied model organism. Examination of the CPO sequences from 47 species reveals a highly conserved motif, RRGRYVEFNL, that may play a crucial role in substrate binding and catalysis. Based on the available data, we have constructed a model to describe how CPO recognizes coproporphyrinogen III and converts it to protoporphyrinogen IX. Diffracting crystals and phase information have been obtained for human and bacterial CPO and structural refinement is in progress.[Supported by Grant from MSMT of Czech Republic (LN 00A079)] (1) Martásek, P. Semin. Liver Dis. 18: 25-32, 1998.

DIAGNOSING PORPHYRIA – A CLINICAL AND BIOCHEMICAL SURVEY IN SWISS PATIENTS WITH ACUTE-INTERMITTENT PORPHYRIA

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Acute-intermittent porphyria is an autosomal dominant inherited disorder with incomplete penetrance. Molecular analysis showed that carriers of identical mutation of the HMB-synthase gene greatly differ in the expression of their disease. Therefore, we performed a survey in Swiss AIP patients and their latent family members to search for possible mechanisms of disease manifestation. The following questions were addressed:

(1) To what extent does the diagnosis of acute-intermittent porphyria affect the quality of life?
(2) Are there any detectable exogenous or endogenous factors that correlate to disease expression?
(3) Does the biochemical profile in urine discriminate between latent and overt disease?
(4) Is there any x-chromosomal marker that correlates to disease expression as suggested by the higher rate of symptomatic disease in females than in males?
(5) Does an allele specific inheritance of the rate limiting enzyme ALA-synthase 1 explain the disease expression?

Twenty-one of the 40 individuals proven by molecular diagnosis to have inherited a mutation of the HMB-synthase were symptomatic. Quality of life was only affected in severely symptomatic patients. The only exogenous factor detected to correlate with overt disease was smoking. Latent mutation carriers showed lower excretion of porphyrin precursors and porphyrins in urine than symptomatic individuals. ALA (Mean ± SD) was 3.68±2.05 vs 9.30±6.55 µmol/mmol creatinine, PBG was 3.51±3.26 vs 19.4±18.7 and porphyrins were 25.1±22.0 vs 102.4± 64.3 nmol/mmol creatinine. Seven of the 19 individuals with latent disease had completely normal urinary porphyrin excretion rates, but none of 21 individuals who had experienced at least one attack in their preceding life time. We found that the gender specific frequency of overt disease is compatible with an dominant x-chromosomal manifestation factor. This was not only true in our patient cohort, but also in recently published series of Swedish and Argentinean AIP patients. However, no significant correlation of x-chromosomal markers (10 cM) was found within the families tested. Further, we also noticed a transmission of overt disease from fathers to daughters. Both findings argue against an x-chromosomal manifestation factor. Lastly, we tested, if an allele-specific inheritance of ALA-Synthase 1 explains incomplete penetrance. Fifteen different polymorphic sites - three of them within the promotor region - of ALA synthase 1 were assayed but none of them were correlated with disease expression. [This work was supported by a grant from Hartmann-Müller Stiftung].

DO THE DIFFERENT MUTATIONS IN THE HFE GENE INFLUENCE THE DISTRIBUTION OF THE PORPHYRIN LEVELS IN PORPHYRIA CUTANEA TARDA?

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Porphyria cutanea tarda (PCT) results from a decreased activity of uroporphyrinogen decarboxylase in the liver. PCT is not a single monogenic disorder with a simple pattern of inheritance. Clinically overt form of any subgroup of PCT is induced by some liver damage caused by a long-term action of hepatotoxic agents like alcohol, estrogen, hepatitis (mainly type C), iron overload of any origin (e.g. hereditary haemochromatosis gene /HFE/ mutations) (1, 2). The goal of the study was to investigate the influence of the mutations H63D and C282Y in HFE gene on the porphyrin levels. PCT patients with and without HFE mutation(s) were examined; all they are registered and cared at our Department. Porphyrins were analysed with HPLC method of Seubert and Seubert (3). HFE mutations were identified by Zs. Nagy et al. (4). Mean values of the urinary porphyrin levels in untreated PCT patients are shown in the Table. In PCT, HFE gene mutation(s) can further increase the total porphyrin levels. Any heterozygous HFE gene mutation can cause only a slight alteration in the porphyrin pattern. In the case of either double heterozygosity (C282Y/H63D) or homozygosity for C282Y, perceptibly increased total porphyrin levels with higher share of the heptacarboxyl porphyrin fraction could be observed.

	HFE negative n=20	C282Y homo-zygous n=2	C282 hetero-zygous n=2	H63D hetero-zygous n=4	C282Y/H63D double heterozygous n=1	Healthy controls n=20
Total porphyrin umol/day	4340	7159	4854	4112	4928	<240
Uro %	66	54	65	68	52	<15
Hepta %	22	28	23	20	28	<5
Hexa %	4	6	3	4	8	<5
Penta %	3	6	4	3	5	<5
Copro %	5	6	5	4	7	>75

Different mechanisms take part in the deterioration of the decarboxylation. Complex contribution of all the possible simultaneous risk-factors may slightly alter the kinetics of the decarboxylation.

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HEPATIC MITOCHONDRIAL RESPIRATORY CHAIN ENZYME ACTIVITIES CORRELATED WITH EXTENT OF LIVER DAMAGE IN A MURINE MODEL FOR ERYTHROPOIETIC PROTOPORPHYRIA

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The Fechm1pas/Fechm1pas murine model for erythropoietic protoporphyria (EPP) affords an excellent system with which to investigate the pathogenesis of severe protoporphyria-induced liver failure. The aim of this study was to identify factors involved in the development of this type of hepatic disease. Interestingly, mice from different strains congenic for the same ferrochelatase germline mutation manifest variable degrees of hepatobiliary injury. Protoporphyric animals bred into the C57BL/6J background showed a higher degree of hepatomegaly and liver damage as well as higher protoporphyrin accumulation than those bred into the SJL/J and BALB/cJ backgrounds. Mitochondrial respiratory chain (MRC) activities remained unchanged in the liver of C57BL/6J mice, whereas they were increased in protoporphyric mice from both SJL/J and BALB/cJ backgrounds. However, no alterations of MRC activities were detectable in spleen, kidney and bone marrow from EPP mice. MRC activities were increased in Hep G2 cell lines after accumulation of protoporphyrin following addition of delta-aminolevulinic acid. As a direct effect of these elevated MRC activities, in both hepatic cells from mutant mouse strains and Hep G2 cell lines, ATP levels significantly increased as the intracellular protoporphyrin concentration was reduced. In conclusion, these results indicate that the cytotoxic effects of protoporphyrin accumulation in the liver may be counterbalanced by increases in ATP suggesting that increased MRC activities protect against hepatocellular injury in EPP mice. It may prove helpful to assess MRC activities as an approach to identifying that subset of EPP patients at risk for developing severe liver injury.

COPROPORPHYRIA IN ARGENTINEAN PATIENTS: GENETIC STUDIES

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Hereditary coproporphyria (HCP) is a metabolic disease produced by a deficiency in coproporphyrinogen III oxidase (CPO), the sixth enzyme of heme pathway, first reported in 1955. Clinically, HCP is a mixed porphyria and patients may present abdominal pain and/or cutaneous symptoms. Biochemically, diminished activity of CPO produces accumulation of coproporphyrin both in urine and faeces and during attacks, excess urinary porphobilinogen and delta-aminolevulinic acid excretion is found. The human CPO gene contains seven exons and spans 14 kb of genomic DNA. The cDNA has an open reading frame of 1062 bp encoding a protein of 354 amino acids and the mature protein consists of 323 amino acid residues. So far, 33 different mutations and 8 polymorphisms have been characterized in the human CPO gene: 23 nucleotide substitutions, 3 splicing mutations, 4 small deletions, 2 small insertions and 1 gross deletion. Genomic DNA was isolated from peripheral blood samples from 4 HCP Argentinean patients and their available relatives using InstaGene Whole Blood Kit (Bio Rad). The 7 exons of CPO gene, including the flanking intronic regions were amplified by the polymerase chain reaction and sequenced using a DNA sequencing kit (Applied Biosystems), reactions were run on an ABI PRISM 310 Genetic Analyzer (Perkin Elmer). In the CPO gene of four unrelated families we have detected 4 novel mutations: one point mutation g/a in intron 6 at position +5 of the donor site of splicing (IVS6 +5g/a), one point mutation in exon 6 changing a tyrosine (TAT) by a cysteine (TGT) (Y392C), one point mutation a/c in the acceptor site of splicing in intron 2 (IVS2-2 a/c) and the deletion of one C in exon 1 (279delC). This is the first genetic study in HCP Argentinean patients and these results are showing again, the genetic heterogeneity of the porphyrias.

MOLECULAR ANALYSIS OF THE PPOX GENE IN ITALIAN PATIENTS WITH VARIEGATE PORPHYRIA (VP): IDENTIFICATION OF 3 NOVEL MUTATIONS

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Variegate porphyria (VP; MIM 176200) is a low-penetrance, autosomal dominant disorder resulting from the partial deficiency of protoporphyrinogen oxidase (PPOX; EC 1.3.3.4), the 7th enzyme of the heme biosynthesis. VP is clinically characterized by photosensitivity and occasional acute neurovisceral attacks. Among the diagnostic criteria, the most powerful is the detection of a plasma fluorescent peak at 630 nm, present in roughly 70 % of the symptomatic VP patients. More than 100 molecular abnormalities have been so far identified in the PPOX gene as responsible for VP showing a high molecular heterogeneity. Few data are presently available on the Italian population. Aim of this study was to identify the molecular defect in the PPOX gene in 9 Italian unrelated subjects (1 male, 8 females) with suspected diagnosis of VP. The plasma peak is detected by fluorometric plasma scan from 580 to 650 nm. The coding region of PPOX gene was amplified by PCR in six fragments and directly sequenced. The plasma

peak at 630 nm was markedly positive in 8 subjects and slightly positive in one. Eight different mutations have been identified among the 9 VP patients and summarized in the following Table.

