The Organic Acidemia Association is proud to present the first edition of Current Research on Organic Acidemias, an educational publication designed to highlight and promote research studies on the organic acidemias.

We need to attract more graduate students, medical students and young physician scientists to the field of inborn errors of metabolism to hasten the development of better treatments and genetic cures for these disorders. Here, we present a broad cross-section of diseases, professionals and their unique ideas and approaches that tackle some hard questions: how do we best develop viable gene therapies for such disorders, and how do we unite the academic and clinical communities to generate successful clinical trials?

The current reality for patients with organic acidemias is tenuous. Expanded newborn screening methods can detect organic acidemias early in infancy and, in the United States, is being legislated by more and more states. Better dietary and clinical management of patients allows children born with these rare disorders to sometimes live longer than in the past, but it is impossible to measure the amount of ongoing damage a child constantly accumulates as he grows. At some point, the number of organ systems affected is too great for metabolic balance to be maintained, and even the mildest of cases can cascade into a toxic crisis that is often devastating to the patient and his family.

For the past twenty years, the Organic Acidemia Association has provided support to families dealing with the incredible life changes that come when a child with an organic acidemia is diagnosed. Our organization has touched the lives of hundreds of people who might otherwise have been left grasping in the dark for information and emotional support to help deal with such life transitions. Our mission would not be complete, however, without trying to inspire more young researchers to take a look at studying organic acidemias as possible career paths. Additionally, as more of our metabolic physicians are retiring we need more clinical professionals to take their places and to understand and adequately treat the long-term complications of these diseases until cures are found. Genetic cures for the organic acidemias urgently need to be developed, but more people need to study these disorders to make this a reality.

The Organic Acidemia Association wishes to thank Juan Carlos López for his editorial support, Simon Fenwick for his work on editorial production and, above all, the distinguished authors who shared our vision and took so much time from their busy schedules to contribute to this publication.

Janice Stoebner Boecker, M.A.
Research Director
Organic Acidemia Association
Propionic Acidemia Research Network (PARnet)
jsboecker@paresearch.org
BOARD OF DIRECTORS

(Propionic Acidemia parent)
Kathy Stagni, Executive Director
13210 35th Avenue North, Plymouth, MN 55441
Email: oaanews@aol.com
Phone: 763-559-1797

(MMA Mut 0 Parent)
Jamie Pitre, Treasurer
Menta Pitre, Secretary
207 E. 14th Place, Cutoff, LA 70345
Email: menta@acadianahouse.com
Phone 985-798-5631

(MMA Cbl C Parents)
Tony Winiarski, Director, Co-Editor
Cindy Winiarski, Director
54 Silver Lake Drive, Agawam, MA 01001
Email: Awiniarski@comcast.net
Phone: 413-786-6768

(Propionic Acidemia parents)
Ruth Milne, Trustee,
Propionic Acidemia Research Fund,
501c3 non-profit corporation. Donations to
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THE ORGANIC ACIDEOMIA ASSOCIATION
(OAA) provides information and support to
parents and professionals dealing with a set of
inborn errors of metabolism collectively called
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Stephen Cederbaum, MD
Mental Retardation Research Ctr/NPI,
635 Charles E. Young Dr., South, Rm 347,
Los Angeles, CA 90095-7332

Piero Rinaldo, MD, PhD
Mayo Clinic, Lab Genetics, 200 First St., S.W.,
Rochester, MN 55905

Richard Hillman, MD
Univ of Missouri Health Sciences Ctr,
G-1 Metabolism Clinic, Department of Child
Health, 1 Hospital Drive,
Columbia, MO 65212

Charles P. Venditti MD, PhD
Genetic Disease Research Branch,
National Human Genome Research Institute,
National Institutes of Health, Bldg 49,
Room 4A56A, Bethesda, MD 20892-4472

Dr. Seymour Packman, MD
Dept of Pediatrics, Box 0748,
University of California San Francisco,
San Francisco, CA 94143-0748

Arthur B. Zinn, MD, PhD
Center for Human Genetics, CWRU/University Hospitals of Cleveland,
11100 Euclid Avenue, Cleveland,
Ohio 44106

Keiko Ueda, MPH, RD, LDN
Metabolic Dietitian, Department of Pediatrics/Division of Metabolism,
Floating Hospital for Children,
New England Medical Center,
750 Washington Street, NEMC #434,
Boston, MA 02111