Pt.	Sex	Mutation	Exon	Protein change	Pl. peak (630 nm)	Ref.
1	M	218 T>C	3	L73P	+	Whatley 1999
2	F	306 insC	4	FS>stop+41	++	This study
3	F	532 C>G	6	L178V	+	De Siervi 2000
4	F	694 G>C	7	G232R	+	Deybach 1996
5	F	745 insG	7	FS>stop+31	++	Deybach 1996
6	F	851 G>T	8	S248I	+/-	This study
7	F	1013 C>G	10	S338X	++	This study
8	F	1013 C>G	10	S338X	++	This study
9	F	1082 insC	10	FS>stop+19	++	Whatley 1999

The 1013 C>G mutations was identified in two unrelated subjects. The 306 insC, (FS>stop+41), 851 G>T (S284I) and 1013 C>G (S338X) mutations have been identified for the first time in this study and cause truncated or unstable proteins. 1082 insC seems to be quite common being found in 12% of VP patients in France and in another Italian study. The molecular analysis has been extended to 10 probands' family members with the finding of 7 mutation carriers. These results, suggest a high molecular heterogeneity for VP in Italy as described for other countries.

STRUCTURAL BASIS FOR TETRAPYROLE COORDINATION BY UROPORPHYRINOGEN DECARBOXYLASE

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Uroporphyrinogen decarboxylase (URO-D) removes a carboxyl group from each of the 4 acetate side chains of uroporphyrinogen (uro'gen) without the need for cofactors to act as proton or electron donors. Subnormal activity of URO-D is the cause of porphyria cutanea tarda (PCT), the most common of the human porphyrias. Approximately 1/3 of patients with PCT are heterozygous for inherited mutations at the URO-D locus, transmitted as an autosomal dominant trait (familial-PCT). We have characterized the structural and functional effects of many of the mutations found in familial-PCT (Blood. 2001; 98:3179-3185). The enzyme is dimeric with a two fold symmetry that lies between the active sites. We have crystallized URO-D under anaerobic conditions in the presence of its fully reduced porphyrinogen substrate. Using the structure of the enzyme product complex we have begun to probe the enzyme mechanism through site-directed mutagenesis. Recombinant His-tagged human URO-D (rhURO-D) was concentrated to approximately 10 mg/ml in buffer with 10% glycerol and crystallized in the presence of enzymatically generated uro'gen I or III in an anaerobic chamber. The structure of the rhURO-D-porphyrinogen complex revealed coproporphyrinogen (copro'gen) in the active site, indicating that the uro'gen substrate had been decarboxylated but the reaction product was not released. The structure revealed no change in the configuration of URO-D compared to the unliganded protein. Copro'gen in the active site was in a domed shape with the 4 pyrrole nitrogens pulled above the plane of the macro cycle through hydrogen bonding to the carboxyl group of Asp86. These data explain why only reduced porphyrinogens can serve as substrates of URO-D as the oxidized porphyrin lacks the flexibility to adopt the domed conformation to orient the macro cycle for catalysis. Three arginine (residues 37, 41 and 50) stabilize the domed macro cycle through hydrogen bonding with the propionate side chains of the porphyrinogen. A hydrophobic domain within the active site, interacting with one quadrant of the macro cycle, is essential for decarboxylation. Mutating either of 2 phenylalanines (residues 154 and 217) to less hydrophobic residues effectively ablated decarboxylation. No residue with the potential to protonate the macro cycle is appropriately positioned within the hydrophobic domain but a domain interacting with the adjacent quadrant contains Tyr164 and Ser219, both capable of protonating the macro cycle. Mutation of either of these residues severely reduces enzymatic activity. Collectively, these data indicate that catalysis requires stabilization of the porphyrinogen substrate in a domed configuration, protonation of the macro cycle and a critical interaction between the hydrophobic domain and an acetate carboxyl group. The specific activity of hepatic URO-D is markedly reduced in humans with PCT and in experimental models of PCT (PNAS. 2001;98, 259-264), strongly suggesting that an inhibitor of URO-D is involved in the pathogenesis of the disease. Defining the structural requirements for URO-D activity may lead to the mechanism by which an inhibitor reduces enzymatic activity.

PROGNOSTIC VALUE OF SCALING IN ACUTE ATTACK OF AIP

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Scaling is an important tool in the modern medicine, necessary for standard clinical evaluation. However, no scales for a severity of acute attacks of AIP exist. Scales designed for other diseases, including those accompanied by peripheral neuropathy, are not suitable for acute porphyrias since many specific features and their combinations are characteristic for acute porphyrias. Clinical manifestations and outcome of an acute attack in 12 Russian AIP patients with severe neurological manifestations during an acute attack were used as a basis for developing a scale by factor analysis. Assessment of muscle strength was performed according to Medical Research Council scale (MRC) from 0 to 5 grades in six muscle groups from both sides (MRC score sum in healthy subject = 60). Intensity of pain was assessed using linear Visual Analogue Scale (VAS, 0 to 10 cm). According to factor analysis, muscle weakness (MRC score sum 25.5±20.2 corresponding to mean 3.6 scores, range 0-6), prolonged mechanical ventilation (from 21.3±20.1 days, mean 2.1 scores, range 0-6), bulbar palsy (mean 2.1 scores, range 0/6), impaired consciousness (mean 1.4 scores, range 0-6), hyponatremia (mean 123.5±9.2 mmol/L, mean 1.9 scores, range 0-6) and arrhythmia (mean scores 0.3, range 0-3) suggested a poor prognosis (max total scores = 33). Other signs and symptoms, such as abdominal pain, tachycardia, hypertension, vomiting, constipation and sensory loss did not affect the prognosis and epileptic seizures correlated with a prognosis only slightly. Thus, they were excluded from the scale (scaled 0). In conclusion, the total score predicted the short and long term-outcome (I:6 months or residual signs; III:>25 scores, critical condition, p=0.0004) and attacks could be classified as mild (=0 scores), moderate (1-4 scores), severe (5-25 scores) or critical (26 -33 scores). In mild attacks, measuring the pulse and following intensity of pain may indicate the activity of the disease. A form to follow-up a patient's clinical condition for evaluation of scoring will be presented.

IDENTIFICATION OF NINE MUTATIONS INCLUDING THREE NOVEL MUTATIONS AMONG RUSSIAN AIP PATIENTS AND DESCRIPTION OF ONE HOMOZYGOUS PATIENT

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Blood samples were collected during 1996-2001 from 11 unrelated Russian AIP patients with severe neurological manifestations who were admitted to the city and local hospitals of the northwestern part of Russia. In addition 31 samples of their relatives and 50 healthy Russian controls were analyzed to exclude the common polymorphisms. Direct sequencing of genomic DNA was performed to find mutations in the PBGD-gene. Digestion with restriction enzymes was used whenever possible. Complementary DNA was synthesized from 5 µg of total RNA extracted from patients Epstein-Barr virus transfected lymphoblastoid cell lines. The mutations were expressed in COS-1 cells

using SV-poly expression vector. The PBGD activity of the mutant transcript was assayed. Nine different mutations, were identified, of which two mutations (77G>A (R26H) and 517C>T(R173W)) were verified in two unrelated families. Of the nine mutations, six were reported previously in AIP patients of Western and Eastern European countries (77G>A(R26H), 517C>T(R173W), 583C>T(R195C), 673C>T(R225X), 739T>C(C247R) and 748G>C(E250A) and three (IVS13+5G>C, IVS13+3_6delAAGT, 770T>C) were novel mutations. Mutations IVS13+5G>C and 770T>C were confirmed by restriction analysis in addition of sequencing and mutation IVS13+5G>C by cDNA sequencing revealing two different transcripts. In all three families the mutation co-segregated with the low erythrocyte PBGD activity or elevation of urinary PBG. One patient, manifesting with an acute attack only at the age of 57, was homozygous for the mutation 748G>C (E250A). The mutation showed a 9% residual PBGD activity in COS-1 cells. In conclusion, total of nine different mutations in 11 unrelated Russian AIP patients were identified. Three of them were novel mutations. As in most other populations, Russian AIP patients are genetically heterogeneous and mutations are family-specific. Out of nine mutations, six were previously reported in Europe. We report a homozygous patient (E250A) with late onset of clinical manifestations. The residual PBGD activity resulting from the mutation is sufficient to explain survival in homozygous patient with the mutation.

UPREGULATION OF CYP1B1, CYP3A, TAP1 AND TAP2 IN CULTURED LYMPHOCYTES FROM PATIENTS WITH PORPHYRIA CUTANEA TARDA AFTER IN VITRO-STIMULATION WITH INTERFERON ALPHA

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Porphyria cutanea tarda (PCT) is the most frequent cutaneous disorder of porphyrin-heme biosynthesis worldwide. At least two forms of PCT are currently distinguished: Sporadic/acquired (type I) PCT without family history, and familial/inherited (type II) PCT, resulting from dominantly inherited mutations in the uroporphyrinogen decarboxylase (URO-D) gene. Both types of PCT arise from decreased activity of the encoded enzyme URO-D, leading to cutaneous porphyrin deposition and photosensitization on the sun exposed areas of the skin. However, the enzymatic deficiency by itself is usually not sufficient for the development of cutaneous symptoms. Clinically overt disease rather requires the involvement of specific triggering factors. These factors are mainly alcohol, estrogens, iron and polychlorinated hydrocarbons. Interestingly, in a 63-year-old male with malignant melanoma we observed the occurrence of PCT after interferon alpha (IFN-alpha) treatment. Mutation analysis of the URO-D gene revealed a heterozygous missense mutation in this individual, indicative of type II PCT. Subsequently, we cultured lymphocytes obtained from peripheral blood of the patient and also from a second patient with clinically overt type I PCT. Lymphocytes from both patients were stimulated in vitro at different concentrations with INF-alpha and phenobarbital, a further drug known to provoke PCT. Interestingly, we observed a significant upregulation of cytochrome P450 (CYP) 1B1 and CYP3A in both individuals. Further, an upregulation of CYP 3A5 was detected in the patient with type II PCT after stimulation with phenobarbital. mRNA-expression of the transport-associated proteins (TAP) 1 and 2 was upregulated in the individual with type II PCT after stimulation with IFN-alpha proving the modulating effect of long term immunotherapy on antigen presentation. However, this effect was not observed in the patient with type I PCT who did not receive INF-alpha. Although previous data obtained in hepatocytes indicated a downregulatory effect of INF-alpha on CYP expression, our results show that it leads to an upregulation of CYP expression in other tissues. Our results suggest that cytokines like INF-alpha, commonly used in low and high dose immunotherapy, represent as yet unrecognized mediators with the potency of triggering PCT.