Stephen G. Kahler, MD
Professor, Division of Clinical Genetics,
Department of Pediatrics, University of Arkansas for Medical Sciences,
Division of Clinical Genetics Slot 512-22,
Arkansas Children’s Hospital,
800 Marshall St., Little Rock,
AR 72202-3591

Dr. Susan Winter, MD
Valley Children’s Hospital, 9300 Valley
Children’s Place, O/C Medical Genetics/Genetic Disease Research Branch,
National Human Genome Research Institute,
National Institutes of Health, Bldg 49,
Room 4A56A, Bethesda, MD 20892-4472

J.V. Leonard, MD
40A Bagley Wood Road,
Kennington, Oxford OX1 5LY,
United Kingdom

Dr. Olaf Bodamer FACMG
Biochemical Genetics and National Screening
Labs, University Children’s Hospital Vienna,
Wahringer Gurtel 18-20, 1090 Vienna, Austria

Mendel Tuchman, MD
Professor of Pediatrics, Biochemistry and Biology,
The George Washington University School of Medicine,
111 Michigan Avenue,
NW, Washington, DC 20010-2970

Elaina Jurecki, MS, RD
Kaiser Permanente Medical Center,
280 W. MacArthur Blvd., Oakland, CA 94611

Jerry Vockley, MD, PhD
Professor of Human Genetics and Pediatrics,
Chief of Medical Genetics, Children’s
Hospital of Pittsburgh, 3705 Fifth Avenue,
Pittsburgh, PA 15213

MARTY MORAN, Director, Trustee,
MMA Research Fund,
455 Thornton Rd., Cheyney,
PA 19319
Email: martinmoran@comcast.net
Phone: 610-354-4861

Lori Sanchez, Director
6260 W. Sumac Avenue, Denver,
CO 80123-2695
Email: LoriASanchez@msn.com
Phone: 303-798-5023

455 Thornton Rd., Cheyney, PA 19319
Email: martinmoran@comcast.net
Phone: 610-354-4861

Lori Sanchez, Director
6260 W. Sumac Avenue, Denver,
CO 80123-2695
Email: LoriASanchez@msn.com
Phone: 303-798-5023
PROGRESS IN ORGANIC ACIDEMIAS

James V. Leonard

Clinical and Molecular Genetics Unit, Institute of Child Health, London, UK

Although organic acidemias were described in the late 1960s, progress in understanding the pathophysiology has been relatively slow. As a result treatment is frequently not satisfactory and the outcome is often disappointing.

It is now clear that these disorders are heterogeneous. With the introduction of extended neonatal screening programmes, the spectrum of these disorders has continued to expand. In their paper, Drs. Vockley and Ensenaerde (page 4), discuss a relatively common mild variant of isovaleric acidemia, of which only one case was known until the introduction of these programmes. However, detection of isovaleric acidemia enables treatment to start at an early stage and the outcome for those with severe disease has thereby improved.

By contrast the outcome after early diagnosis of methylmalonic (MMA) and propionic acidemia (PA) with conventional therapy is less satisfactory. These patients continue to have major complications including recurrent episodes of ketoacidosis, cognitive disabilities, pancreatitis, stroke-like episodes, cardiomyopathy, optic atrophy and renal failure. As a result the prognosis for patients with severe disease remains guarded. Although some patients do unexpectedly well, most do not. Unfortunately, as Dr. Tuchman discusses (page 13), we do not understand the cause of these complications, but it is hoped that long-term studies and detailed metabolic investigations in both patients and animal models will cast light on these problems.

Such studies are already under way in the USA, as described by Dr. Venditti and his colleagues (page 10), and in Europe led by investigators in Heidelberg and London. There is a particular need to develop treatment that is evidence-based, as almost none has been tested to current standards (Leonard, 2006).