DEMYSTIFICATION OF CHESTER PORPHYRIA: A NONSENSE MUTATION IN THE PORPHOBILINOGEN DEAMINASE GENE

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The porphyrias are disorders arising from predominantly inherited catalytic deficiencies of one of the eight enzymes along the heme biosynthetic pathway. All genes encoding these enzymes have been cloned and several mutations underlying the different types of porphyrias have been reported. The diagnosis of porphyria is traditionally made on the basis of clinical symptoms, characteristic biochemical findings, and specific enzyme essays. In some cases however, these diagnostic tools reveal overlapping data, indicating the existence of dual porphyrias with two enzymes of heme biosynthesis being deficient simultaneously. Recently, it was reported that the so called Chester porphyria shows attributes of both variegate porphyria and acute intermittent porphyria. Linkage analysis revealed a novel chromosomal locus on chromosome 11 for the underlying genetic defect in this disease. For the first time, these data suggested that a gene, which does not encode one of the enzymes of heme biosynthesis might be involved in the pathogenesis of the porphyrias. After excluding several candidate genes within the originally published new linkage interval, we identified a nonsense mutation in the porphobilinogen deaminase gene, which harbors the mutations causing acute intermittent porphyria, as the underlying genetic defect in Chester porphyria. However, we could not detect a mutation in the coding or the promotor region of the protoporphyrinogen oxidase gene that is mutated in variegate porphyria. Our results indicate that Chester porphyria is neither a dual porphyria, nor a separate type of porphyria, and also exclude the possibility that a hitherto unknown gene is involved in the pathogenesis of this disorders. We suggest that all so-called dual porphyrias should be studied on the molecular genetic level before claiming their existence.

ACUTE PORPHYRIA - BUT WHICH ONE?

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A 36-year-old woman was admitted to our center with tetraparesis, assisted breathing, red urine and hyponatremia (113 mmol/l). Starting one month earlier she experienced weakness, increasing colicky abdominal pain and developed paralytic ileus and tetraparesis. Biochemical studies revealed increased porphyrin precursors (porphobilinogen: 348 µmol/l, aminolevulinic acid: 114 µmol/l) and porphyrins (uroporphyrin: 237 nmol/l, coproporphyrin: 3161 nmol/l) in the urine indicating acute porphyric attack. The patient improved for hematine therapy, after 6 months of physical therapy she was able to walk again and had no symptoms. To identify the type of her acute porphyria HPLC analysis of the feces was carried out. The result did not indicate the presence of coproporphyria or variegate porphyria. Later the coproporphyrinogen oxydase and protoporphyrinogen oxydase activities were measured in the French Porphyria Center in Paris and were within the normal range. The aminolevulinic acid dehydratase activity was normal too. The hydroxymethylbilane synthase activity was high (150% of the normal level) which occurs during an acute attack as it was reported in the literature, but it remained high even years later (97% of the normal level). The porphobilinogen level in the urine also remained elevated. These findings suggested the non-erythroid form of

acute intermittent porphyria. According to this exon 1, its boundaries and the promoter region of the hydroxymethylbilane synthase gene were sequenced in both direction but no mutation was detected. The aim of this presentation was to show a case where the usual diagnostic laboratory methods failed to identify the type of acute porphyria making impossible to screen the family members for this disease. [The genetic study of this work was supported by the Hungarian Ministry of Welfare (ETT 025/2000)].

AN ALTERNATIVELY-SPLICED 5'UNTRANSLATED EXON IN HUMAN ALAS1 INHIBITS TRANSLATION AND HAEM-REGULATED mRNA DESTABILISATION

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Heme controls its own supply in non erythroid cells by regulating the stability of the mRNA encoding 5-amino-levulinate synthase (ALAS1), the first enzyme of the heme biosynthetic pathway. The human ALAS1 gene contains two non coding exons (1A and 1B) that are alternatively spliced to generate two mRNA species: a major one in which exon 1B is omitted and a ubiquitously expressed minor one, representing approximately 10% of the steady state concentration of ALAS1 mRNA, which contains exons 1A and 1B. We have investigated the role of this spliced form in regulated ALAS1 expression. Firstly, heme minimally affects the concentration of the minor mRNA species at a concentration that destabilises the major form. In addition, the 5' UTR of the minor mRNA inhibits, 8 fold, the translation of a heterologous reporter gene compared to the 5' UTR of the major mRNA species in HepG2 cells. These data suggest that the mechanism of heme-dependent destabilisation of ALAS1 mRNA may require its active translation. We have also investigated the sequence elements within of the minor mRNA that inhibit translation. Notably the 5' UTR contains 4 short overlapping upstream open reading frames (uORFS; U1-U4) that may inhibit cap-mediated translation by regulating the scanning of ribosomes to the ALAS1 ORF. Alternatively, the 5' UTR may contain an Internal Ribosomal Entry Site (IRES) that allows translation under stress conditions in which cap-mediated translation is compromised.

PRO- AND ANTIOXIDANT FACTORS IN ACUTE INTERMITTENT PORPHYRIA

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Proven the crucial role of iron and porphyrins in the oxidative cellular damage in chronic porphyrias, we have undertaken an extensive study in families with acute porphyrias in order to evaluate the possible role of similar oxidative damage even in these diseases, whose natural history is often complicated by neoplastic evolution (hepatocellular carcinoma), as well. Four unrelated patients affected by Acute Intermittent Porphyria (AIP) were studied together with 37 members from correspondent different families. All subjects have been assessed for: serum δ-aminolevulinic acid and porphobilinogen; urinary, erythrocyte and fecal porphyrins; porphobilinogen-deaminase enzymatic activity; iron status (plasma iron, transferrin and ferritin); serum different antioxidants (ascorbic acid, retinol, tocopherol, α- and β-carotene) and urinary and plasma metabolites of nitrous oxide. No significant increase in plasma markers of oxidative damage was observed in PAI patients. No significant correlation between porphyrin precursors accumulation and decrease in the vitamin antioxidant potential was observed; when present, oxidative damage markers resulted significantly related to spontaneous or iatrogenic iron accumulation. Family studies in AIP must also include evaluation of iron stores in order to prevent a further oxidative damage and probably a neoplastic evolution of the disease. The mechanism underlying the occurrence of neoplastic evolution in PAI in absence of iron accumulation seems however to depend on pathophysiological mechanisms different from those involved in chronic porphyrias (and probably not involving free radicals generation).

EVALUATION OF NEUROTOXICITY OF PORPHYRINOGENIC AGENTS THROUGH DIFFERENT HEME METABOLISM PARAMETERS

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Several drugs are mainly involved in the triggering of neurological attacks in acute porphyrias, however, the neurological mechanism involved has not been yet elucidated. We have previously demonstrated that some porphyrinogenic agents altered brain cholinergic system. Heme oxygenase (HO), the rate-limiting step in heme degradation exists in three isoforms HO-1, HO-2 and HO-3. Brain HO activity exceeds that of various systemic organs including liver, this activity is largely attributed to HO-2. Cytochrome P-450 (CYP) was found to be mainly localized in mitochondria but only a small quantity of the enzyme was also found in the microsomal fraction. Despite their relatively low content in the brain tissue, cytochromes P450 have been identified as functional enzymes, allowing central nervous system to metabolize a variety of substrates of both exogenous and endogenous origin. The aim of this work was to investigate how known porphyrinogenic drugs affect δ-aminolevulinic acid synthetase (ALA-S) and HO and which is the role of CYP in metabolizing these drugs in brain mice. ALA-S activity was more than 100% induced after anaesthetic, veronal and ethanol administration. HO activity was increased after chronic Enflurane and Isoflurane administration, dietary griseofulvin and after starvation. ALA-S and HO gene expression were also evaluated. Mitochondrial CYP levels were 67% induced after dietary griseofulvin, but it was 20-40% reduced after starvation and chronic isoflurane, allylisopropyl-acetamide and cutaneous griseofulvin application. Data indicate that the effects of the drugs studied on brain parameters differ from those reported for liver tissue and depend on the agent examined.

OVERT PCT IN A SYMPTOMATIC AIP PATIENT ON HEMODIALYSIS

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The patient is a woman born in 1953 who had her first AIP attack at the age of 24 years. Since the age of 40 years she was continuously affected by recurrent serious acute attacks. On each occasion she was treated during 3-4 days with glucose and electrolyte infusions, analgetics and heme infusion (NormosangR). At the age of 50 years she has received 220 NormosangR cures, corresponding to 110 g hemarginate containing 9.5 g iron. During the last years renal impairment has progressed to end-stage renal disease (ESRD) requiring hemodialysis (HD), with three dialysis occasions per week, and administration of erythropoietin and parenteral iron. After the start of hemodialysis treatment the clinical picture has changed. The acute attacks have diminished in intensity and frequency, but the incipient motoric neuropathy has rapidly progressed and she is now bound to wheelchair. She has developed intensive PCT symptoms with skin blisters on sun exposed areas of the body. The porphyric biochemical pattern has changed as well. Plasma porphyrins are increased (>1000 nmol/L, normal <10 nmol/L) and the porphyrin pattern is dominated by polycarboxylated porphyrins in accordance with PCT. The levels of plasma porphyrins are not, or only slightly, affected by HD. The plasma levels of the porphyrin precursors ALA and PBG are affected by dialysis, especially PBG that is shown to be much lowered by dialysis. Clinical symptoms of AIP, although less common than before, are treated as usual. In contrast, attempts to treat PCT symptoms are impeded by the concurrent metabolic conditions, AIP and ESRD. Different aspects of the management are discussed in the presentation.