Despite the difficulties, progress is being made in some areas. There is now a better understanding of glutaric aciduria type 1. An international cross-sectional study (Köllker et al., 2006) combined with some individual clinical and pathological observations and laboratory work, including studies of a mouse model, has led to a better understanding of this disorder. It is now clear that the dicarboxylic acids that accumulate in this disease are neurotoxic and responsible for the acute neurological damage. These compounds are ‘trapped’ in the brain reaching high concentrations (Köllker et al., 2004). The cross-sectional study confirmed the value of the low lysine diet, lysine being the main precursor of glutaric and 3-hydroxyglutaric acid. Restricting lysine intake reduces uptake by the brain and hence the accumulation of the toxic metabolites. Guidelines have now been published for the management of this disorder (Köllker et al., 2007) and we hope that these guidelines, combined with early detection in neonatal screening programmes, will greatly improve the outcome of this devastating disorder.

The recent work on glutaric aciduria type 1 on the mechanisms of brain damage may have wider implications in related disorders including MMA and PA (Köllker et al., 2006). This may help to reduce the burden of neurological disability. However, given the poor outcome of PA and MMA, alternatives to conventional dietary treatment have been explored. The role of liver and kidney transplantation for MMA is uncertain and, although widely discussed, both the indications and timing remains unclear (Barshe et al., 2006, Kasahara et al., 2006). It has to be recognised that the enzymes affected in most organic acidemias are expressed in almost all tissues, but liver transplantation only replaces them in that organ. Although this reduces the metabolic effects of the disease, it does not correct the enzyme deficiency in the brain. This seems to matter less in patients with PA than with MMA, who may have stroke-like episodes years after successful transplantation (Chakrapani et al., 2002).

Better forms of treatment are urgently needed with the aim of correcting the deficiency in all tissues. Although the work on viral vectors for gene transfer discussed by Drs. Holherr and Barry (page 20) is promising, many hurdles have yet to be overcome. Other methods of genetic manipulation hold out more immediate promise. As discussed by Dr. Kraus (page 17), chemical chaperones could be used in people with missense mutations to stabilize the mutant enzyme, correct misfolding and ensure that the enzyme reaches the intended site in the cell. This should increase the residual enzymatic activity sufficiently to ameliorate the disease, even if it does not cure it (Frustaci et al., 2004, Ishii et al., 2004).

Several of these chemical chaperones are already in use in metabolic patients. For example, sodium phenylbutyrate is used as a nitrogen scavenger in organic acidemias and urea cycle disorders.

Altering the activity of aberrant splicing also looks promising, as highlighted by Dr. Ugarte and her colleagues (page 24). Sodium phenylbutyrate can also be used to this end as well, as it is also a histone deacetylase inhibitor. As several of these compounds are already in clinical use, trials could be imminent.

Oligonucleotides may prove to be useful in correcting splice site mutations and another possible line of treatment is the correction of mutations in situ. Whilst some of these have promise for correcting monogenic diseases (for a brief review of both gene targeting and delivery, see Tachikawa and Briggs, 2006), there are many problems and a long way to go before they can be applied to those with organic acidemias.

Address for correspondence
Dr. J. V. Leonard, 4OABagley Wood Road, Kennington, Oxford, OX1 5LY, UK. Email: J.Leadenl@icho.uch.ac.uk

References
ISOVALERIC ACIDEMIA: NEW ASPECTS OF GENETIC AND PHENOTYPIC HETEROGENEITY

Jerry Vockley¹ and Regina Ensenauer²

¹Professor of Pediatrics and Human Genetics at the University of Pittsburgh School of Medicine and Graduate School of Public Health, and Chief of Medical Genetics Children's Hospital of Pittsburgh, Pittsburgh, PA, USA. ²Children's Hospital of the Ludwig-Maximilians University, Munich, Germany