FIRST CLINICAL TRIAL OF I.V. rhPBGD IN HEALTHY SUBJECTS WITH AND WITHOUT DIAGNOSED MANIFEST ACUTE INTERMITTENT PORPHYRIA (AIP)

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The study was a combined phase I and II study with the primary objective to study the safety and tolerability of a single and repeated dose(s) of i.v. rhPBGD and the pharmacokinetics of a single and repeated dose(s) of i.v. rhPBGD. The secondary objectives were to study the biochemical efficacy by measuring the change in plasma concentration of PBG over time for both single and repeated dose(s) of rhPBGD. The trial was an open label, dose escalating, rhPBGD single dose (Part A), and a double blind, randomised, placebo controlled repeated dose (Part B) study. There were 4 doses (0.5 mg/kg, 1 mg/kg, 2 mg/kg, 4 mg/kg) and Part A of the trial involved 12 AIP subjects with 3 subjects at each dose level. Part B involved 20 healthy AIP subjects and 20 healthy male (non-AIP) subjects with 10 subjects at each dose level, one AIP subject and one healthy male (non-AIP) were randomized to placebo at each dose level. The results of this trial showed that rhPBGD seem to have biphasic pharmacokinetic characteristics. The terminal half-life was about 2.5 hours. Approximate dose proportionality was observed.. The i.v. bolus injection of rhPBGD produced an instant, almost complete, removal of plasma PBG lasting for at least 2 hours even at the lowest dose of 0.25 mg/kg bid. After 2 hours, the plasma PBG level increased gradually at slower rates for higher doses and the initial level is reached after 12 hours. No reduction was produced by placebo. No safety issues were raised during the trial and rhPBGD was well tolerated.

IDENTIFICATION OF KEY ELEMENTS THAT ARE RESPONSIBLE FOR HEME-MEDIATED INDUCTION OF THE CHICK HEME OXYGENASE-1 GENE

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Heme oxygenase (HO) catalyzes the conversion of heme to biliverdin with the release of iron and carbon monoxide. HO-1, the first isoform of HO to be identified, is highly inducible by a large number of physical and chemical factors. Many of these factors cause oxidative or other stresses to cells. In this work, we studied the regulation of the chick HO-1 gene by metalloporphyrins, using selected promoter-reporter constructs of the gene transiently or stably transfected into the chicken hepatoma cells (LMH) line. We identified and characterized the key regulatory elements in the 5'-flanking of the chick HO-1 gene which confers up-regulation of luciferase reporter gene expression in the presence of heme and cobalt protoporphyrin (CoPP). LMH cells were maintained in with 10% fetal bovine serum Waymouth's medium. Transfections of the constructs were carried out using Lipofectamine. Reporter gene expression and activation were assessed by quantitation of luciferase activity, normalized to β-galactosidase activity and protein content. To establish stable transfections, the transfected cells were incubated with 200 μg/ml geneticin beginning the day after transfection, and resistant colonies were selected over a 2-3-week period. The largest reporter plasmid, called pcHO7.1Luc, was constructed by cloning 7.1 Kb of the chick HO-1 proximal promoter into the pGL3 vector. The smaller plasmids, pcHO7.1-4.6Luc and pcHO4.6Luc, were constructed by deletion from pcHO7.1Luc. The plasmids, pcHO7.1-5.6Luc and pcHO5.6-4.6, were constructed by further deletion from pcHO7.1-4.6Luc. Site-directed mutagenesis of heme responsive elements (HeREs) was carried out using pcHO7.1-4.6Luc as template. The HeREs, located -4600, and -4676 from the transcription start site, were mutated from 5'- **tgc**TGTGTCA to 5'- **gag**TGTGTCA. The mutants were confirmed by DNA sequencing. Deletional analysis of chick HO-1 promoter/enhancer induction by heme and CoPP in transiently transfected LMH cells showed that heme and CoPP significantly up-regulated activity in pcHO7.1Luc (4.2, 5-fold), pcHO7.1-4.6Luc (3.1. 3.5-fold), and pcHO5.6-4.6Luc (4.1, 4.2-fold), but not pcHO7.1-5.6Luc (1.6, 0.6-fold), (Fig 1). These results suggested that the key regulatory elements of heme- and CoPP-mediated inductions are located 5.6 to 4.6 Kb upstream from transcription starting point of the chick HO-1 gene. Within this region, we identified two key "expanded" AP-1 sites at 4600 bp and 4676 bp upstream of the transcription starting point. The maximal induction responded particularly to heme and CoPP were 10 μM concentrations and 16 hours of exposure. Single or double mutations of these sites within pcHO7.1-4.6Luc significantly abrogated the heme-dependent, and the CoPP-dependent, up-regulation of reporter gene expression in transient transfection (Fig 2). Single and double mutations of these sites within pcHO7.1Luc in stable transfection showed the same results (data not shown). In conclusion, the chick HO-1 promoter region contains "expanded" AP-1 sites that are important for up-regulation of the gene by heme or CoPP. These key regulatory elements consist of consensus AP-1 binding sites that have been extended by three bp. [Supported by RO1DK38825 from NIH].

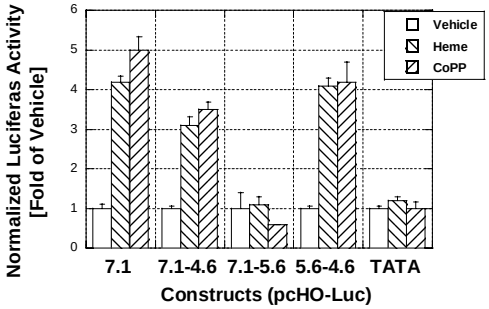

Figure 1. Deletional Analysis Chick HO-1 promoter/ enhancer indution by heme and CoPP in LMH Cells.

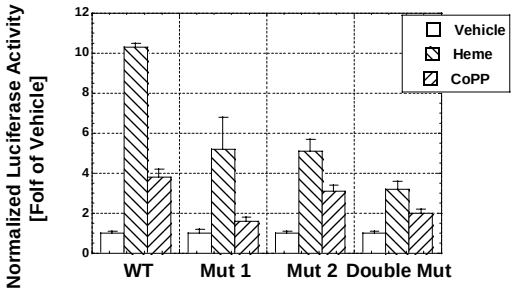

Figure 2. Effects of heme and CoPP on pcHO7.1-4.6Luc wild type and mutants in transiently transfected LMH cells.

NON-HEME INDUCTION OF HO-1 DOES NOT APPARENTLY ALTER CELLULAR IRON LEVELS

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The catabolism of heme is carried out by members of the heme oxygenase (HO) family. The predominant isozyme in the digestion of erythroid heme is the inducible form, HO-1. The products of heme catabolism by HO-1 are ferrous iron, biliveridin (subsequently converted to bilirubin), and carbon monoxide (CO). In addition to its function in the recycling of erythrocyte iron, this microsomal enzyme has been implicated in a number of cytoprotective mechanisms mainly in the context of oxidant insult. HO-1 can be induced not only by heme, but also by cytokines, heat shock, metals, and other cellular stressors, especially those that generate reactive oxygen species (ROS). Implicit in all the reports of HO-1 cytoprotection to date are effects on the cellular handling of heme/iron. While bilirubin is an antioxidant, ferrous iron is known to catalyze the production of hydroxyl radicals through Fenton chemistry. Thus, the release of iron from heme could be counterproductive in protecting the cell from oxidative stress. Furthermore, there are a number of non-heme stimulators of HO-1 induction, bringing to question the source of substrate for this enzyme in these paradigms. In the present study, HO-1 was induced by either sodium arsenite or hemin in the murine macrophage-like cell line,

RAW264.7. Both of these inducers elicited a transient increase in both the mRNA and protein levels of HO-1, however, only hemin exposure induced an increase in the synthesis rate of the iron storage protein, ferritin. This increase in ferritin production rate, as measured by ^{35}S-methionine incorporation, was attenuated by the HO inhibitor, tin-protoporphyrin IX (SnPP) as well as the cell permeable iron chelator, salicylaldehyde isonicotinoyl hydrazone (SIH). Additionally, treatment of the cells with hemin was able to elicit a decrease in the activity of iron regulatory proteins (IRPs) that could be blocked by preincubation with SnPP. Sodium arsenite had no effect on IRPs. These results suggest that iron released from hemin by HO-1 stimulates ferritin synthesis via the IRE/IRP system, while an increase in the enzyme via a non-heme inducer does not lead to iron release from endogenous heme sources.

HIGHLY EFFICIENT HEME SYNTHESIS IN ERYTHROID CELLS REQUIRES A DIRECT TRANSFER OF IRON FROM TRANSFERRIN ENDOSOMES TO MITOCHONDRIA

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During differentiation, immature erythroid cells acquire vast amounts of iron at a breakneck rate. Proper coordination of iron delivery and utilization in heme synthesis is essential and disruption of this process likely underlies iron loading disorders such as sideroblastic anemia and myelodysplastic syndrome with ringed sideroblasts. Iron is taken up by the cells via receptor mediated endocytosis, a process whereby diferric transferrin (Tf) binds to its cognate receptor (TfR) on the erythroid cell plasma membrane, followed by internalization of the Tf-TfR complex. Subsequent to endocytosis, the endosome is acidified by a H^+-ATPase, allowing the release of iron from Tf. Through an unknown mechanism, iron is targeted to the inner membrane of the mitochondria, where the enzyme that inserts Fe into protoporphyrin IX, ferrochelatase, resides. Although it has been demonstrated that the divalent metal transporter, DMT1, is responsible for the egress of reduced Fe from the vesicle, the immediate fate of the iron atoms after their transport across the vesicular membrane remains unknown. Because reduced iron is a strong pro-oxidant, contributing to free radical formation through Fenton chemistry, it has been predicted that an iron binding molecule shuttles Fe from the endosome to mitochondria. However, this much sought iron binding intermediate, that would constitute the labile iron pool (LIP), has yet to be identified. Thus, we hypothesize that, in Hb-producing cells, there is a direct relaying of Fe from the endosomal machinery to that of the mitochondria. We have taken two strategies in examining this supposition: 1) a biochemical approach by which the cytoplasm of cells was loaded with an impermeant iron chelator, thus intercepting the delivery of Fe by the putative LIP intermediate, and 2) a morphological approach employing time-lapse confocal microscopy which permits the tracking of iron-loaded endosomes and mitochondria with high spatial and temporal resolutions. To examine whether iron delivered by Tf for heme synthesis can bypass the cytosol, we have loaded reticulocytes with a high-molecular weight version of desferrioxamine, hDFO, prior to incubation with ^{59}Fe-Tf. The incorporation of transferrin iron into heme was unaffected by hDFO when compared to controls. Importantly, iron delivered to these cells in a form that freely diffuses across the membrane, iron-salicylaldehyde isonicotinoyl hydrazone (^{59}FeSIH2), was significantly prevented from being used for heme synthesis in hDFO-laden reticulocytes. Using confocal microscopy, as well as polarized light microscopy, we found that endosomes are very mobile organelles. Immediately following budding from the plasma membrane, these organelles continuously traverse the cytosol and touch a number of mitochondria multiple times. Experiments using various pharmacological agents indicate that these movements are mediated by components of the cytoskeleton which are essential for proper iron delivery for use in heme synthesis. Together, these data suggest that iron is directly delivered to mitochondria by endosomes in a "kiss and run" paradigm. Our current studies will examine the required components and regulation of this interaction using the same experimental strategies as well as a cell free system consisting of isolated organelles.

MOLECULAR CHANGES IN PORPHOBILINOGEN DEAMINASE IN AIP

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Acute intermittent porphyria is essentially a hepatic disease in which porphobilinogen deaminase is approximately halved in heterozygote carriers. This reduction in the level of porpobilinogen deaminase is also reflected in erythrocytes that also show approximately 50% of the normal value. However, during an acute attack, levels of hepatic porphobilinogen deaminase are reduced to values as little as 25% causing a further drop in haem synthesis, whereas the erythrocyte enzyme level remains relatively unchanged. The structural basis for the lowered levels of hepatic porphobilinogen deaminase during the acute attack, noted by Marver in the 1970s, has been traced to the post-translational step involving the assembly of the dipyrromethane cofactor. This is achieved by the reaction of apo-porphobilinogen deaminase with the 1-hydroxymethylbilane, preuroporphyrinogen. We have found that this process is strongly inhibited by porphobilinogen. During an acute attack, it is therefore likely that the level of porphobilinogen is sufficiently high to prevent the formation of active porphobilinogen deaminase holo-enzyme. In hepatic tissue where there is a rapid turnover of porphobilinogen deaminase the effect is magnified compared with erythrocytes that have a half life of several weeks.

PARTIAL CHARACTERISATION OF RECOMBINANT *MYXOCOCCUS XANTHUS* AND HUMAN PROTOPORPHYRINOGEN OXIDASES, AND THEIR PHYLOGENETIC ANALYSES USING PROTEIN SEQUENCES FROM DIVERSE SPECIES

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A kinetic and inhibitor characterisation of PPOX from the prokaryote *Bacillus subtilis* has previously been reported from our laboratory. Here, in an extended study, a partial characterisation of PPOX from another prokaryote *Myxococcus xanthus*, in addition to the human form, is reported. The required recombinant PPOXs were expressed in *E. coli* cells and purified to apparent homogeneity by rapid metal chelate affinity chromatography. Thereafter, detailed kinetic and inhibitor profiles were established for each form of the enzyme. Sequence data (nucleotide and/or protein/polypeptide) accessed from publicly available databases allowed us to perform multiple protein sequence alignment and phylogenetic analyses in order to further analyse the possible relationships and/or differences between the biochemical properties amongst these PPOXs and those from several diverse species. Detailed kinetic analysis showed that *M. xanthus* PPOX behaved more like human PPOX than the *B. subtilis* enzyme - *M. xanthus* had a more stringent substrate specificity compared to that of the *B. subtilis* enzyme and, secondly, both *M. xanthus* and human PPOXs were were very sensitive to diphenylether inhibition, unlike *B. subtilis*. Our data suggests that differences in inhibitor profiles are not simply general differences between prokaryotic and eukaryotic forms of PPOX, nor does it simply reflect the Gram positive/negative status of the organism. Sequence alignment analyses identified, in most cases, two conserved domains (A and B), both of which were not present in the human monoamine oxidase (MAO-B) sequence, used to root the PPOX proteins for phylogenetic analysis. From this work it is clear that the N-terminal domain (A) appears to be better conserved in most of the sequences, than the C-terminal domain (B). These differences shed

some light on their behaviour, particularly with respect to the different inhibitors - PPOXs containing both the conserved domains (A and B), appear to have greater affinity for the diphenylether inhibitors and tighter substrate specificity. The phylogenetic tree showed interesting relationships and clustering. For example, all plant PPOXs were clustered together, but two distinct branches were formed, which separated these proteins on the basis of the organelle in which the PPOX isoform is located (mitochondria or chloroplast). Amongst the prokaryotic PPOXs there was an important deviation from the bacterial clustering. *M. xanthus* PPOX was more closely associated with the eukaryotic (yeast and mammalian) proteins than other prokaryotic PPOXs. Our biochemical findings add weight to this anomaly in that the kinetic behaviour of this enzyme was similar to that of eukaryotic enzymes. In conclusion, we provide evidence that differences in the biochemical properties of various PPOXs, although sometimes relatively subtle, probably relate to their differences at the primary structural level which have evolved over time. PPOXs containing both conserved sequence homologies (domains A and B) appear to have greater affinity and catalytic activity towards the substrate, protoporphyrinogen-IX and are also more sensitive to the diphenylether inhibitors.

AN AIP FAMILY WITH NO HMBS SEQUENCE VARIANT

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In a Norwegian family with typical clinical and biochemical acute intermittent porphyria, no mutations were found by sequencing the entire HBMS gene. While awaiting a mutation find, haplotype analysis was employed as a diagnostic tool. DNA was isolated from blood and all coding exons of HMBS of the index case were sequenced (capillary electrophoresis, ABI 3100). No sequence variants, thought to be associated with AIP, were found. Also the non-coding exon 2, as well as segments of 580 and 660 bp at the 5' and 3' UTR, respectively, were sequenced without suspicious findings. To reduce the possibility of interfering polymorphisms in the primer sites, a second set of sequencing primers was designed. No mutations were found. The sequence variants observed were apparent homozygosity for 5'UTR-430G>A, IVS2-96T>G, IVS3-14A>G, and V202V. Proof of hemizygosity was not found. We also observed heterozygosity for 3`UTR+337G>A. We tested family members for five chromosome 11 dinucleotide markers covering 1.8 Mb surrounding the HMBS locus (ABI 310, markers given in Fig.1). The affected haplotype was established among the definitely affected family members, and was subsequently used for identifying those sharing the HBMS locus carrying the putative AIP-allele. Apart from unconfirmed reports of a "Chester locus", all patients with AIP identified so far has been related to deficits of the HMBS locus. The affected haplotype co-segregated with AIP in this pedigree, a finding supporting the diagnosis. Thus, even though no DNA abnormalities have been found, true AIP families may be worked up and individual family members may be reliably classified, using indirect genetic haplotyping tools. By sequencing, we have so far identified the causative AIP mutation in 22 different Norwegian families. However, the putative mutation in this family defies revelation. Sequencing may not readily disclose whole exon deletions and large structural rearrangements or mutations afflicting control elements on the same (or other) chromosomes. We will continue to search for the putative AIP mutation in this family.

MOLECULAR CHARACTERIZATION OF A SPANISH PATIENT WITH X-LINKED SIDEROBLASTIC ANEMIA

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X-linked sideroblastic anemia is caused by mutations in the gene of the erythroid form of 5-aminolevulinate synthase (ALAS2), the first enzyme of the heme biosynthesis pathway. We report the first molecular study in Spain of this anemia, carried on a 19 years old patient, who presented with a microcytic, hypochromic anemia (Hb 8g/dl, MCV 58 fl, MCH 18 pg). Bone marrow examination showed 64% ring sideroblasts, as well as erythroid hyperplasia, which was accompanied by a low reticulocyte count (0.75%). Ferropenic anemia was discarded in view of his serum iron (119 µg/dl), serum ferritin (432 ng/ml) and serum transferrin saturation (80%). No mutations were found in the HFE gene. Direct sequencing of the ALAS2 gene revealed that the patient and his mother had the mutation R452C in exon 9, in this exon is also located the pyridoxal 5´-phosphate binding lysine. The patient is now on treatment with pyridoxine, and after a month treatment the response has been satisfactory (hemoglobin increased from 8 to 9 g/dl).

ACUTE HEPATIC PORPHYRIAS WITHOUT DETECTABLE GENETIC DEFECTS

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Acute hepatic porphyrias (AHP) are characterized by a complex acute clinical syndrome in conjunction with excessive excretion of heme precursors in urine and/or feces, with enzyme deficiencies based on mutations in the corresponding genes of the heme biosynthetic pathway. We report on two male patients with an polysympto-matic manifestation of an acute porphyria syndrome which was first diagnosed as acute intermittent porphyria (AIP). In both cases porphyrin precursor ALA and PBG as well as porphyrin excretion was extremely elevated.