Isovaleric acidemia (IVA) was the first of the organic acidemias to be described and its nature was elucidated due to a combination of astute clinical acumen and new technology. The two original patients were 4 and 2 1/2 years old, respectively, and had similar histories of recurrent episodes of vomiting and lethargy that resolved with supportive therapy including glucose infusions (Tanaka et al., 1966; Budd et al., 1967; Efron, 1967). A “smell specialist” suggested that the unusual odor associated with their acute episodes was likely a short chain fatty acid, and isovaleric acid was identified in patient plasma through the then novel approach of gas chromatography followed by mass spectrometry (Tanaka, 1990). Urine was subsequently shown to contain isovalerylglucose and 3-hydroxyisovaleric acid as well as other metabolites (Tanaka and Isselbacher, 1967; Tanaka et al., 1968). Oxidation of labeled isovaleric acid by patient white blood cells was reduced compared to control cells, and a defect of leucine metabolism was postulated (Shih et al., 1973; Rhead and Tanaka, 1980; Yoshida et al., 1985; Hyman and Tanaka, 1986). It must be remembered that until the characterization of these patients, the oxidation of isovaleryl-CoA was assumed to be catalyzed by the newly identified short chain acyl-CoA dehydrogenase (Crane et al., 1956; Engel and Massey, 1971; McKeen et al., 1979; Freerman et al., 1980). Over the next 20 years IVD was separated from the other acyl-CoA dehydrogenases, purified, cloned, and the molecular nature of the defects responsible for loss of IVD activity elucidated. The use of gas chromatography/mass spectrometry (GC-MS) ultimately became the mainstay of the identification and routine clinical diagnosis of a new class of inborn errors of metabolism resulting in the abnormal accumulation of organic acids in urine, and it remains a valuable tool for biochemical geneticists today (Tanaka et al., 1980; Burke et al., 1983). The more recent application of tandem mass spectrometry (MS/MS) for analysis of the acylcarnitine profile of blood spots from newborn screening filter paper cards has allowed a significant expansion of the recognition of mildly affected and potentially asymptomatic individuals with IVD deficiency through newborn screening (Ensenauer et al., 2004).

Disease spectrum

Early literature on IVA, an autosomal recessive disorder, emphasized two apparent phenotypes (Tanaka, 1990). The first was an acute, neonatal presentation with patients becoming symptomatic within the first 2 weeks of life (Tanaka et al., 1966; Budd et al., 1967; Efron, 1967; Lott et al., 1986; Levy et al., 1973; Elsas and Naglak, 1988). Patients appeared initially well, then developed vomiting and lethargy, progressing to coma. The second group presented with relatively non-specific failure to thrive and/or developmental delay (chronic intermittent presentation) (Levy et al., 1973; Shih et al., 1984; Berry et al., 1988; Elsas and Naglak, 1988; Mehta et al., 1996). Patients who survived an early acute presentation subsequently were indistinguishable from those with the chronic phenotype, and both groups of patients were prone to intermittent acute episodes of decompensation with minor illnesses (Tanaka, 1990). In reality it is now apparent that patients can fall anywhere on the spectrum of acute to chronic presentation and that there is probably little predictive value to the initial presentation. Moreover, with the application of MS/MS in newborn screening, potentially asymptomatic patients with one recurring IVD gene mutation and a mild biochemical phenotype are being identified in increasing numbers, representing an additional phenotype of IVA (Ensenauer et al., 2004). This type may represent a biochemical phenotype only without the expression of any clinical symptoms (such as in benign hyperphenylalaninemia) and, therefore, needs to be differentiated from the classic forms of IVA. For practical purposes, we suggest classifying patients with IVA as “metabolically severe” and “metabolically mild or intermediate”, giving consideration to the broader spectrum of IVA detectable by newborn screening.

Clinical presentation

Nearly all published clinical information on patients with IVA is retrospective (Sweetman and Williams, 2001), and thus the following discussion of clinical symptoms is limited to the classic presentations of IVA prior to newborn screening, that is, manifestation in the neonatal period versus later in childhood.
In one summary of 37 patients compiled from different publications, 28 presented in the first 2 weeks of life, 7 between 2 weeks and 1 year of age, and the remaining 2 after 1 year of age (Tanaka, 1990). Sixteen of the patients were deceased, and of those still alive, seven were reported to have mild to moderate mental retardation.

Neonatal symptoms are non-specific and include poor feeding, vomiting, decreased level of consciousness, and seizures (Tanaka et al., 1966; Budd et al., 1967; Efron, 1967; Lott et al., 1972; Levy et al., 1973; Spiteri et al., 1975; Elsal and Naglak, 1988). Infants may develop hypothermia and appear to be dehydrated. A characteristic smell of “dirty socks” may be present when the patient is acutely sick though, unlike other organic acidemias, the urine has no odor since the unconjugated isovaleric acid responsible for the odor is not excreted in urine in appreciable quantity (Tanaka et al., 1966; Tanaka, 1990). The odor may be best appreciated in body sweat or cerumen from the ear. Acidosis with an unexplained anion gap is characteristic, and hyperammonemia, hyper- or hypoglycemia and hypocalcemia may be present (Tanaka et al., 1966; Budd et al., 1967; Lott et al., 1972; Levy et al., 1973; Yoshino et al., 1982; Mendiola et al., 1984; Tanaka, 1990; Worthen et al., 1994). Secondary hyperammonemia is presumed to be due to inhibition of N-acetylglutamate synthetase by isovaleryl-CoA and/or intracellular depletion of acetyl-CoA leading to reduced N-acetylglutamate synthesis and impairment of the urea cycle (Coude et al., 1979; Stewart and Walser, 1980). Pancytopenia, as well as isolated neutropenia and thrombocytopenia, can occur due to bone marrow suppression (Kelleher et al., 1980). Left untreated, patients may progress to coma and death often due to cerebral edema or hemorrhage (Fischer et al., 1981). Overall, the clinical picture overlaps other organic acidemias including the α-oxidation defects as well as the urea cycle disorders and other primary causes of hyperammonemia, all of which must, therefore, be considered in the differential diagnosis. Patients who survive a neonatal crisis may be clinically indistinguishable from children diagnosed later in life (Tanaka, 1990).

Children diagnosed outside the newborn period may present with more chronic, relatively non-specific findings of failure to thrive and/or developmental delay or mental retardation (Tanaka, 1990). Minus the “sweaty feet odor” of isovaleric acid, which is not present when a patient is otherwise well, there is little to suggest a specific diagnosis in these children and thus, it must be considered in all patients with this clinical picture. They also are at risk of episodes of acute acidosis and metabolic decompensation, usually due to intercurrent illnesses or other physiologic stress including fasting (Berry et al., 1988; Tanaka, 1990). Acute episodes may be misdiagnosed as diabetic ketoacidosis due to hyperglycemia, acidosis and the apparent presence of blood and urinary ketones (Attia et al., 1996). Acute pancreatitis, myeloproliferative syndrome, Fanconi syndrome, and cardiac arrhythmias have been reported (Arnold et al., 1986; Kahler et al., 1994; Weinberg et al., 1997; Gilbert-Barness and Barness, 1999); abnormalities of the globus pallidus can be seen (Sogut et al., 2004). Age of crises can be variable as highlighted by the report of a well-controlled 18-year-old man with IVA who developed acute nausea, vomiting, and mental status changes during basic training camp for the United States Air Force (Feinstein and O’Brien, 2003).

There have been reports of successful pregnancies in women with IVA resulting in apparently well infants (Shih et al., 1984; Spinty et al., 2002).

**Biochemical diagnosis and follow-up**

The majority of patients with IVA today are diagnosed pre-symptomatically through newborn screening by use of MS/MS, which reveals elevations of the marker metabolite C5 acylcarnitine in dried blood spots. Because C5 acylcarnitine represents a mixture of isomers (isovalerylcarnine, 2-methylbutyrylcarnitine, and pivaloylcarnitine), further diagnostic evaluation is required (Table 1). Elevations of 2-methylbutyrylcarnitine are seen in patients with 2-methylbutyrylglycinuria caused by a deficiency of short/branched-chain acyl-CoA dehydrogenase (SCBAD), an inborn error of isoleucine catabolism (Andresen et al., 2000; Gibson et al., 2000), whereas pivaloylcarnitine is derived from pivalic acid, a component of several antibiotics (Abdenur et al., 1998).

<table>
<thead>
<tr>
<th>Test</th>
<th>Determination of</th>
</tr>
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<tbody>
<tr>
<td>Urine organic acid analysis</td>
<td>Multiple abnormal metabolites; isovalerylglucose concentration</td>
</tr>
<tr>
<td>Plasma acylcarnitine analysis</td>
<td>Isovalerylcarnine concentration</td>
</tr>
<tr>
<td>Plasma carnitine analysis</td>
<td>Free carnitine concentration</td>
</tr>
<tr>
<td>Molecular genetic analysis</td>
<td>Common 932C&gt;T (A282V) IVD gene mutation associated with a mild biochemical phenotype; otherwise heterogeneous mutations</td>
</tr>
<tr>
<td>Enzymatic analysis, optional (fibroblasts, lymphocytes)</td>
<td>Residual enzyme activity</td>
</tr>
</tbody>
</table>

*Evaluation following abnormal newborn screening with an elevated C5 acylcarnitine concentration*
Mohsen et al., 1998; Tajima et al., 2005). Fibroblasts, lymphocytes, and amniocytes all have measurable amounts of IVD activity and serve as ready sources of tissue for this purpose (Vockley et al., 1991; Kleijer et al., 1995; Mohsen et al., 1998; Ensenauer et al., 2004). While significant residual activity blunts the level of abnormal metabolites, correlation between clinical presentation and enzyme activity has been poor (Ikeda et al., 1985b; Hyman and Tanaka, 1986; Vockley et al., 1991).