Case 1: A 23-year-old male patient was diagnosed as AIP due to high excretion of ALA (378 µmol/24h, normal <49), PBG (559 µmol/24 h, normal <8) and porphyrins (6878 µg/24 h, normal <145), Uro/Copro-ratio 4:1. Fecal porphyrins were normal. The activity of PBG-deaminase was 71% of controls. Molecular genetic analysis of the PBG deaminase gene (Intron/Exon 1–14) did not exhibit any mutation. Analogous investigations of the coproporphyrinogen oxidase and protoporphyrinogen oxidase genes did also show no deviations of the normal base sequence. The patient was treated successfully with heme arginate both by the acute and the interval application treatment type.

Case 2: A 12-year-old boy sufferd from severe neurological symptoms which were probably induced by antiepileptic drugs. He excreted high amounts of ALA (358 µmol/24h), PBG (311 µmol/24h) and porphyrins (4721 µg/24h), Uro/Copro-ratio 1:5. Urinary Copro III Isomer was 96% (normal 69-83%). Fecal porphyrins were normal (34 µg/g, normal <85) as well as the PBG deaminase activity (70%). Mutation analyses of PBG deaminase and protoporphyrinogen oxidase genes did not show any aberrations. However the sequence of the coproporphyrinogen oxidase gene shows in codon 272 of the exon 4 the transversion AAC>CAC leading to a substitution of the amino acid asparagine by histidine at this position. We think that this mutation can be interpreted most likely as a genetic polymorphism without effect on the enzyme activity. Nevertheless, the intense metabolic dysregulation enforces a weekly application of heme arginate. Under this therapy the excretion values returned to subclinical levels: ALA 77 µmol/24 h, PBG 95 µmol/24 h and porphyrins to 428 µg/24 h. In conclusion, these studies illustrate that a clear molecular genetic explanation of the porphyrias still remains difficult in some cases. Furthermore the value of genetic analyses should not be overestimated. These data do not serve for clinical explanation of the porphyria process. Also in the future the clinical biochemical analyses of the excretory constellations of porphyrin precursors and porphyrins remain of decisive importance for diagnostic and therapeutic purposes.

ESTABLISHMENT OF A NATIONAL REGISTER OF PORPHYRIA PATIENTS - CAN IT BE EXTENDED TO A EUROPEAN REGISTER?

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The Norwegian Porphyria Centre (NAPOS) established a national register of patients with porphyria in 2002. The main object of the register is research, but it will also serve as an important link between the patients and the Porphyria Centre, for example in prophylaxis or treatment of individual patients. Data collection is based on standardised questionnaires filled in by the patients, who also have to sign an informed consent in order to be registered. The questionnaire includes personal data and questions concerning diagnosis, symptoms, treatment, medication, other health information, living habits, disease-related problems, quality of life, and family information. The data from all questionnaires are stored in a Microsoft Access computer database. Patients also receive a personal porphyria ID card along with the questionnaire. The card contains a brief description of the disease, in both Norwegian and English. We have recently extended our register permission from the Norwegian Data Inspectorate to also include patients from other Nordic countries. In co-operation with the Danish Porphyria Centre, Danish patients will soon be included in the database. We propose that the next step could be to establish a European porphyria register. The main advantage of a common European database would be the possibility to gather a larger amount of data for epidemiological studies. A European porphyria register would also fit well within the primary objective of the European Porphyria Initiative (EPI), which is to compare experience between countries, attempt to develop a common approach to the management of these diseases and to facilitate international collaborative clinical research and development. Porphyria centres or countries are encouraged to take contact to participate in this international co-operation.

HFE-MUTATIONS AND RESPONSE TO CHLOROQUINE IN PORPHYRIA CUTANEA TARDA

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The role of HFE gene mutations, which are associated with porphyria cutanea tarda (PCT), on the therapeutic response to chloroquine is unknown. We retrospectively analyzed a data base of chloroquine-treated patients with PCT on whether HFE mutations (C282Y and H63D) might have influenced the clinical response, urinary porphyrin excretion, liver enzyme activities and serum iron markers. Sera and corresponding complete sets of data before and after therapy were available in 62 of 207 patients with PCT who were treated exclusively with chloroquine. For treatment low dose chloroquine diphosphate, 125-250 mg twice weekly, was used during a median time of 16 months (range, 12-26). The majority (37/62=61%) of German PCT patients carry HFE mutations. Chloroquine therapy was accompanied by clinical remission and reduced urinary porphyrin excretion ($p<0.001$) in the 24 (39%) patients with HFE wild type as well as in 34 (55%) HFE heterozygous patients with PCT. Decreases of serum iron markers following chloroquine therapy were limited to patients with PCT and HFE wild-type. All patients homozygous for the C282Y mutation (3/62=5%) had high serum iron, ferritin and transferrin saturation and failed to respond to chloroquine treatment. In conclusion, C282Y heterozygosity and compound heterozygosity of HFE mutations did not compromise the therapeutic response to chloroquine. Since HFE C282Y homozygotes (+/+) did not respond to chloroquine and decrease of serum iron markers was limited to patients with PCT and HFE wild-type, phlebotomy should be first line therapy in patients with PCT and HFE-mutations.

EFFECTIVENESS OF ALPHA-LIPOIC ACID IN PORPHYRIA CUTANEA TARDA

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Alpha-lipoic acid a coenzyme of pyruvate and alpha-ketoglutarate dehydrogenase, is unique in its ability to act as a fat-and water-soluble antioxidant in tissues in both its oxidized and reduced forms, therefore it is considered to be an ideal antioxidant. It is readily absorbed from an oral dose. This natural cofactors has long been suggested to improve glucose oxidation. Therapeutic application of it was justified in diabetic polyneuropathy, cataracts, glaucoma, ischaemia-reperfusion injury, and Amanita mushroom poisoning. Our aim was to establish the effectiveness of alpha-lipoic acid on the porphyria cutanea tarda (PCT). PCT is disorder of hem biosynthesis resulting from a decreased activity of uroporphyrinogen-decarboxylase enzyme. Hem precursors accumulate in the blood, liver, urine, stool and skin. Iron toxicity is associated with pathological free radical reactions. Patients were treated with alpha-lipoic acid (Thiogamma 600R) capsule for 8 weeks. We analysed the data of 24 PCT patients (M:18;F:6) (average age: M:60±15; F:58±12). The diagnosis of PCT was based on the results of blood, urine and faeces analyses. Rutin laboratory methods: AST, ALT, GGT, ALP, TG, chol, HDL-chol, LDL-chol, glucose HgA1c, Fe, transferrin and ferritin measurements were carried out. H-donor activity, reducing power were measured by spectrophotometry and chemiluminescent intensity of plasma and erythrocytes were measured with LB 9501 luminometer. These results of treatment signaled moderate beneficial changes of enzyme activities of liver. Significant decrease of GGT activity and concentration of bile acid were detected as well. Non-significant elevation of uric acid level of the plasma was measured after the treatment. The concentration of glucose and HgA1c were decreased non-significantly. The antioxidant property of alpha-lipoic acid was enforced in plasma and erythrocyte. Statistical analysis of data showed more favourable effects of alpha-lipoic acid on PCT in male patients. In conclusion, per os treatment with alpha-lipoic acid (600 mg/day) over 8 weeks is safe and effective in reducing symptoms of PCT. [This study was supported by Wörwag Pharma, 1/016 NKFP and 002/2003 ETT Projects].

EXTENDED AND REVISED PORPHYRIA DRUG LIST: SCHEME FOR PREVENTIVE MEASURES IN PORPHYRIA PHARMACOTHERAPY

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Background. An inquiry among Swedish carriers of acute porphyria (102 persons, 79 women, 23 men; 20-84 years) yielded 371 reports of intake of 171 different pharmaceutic drugs during the last twelve months. Twenty-four of the drugs were previously unclassified with regard to porphyrinogenicity and 25 classified as dangerous. In no case porphyria symptoms prompting medical attention developed. Conclusion. As previously observed by other workers, most carriers of acute porphyria seem to tolerate most drugs most of the time. The problem in porphyria drug counselling may primarily be to single out the occasional combination of a porphyrinogenic drug and a vulnerable patient that can result in the rise of a porphyria crisis. The "better safe than sorry" principle when adopted in porphyria pharmacotherapy with all probability will result in uncalled for undertreatment. If also potentially porphyrinogenic drugs are considered for use under specific precautions adapted to the actual porphyric vulnerability of the patient, this may be avoided. Extended and revised drug list. By undertaking a porphyrinogenicity classification of all pharmaceuticals included in the Swedish Pharmacopoeia 2003, a larger selection of drugs to consider for use in the acute porphyrias was aimed at. A total of 970 substances were categorized, of which 674 were classified as not or probably not porphyrinogenic, 182 as possibly porphyrinogenic and 113 as probably or most certainly porphyrinogenic. The general criteria used in the classifications will be presented in detail. The list, adopted for patient's

use by also including commercial names of the drugs, will be published by the Swedish Porphyria Association, and will also be available in the drug database of the Norwegian and Swedish National Porphyria Centres, where the rational for the categorization of each substance will be given as well. Guide to precautions in drug treatment. In cases of strong or vital indication for drug therapy, by administration of a potentially porphyrinogenic drug unfortunate undertreatment is avoided where no safe alternative is at hand. A prerequisite for this strategy is the undertaking of preventive measures dimensioned to which extent the porphyria gene carrier is open to porphyric bye-effects of the drug. Five vulnerability classes can be deduced from age, gender, previous and current disease activity and current porphyrinogenic burden of the patient. A scheme over precautions to take will be presented in the form of a matrix where recommended measures are presented for each combination of drug porphyrinogenicity and patient vulnerability. The recommendations will also be available through the Norwegian and Swedish National Porphyria Centre's database, calling upon the generic or the commercial name of the substance.

A NOVEL MUTATION IN THE FERROCHELATASE GENE AMONG FINNISH EPP PATIENTS

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Patients and clinical manifestations: We have studied altogether 14 Finnish EPP families including 40 patients, of whom 24 experienced photosensitivity. The pattern of inheritance is autosomal dominant in these families. The proband with a novel mutation was a 7-year-old boy experiencing stinging sensations and painful edema of the sun-exposed skin accompanied by severe hemorrhagic bullae on the toes after strong sun-exposure. The same mutation was identified in his father, who was symptom-free, and in his 72-year-old grandfather, who had experienced characteristic skin symptoms since childhood without a proper diagnosis. The activities of reticulocyte ferrochelatase in the proband, the father and the grandfather were considerably higher (90%, 80% and 50 % of the normal) compared to the mean activity of Finnish EPP patients (20% of the normal), while erythrocyte protoporphyrin levels (28 000, 1000, 42 000 nmol/l, respectively) correlated well with the clinical condition and were parallel to those of other EPP patients. *Molecular genetics:* To date we have identified a novel mutation (E413X) in the ferrochelatase gene in addition to eight previously described mutations (K86E, Q96X, 751delGAGAA, R115X, H157R, 1122delT, IVS8+1g-a, IVS4+1delg). Two mutations cause a splicing defect resulting exon skipping, two point mutations cause an amino acid change and four mutations cause early stop codon and truncated polypeptide. The outcome of the mutations have been determined by sequencing of the RT-PCR product amplified from total RNA extracted from the patients' lymphoblast cell lines. E413X is predicted to interrupt dimerization of ferrochelatase by disrupting α-helix number 17 at the C-terminus. Even in prokaryote expression, where dimerization of human ferrochelatase cannot occur, the remarkable difference in the activity between the wild-type (100%) and mutants (0%) indicates the complete loss of function of the mutated allele. The genotype frequencies of the IVS3-48 T/C polymorphism in different Finnish EPP patient groups (symptomatic EPP patients T/T 0.0; T/C 0.67; C/C 0.33, asymptomatic carriers 0.5; 0.5; 0.0, healthy relatives 0.22; 0.78; 0.0, controls 0.86; 0.14; 0.0, respectively) demonstrated over expression of a C-allele among symptomatic patients.

LONG-STANDING CHANGES IN THE URINARY PROFILE OF PORPHYRINS AFTER CLINICAL REMISSION OF PORPHYRIA CUTANEA TARDA

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Patients with overt porphyria cutanea tarda (PCT) show a very distinctive abnormal urinary profile of porphyrin excretion. It is not known, however, if the clinical remission of the disease does always conclude with a complete normalisation of this profile. We have selected 46 patients early diagnosed of PCT that after a period of treatment, present low values of total porphyrins in urine (<35 nmol/mmol creatinine). We have analyzed the urine by HPLC, identified and quantified the different porphyrins and compared the urinary profile with that of a group of healthy volunteers. The results showed that while the healthy volunteers presented a pattern dominated by the excretion of coproporphyrin III, a large proportion of the patients in clinical remission (80%) still presented a characteristic profile of PCT, with decrease of the coproporphyrin-to-uroporphyrin ratio and/or inversion of the normal coproporphyrin III-to-coproporphyrin I ratio. Detectability of uroporphyrin III and heptacarboxyl III intermediates was also significantly higher among these patients than among the controls ($P<0.05$). These results show that patients with PCT present persistent subtle changes in their urinary porphyrin profile even after a period of clinical remission and even if the total bulk of porphyrins excreted have fallen to low normal values.

"BEING PREPARED, BUT PREPARED FOR WHAT?" A QUALITATIVE STUDY ON THE EXPERIENCE OF GENETIC COUNSELLING AND LIVING WITH A PREDISPOSITION FOR ACUTE INTERMITTENT PORPHYRIA

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In Norway genetic counselling is mandatory before testing patients without symptoms and with a genetic risk of acute intermittent porphyria. The present study deals with how individuals with a positive test result handle their situation. The study comprised 11 individuals: 7 women and 4 men who had a genetic mutation of AIP. Some of them described symptoms related to AIP, others had no symptoms. They described themselves as healthy. The age of the participants varied from 20 to 60 years. A qualitative interview were performed. Their experiences were qualitatively analysed to identify central themes. The study indicates that the participants, knowing they have a genetic risk for AIP, live with uncertainty whether if-or when-they will get symptoms of AIP, how they can manage the symptoms and if their children will get the disease. Support from the professional health staff with information that enables them to interpret their own experiences and reactions in a situation of uncertainty are more important for them than basic medical knowledge. In spite of uncertainty; they are satisfied with the information especially because they now know that treatment can be given if they get an attack. In conclusion, the study may contribute to a better understanding of the complexity that follow genetic counselling and testing and how individuals react on knowing their possibility for getting the disease.

MITOCHONDRIAL TARGETING MECHANISMS OF PROTOPORPHYRINOGEN OXIDASE: IDENTIFICATION AND CHARACTERIZATION OF INTERNAL TARGETING SEQUENCES

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Recent studies have revealed that a relatively short 28-amino acid segment in the amino terminus of protoporphyrinogen oxidase (PPOX) contains information, which is sufficient to direct the protein into mitochondria. Furthermore, our experiments with amino-terminally truncated green fluorescent protein (GFP)-fusion polypeptides showed that amino acids 25-477 of PPOX contained an additional mitochondrial targeting signal(s), which could direct the reporter protein into mitochondria even in the absence of the amino-terminal signal. We have investigated the internal signal sequences for mitochondrial transport of PPOX by performing a secondary structure analysis of the polypeptide and studying the intracellular localization of mutated PPOX-GFP-fusion proteins in COS-1 cells. Since the model for the interaction between the amino terminus of PPOX and the putative mitochondrial receptor

protein Tom20 suggested that leucine and isoleucine residues forming an alpha-helical hydrophobic motif LXXXIXXL were crucial for the recognition of the targeting signal, we searched for helical, leucine-rich segments with net positive charge and chose four most promising candidate segments. Three of the segments could direct PPOX into mitochondria when replacing the authentic amino-terminal targeting signal. Substituting polar glutamine for non-polar leucine residues of these segments could impair the mitochondrial transport in the absence of the amino-terminal targeting signal indicating that these regions contribute to internal mitochondrial targeting signaling of PPOX. The importance of leucine and isoleucine residues for the mitochondrial targeting suggests that hydrophobic interactions are involved also in the recognition of internal targeting signals, possibly through interaction with Tom20 or other mitochondrial receptors.

VARIEGATE PORPHYRIA IN SOUTH AFRICA: A POPULATION-BASED STUDY TO DETERMINE THE FREQUENCY OF THE FOUNDER GENE MUTATION (R59W)

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The incidence of variegate porphyria (VP) in the South African population of European descent is the highest in the world because of a founder gene effect and the gene frequency was estimated to be 0.3%. However, this estimate is questionable on the grounds of genealogical and biochemical methods used and as indicated by more recent records from diagnostic laboratories. According to earlier reports more than 80% of the estimated 10 000-20 000 South African individuals with VP remain undetected and are therefore at risk of potentially lethal acute neurological attacks. This poses a serious question regarding the actual frequency of VP, and specifically the founder gene mutation, in South Africa. Are there thousands of undiagnosed VP carriers in South Africa as genealogical studies and population growth curves have predicted, or is the allele frequency much lower than 0.3%? The aim of this study was thus to determine the prevalence of the founder mutation (R59W) with a highly specific DNA test. Blood samples were obtained at blood transfusion clinics, pathology clinics and maternity wards. For the initial screening we used SSCP analysis, which proved to be consistent and cost-effective. All R59W positive samples were re-tested using restriction enzyme analysis. The R59W mutation was detected in five of the 4637 samples collected to date. The estimated frequency of 0.15% (5/3311) for the founder mutation in South Africans of European descent is much lower than the frequency of 0.3% estimated previously. However, different frequencies were obtained within the various population samples and we suggest that a newborn baby population sample be used in the future, as it is the most unbiased sample source. Interestingly, the highest frequency for the R59W mutation was obtained in this sample. One of the two adult R59W positive participants was unaware of her carrier status, in accordance with the incomplete penetrance of the trait. Two of the mothers of the three newborn babies found to be R59W positive were also not aware of VP in their families, indicating that ignorance regarding VP status in South Africa is a matter of some concern.

NON-PORPHYRIC SKIN DISEASE IN THE PORPHYRIAS

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Although the skin manifestations of the porphyrias are well described, little attention has been paid to non-porphyric skin diseases in this group of patients.

Acne - Topical treatments for mild to moderate acne are generally safe in patients with porphyria, though there is scant evidence in the literature. Unfortunately, none of the commonly-used antibiotics are safe. Similarly, Isotretinoin, the most effective treatment for acne, is unsafe in the acute porphyrias and should be avoided.

Eczema - Eczema is often controlled with a combination of moisturizers and steroid creams or ointments. These treatments are generally safe. Moisturizers keep the skin in good condition which may help to avoid minor injuries of the skin which may lead to scarring. Anti-histamine tablets are sometimes given to help reduce itching. Chlorpheniramine (Piriton) and Promethazine (Phenergan) are considered safe. Most other antihistamines should be avoided. Considering systemic treatments for eczema, Prednisolone and Azathioprine are both safe in all forms of porphyria. Ciclosporin, another agent occasionally used to treat eczema, is not safe. For ultraviolet treatment of eczema, see below under psoriasis.

Psoriasis - Treatment with moisturizers and steroid creams/ointments is safe. Topical treatments such as Calcipotriol, Dithranol and coal tar are not known to be harmful. Systemic treatments for psoriasis include Methotrexate which must be used with caution; Acitretin and Ciclosporin are both unsafe. Ultraviolet light may be used for the treatment of both psoriasis and eczema. This may be with uv-B, or uv-A with psoralens tablets (PUVA). This treatment must be avoided in photosensitive porphyrias. Psoralens tablets are known to be unsafe in porphyria.

Skin surgery - Sometimes the skin needs to be sampled either to help make the diagnosis or to remove benign or malignant tumours. This is usually done under local anaesthetic. Most local anaesthetic agents are safe in porphyria, but Bupivacaine (Marcaine) is preferred.

Warts - Preparations such as salicylic acid (cream or paint) are safe but care should be taken since the skin in porphyria is fragile. This is also the case for cryosurgery.

Summary - Sun avoidance and general skin care measures such as the use of moisturizers are important in keeping the skin in good condition in those with cutaneous porphyrias. For skin problems unrelated to porphyria, cream/lotion treatment is generally safe. Tablet treatments can be unsafe and should be checked with the doctor prescribing the treatment. Monitoring urinary ALA and PBG may be useful in predicting a relapse to porphyrinogenic drugs.

IDENTIFICATION OF A RECURRENT MUTATION IN THE PORPHOBILINOGEN DEAMINASE GENE IN GERMAN PATIENTS WITH ACUTE INTERMITTENT PORPHYRIA

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The porphyrias are a group of metabolic disorders arising from catalytic deficiencies of specific enzymes along the heme biosynthetic pathway. Acute intermittent porphyria (AIP) is the most frequent type of acute porphyria worldwide and results from a decreased activity of porphobilinogen deaminase (PBGD), the third enzyme in heme biosynthesis. Clinically, the disease is characterized by life-threatening acute neurological attacks that can be provoked by porphyrinogenic drugs. AIP is transmitted as an autosomal trait with incomplete penetrance and, to date, several disease causing mutations have been reported. In an effort to characterize the molecular basis of AIP in Germany, we identified seven different mutations in ten AIP families by PCR, heteroduplex analysis, automated sequencing, and restriction enzyme digestion. Interestingly, a mutation located at the donor splice site of exon 1 was found in four unrelated families of German origin thus raising the possibility that this mutation might represent a mutational hotspot or a novel founder mutation in the PBGD gene. Our data emphasize the molecular heterogeneity in AIP and confirm the advantages of DNA analysis as a diagnostic tool and for the detection of clinically asymptomatic mutation carriers within AIP families.

A NEW VARIANT OF ERYTHROPOIETIC PROTOPORPHYRIA WITH NORMAL FERROCHELATASE ACTIVITY

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Erythropoietic protoporphyria (EPP) in most patients is associated with raised erythrocyte protoporphyrin (PP) levels, ferrochelatase (FECH) activity < 30% of normal and a combination of an inactivating mutation in the FECH gene with a less active allele, or compound heterozygosity for two inactivating mutations. We recently identified a mother and son with the classic EPP skin symptoms and raised PP levels who have normal FECH activity in lymphoblastoid cells. A 52 year old woman has had complaints of painful erythema and swelling after brief sun exposure since late childhood. Her parental grandmother and an aunt had been photosensitive. Her 23-year-old son has had the same symptoms from the age of 2 years. Both mother and son had raised erythrocyte PP levels, but in contrast to classic EPP they also had raised Zinc PP levels and normal FECH activity levels on three occasions. The mother, but not the son, also had markers of iron deficiency with a low serum iron, high ferritin and high transferrin. Erythrocyte porphobilinogen deaminase levels were increased in both, whereas uroporphyrinogen decarboxylase and protoporphyrinogen oxidase levels were normal (see table). No mutations were detected in the exons, flanking intron regions or mitochondrial targeting region of the FC gene, nor in the iron responsive element of the erythrocyte aminolevulinic acid synthase gene.

Test	Mother	Son	Controls
FECH pmol/mg prot/hr	770 - 804	769 - 850	> 350
Ery. PP µmol/l	25,7 - 242	10,4 - 15,5	< 1,5
Ery. Zn PP µmol/l	24,8 - 45,8	15,0 - 17,9	< 1,5
Ery. PBG-D	195	167	> 64
Uro-Decarb uro copro ratio	1,68	1,45	< 1,95
PP-Ox pmol/mg prot/hr	3785	3310	>2200
Plasma ALA nmol/l	609	75	< 74
Plasma PBG nmol/l	41	9	< 12
Plasma porphyrin	PP +, Uro -, Copro -	PP +, Uro -, Copro -	PP -, Uro -, Copro -

In conclusion, this family appears to have a variant of EPP, presenting with similar photosensitivity and raised PP levels, but differing from classic EPP in having normal lymphoblastoid FECH activity and in addition to PP, raised Zinc PP levels. When associated with iron deficiency the PP and ZnPP levels increase further and raised ALA levels are found. The underlying molecular genetic mechanism remains to be determined.

NEUROPSYCHIATRIC PORPHYRIA IN PATIENTS WITH REFRACTORY EPILEPSY: REPORT OF THREE CASES

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Acute symptomatic seizures are a well recognised symptom of acute porphyria in relapse. We report three patients with a long history of chronic refractory epileptic seizures who were subsequently found to suffer from one of the neuropsychiatric porphyrias. Patient 1 had an eight-year history of seizures since the age of 12, probably due to primary generalised epilepsy. Past medical history showed chronic constipation requiring enemas. The seizure frequency increased despite high doses of Sodium Valproate and the patient developed abdominal pains when Lamotrigine was added which caused a marked worsening of the seizures. A diagnosis of acute intermittent porphyria was established. Patient 2 had a three-year history of intractable generalised convulsions. Carbamazepine and Phenytoin were unable to control the seizures. Blood tests showed abnormal liver toxicity tests and hyponatraemia. Revisiting her medical history she admitted to frequent abdominal pains with constipation and sun sensitive skin. The patient was found to suffer from variegate porphyria. Patient 3 had an 18-year history of mainly simple partial sensory and motor seizures and then atonic seizures. She was unsuccessfully tried on a variety of anti-epileptic drugs and after starting Topiramate developed an acute neurological disorder with reduced level of consciousness, bulbar disturbance, an asymmetric spastic quadriparesis, negative imaging with only partial recovery. At this time her urine was noted to be dark and a diagnosis of variegate porphyria was established. Porphyria may be an aetiological factor in some cases of chronic refractory partial or generalised epilepsy. Porphyria should also be considered if addition of a new antiepileptic medication causes a major deterioration in epilepsy.

ERYTHROCYTE AND LIVER PORPHOBILINOGEN DEAMINASE ACTIVITY IN CIRRHOSIS AND CLINICAL OR EXPERIMENTAL CHOLESTASIS

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Porphobilinogen deaminase (PBG-D), the third enzyme in the haem synthetic process, mainly expressed in the erythrocytes and the liver, has a key role in the pathogenesis of the acute porphyrias. In the present study we investigated the effect of cirrhosis or cholestasis on this enzyme. We have found that: 1) Erythrocyte PBG-D activity was significantly increased (p=0.0003) in 27 patients with non-alcoholic liver cirrhosis (19.89±6.65 nmoles/h.l) and in 24 patients with extrahepatic cholestasis (20.69±11.17 nmoles/h.l) as compared to 30 healthy controls (12.77±4.76 nmoles/h.l). The enzymic activity was positively correlated to the prothrombin time in both patient groups and negatively to the alkaline phosphatase in the cholestatic group. 2) Erythrocyte PBG-D activity in blood samples from normal subjects significantly increased when their homologous plasma was substituted by that of patients with cholestasis (p<0.001) or cirrhosis (p=0.05) although it remained lower than that of the respective patients' blood samples. 3) In 8 rabbits, after ligation of the common bile duct, PBG-D activity significantly increased both in the erythrocytes (from 30.26±10.33 to 48.87±15.82 nmoles/h.l, p=0.002) and the liver (from 13.27±4.79 to 17.68±5.42 nmoles/h.g, p=0.035). In 8 sham-operated rabbits erythrocyte PBG-D also increased (from 29.60±9.85 to 33.32±12.23 nmoles/h.l, p=0.016) but in a significantly lower degree than in the "cholestatic" group (111.10±10.95% versus 167.96±41.64%, p=0.006) while the hepatic enzyme remained unchanged (from 13.40±3.85 to 13.83±7.21 nmoles/h.g, p=0.80). 4) In 5 patients operated for extrahepatic cholestasis the mean hepatic PBG-D activity was higher than in other 5 who underwent cholecystectomy without having cholestasis (12.63±3.24 versus 9.64±1.17 nmoles/h.g) but the difference was not statistically significant (p=0.11). In conclusion, erythrocyte and liver PBG-D activity is considerably increased in cholestasis and cirrhosis and plasma factors seem to play a role in it.

MODIFICATION OF GRISEOFULVIN-INDUCED PORPHYRIA BY THE DEVELOPMENT OF EXPERIMENTAL DIABETES MELLITUS

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Diabetes is known to affect the activity of some haem synthetic enzymes. In clinical practice the coexistence of diabetes and porphyria is not uncommon and as we have previously indicated (BMJ 1987; 295:1237-8) may alter the natural course of porphyria. The aim of our study was to investigate whether the development of diabetes in porphyric animals could change their fecal porphyrin excretion, which can be used as a reliable index of porphyric activity. Four white mice became porphyric by the per os administration of high-dose griseofulvin (210 mg/kg b.w. daily). Fecal porphyrins (FP) increased from 1.63±1.07 to 70.91±43.74 µg/g (p<0.001). When the mice were made diabetic by streptozotocin a significant further increase of FP was observed (105.91±34.34 µg/g, p=0.001). In another 12 mice the administration of a low dose griseofulvin (70 mg/kg b.w. daily) produced a less pronounced but also significant increase in FP (6.84±3.78 µg/g,

p<0.001). In 8 of these animals streptozotocin-induced diabetes led to a further increase of FP (8.28±3.40 μg/g, p=0.024). In 4 porphyric (with low-dose griseofulvin) and in 4 porphyric/diabetic mice phenobarbital, a potent inducer of porphyrin synthesis, was also administered (200 mg/kg b.w. daily) to aggravate their mild porphyria. FP significantly increased in both groups, but more in the diabetic than in the non-diabetic porphyric mice (14.6±1.35 versus 8.71±1.35 μg/g, p<0.001). It is concluded that diabetes significantly increased the porphyric activity (as estimated by FP) in mice with severe or mild porphyria and made them more responsive to an aggravating factor.

Autor index