Regarding routine follow-up visits, there is no established laboratory marker for monitoring therapeutic control or disease state. Weight gain, growth and development should be age-appropriate and thus, body measurements are key parameters to follow on a routine basis. Specifically, protein malnutrition must be avoided if the patient is protein restricted. Analysis of amino acids, acylcarnitine concentrations, most if not all patients with IVA have reflected the effects of these mutations but have not provided insight into the clinical variability seen in the disorder (Ikeda et al., 1985b; Vockley et al., 1991). In general, genotype and phenotype have not been well correlated.

Newborn screening and isovaleric acidemia

With the advent of the use of MS/MS to screen newborn blood spots for acylcarnitine concentrations, most if not all patients with IVA should be identified as newborns prior to the development of symptoms. One unexpected finding to arise from newborn screening studies is the identification of individuals with only mild elevations of isovaleryl-CoA related metabolites in plasma and urine, orders of magnitude lower than in the classic forms of IVA, and apparently only partial reduction in IVD activity (Table 2) (Ensenauer et al., 2004). Nearly half of the mutant IVD alleles sequenced from infants diagnosed by newborn screening have been found to contain a common recurring missense mutation (932C>T; A282V; FIG. 2) (Ensenauer et al., 2004). This specific mutation was present in approximately two thirds of newborns in this cohort, mostly in a compound heterozygous fashion. All of the affected newborns carrying the common mutation have remained asymptomatic with

![FIGURE 1. The catabolic pathway of leucine. Isovaleryl-CoA dehydrogenase catalyzes the conversion of isovaleryl-CoA to 3-methylcrotonyl-CoA. Enzyme deficiency results in the accumulation of isovaleryl-CoA derivatives.](image1)

![FIGURE 2. Molecular defects in the IVD gene on chromosome 15q14-15 (Mohsen et al., 1998; Ensenauer et al., 2004). The nucleotide change 932C>T (A282V) in exon 9 has been identified in a significant proportion of individuals diagnosed by newborn screening (Ensenauer et al., 2004).](image2)

and the 2'-hydroxyl hydrogen of the FAD ribityl moiety is crucial for activation of the acyl moiety (Ghisla et al., 1992; Nishina et al., 1995; Miura et al., 2004). The nucleotide change 932C>T (A282V) in exon 9 has been identified in a significant proportion of individuals diagnosed by newborn screening (Ensenauer et al., 2004). The molecular defects in the IVD gene on chromosome 15q14-15 (Mohsen et al., 1998; Ensenauer et al., 2004). The nucleotide change 932C>T (A282V) in exon 9 has been identified in a significant proportion of individuals diagnosed by newborn screening (Ensenauer et al., 2004).
mild or no dietary protein restriction and carnitine supplementation if necessary over a maximum duration of follow-up of up to 5 years of age. Subsequently, asymptomatic siblings of patients identified through newborn screening (ages 3–11 years at the time of diagnosis) have been found to be homozygous or compound heterozygous for the same mutation with a similarly mild biochemical phenotype (Ensenauer et al., 2004). They have remained without symptoms during episodes of febrile illnesses.

Prior to newborn screening, this mutation was identified only in a single patient with mild IVD deficiency originally evaluated for a tic disorder and slight developmental delay (Vockley et al., 1992b; Mohsen et al., 1998). The mutant A282V protein is stable in vitro but is kinetically impaired, exhibiting an increased K_0 and a reduced catalytic efficiency, and has diminished thermal stability (Mohsen et al., 1998; Nasser et al., 2004). It is clear that the newborn screening patients who carry the common mutation either in a homozygous or compound heterozygous state and their sibs skew the spectrum of IVA with more than half of individuals representing a new mild phenotype and potentially remaining asymptomatic. This is an expansion of our view of the natural history of IVA prior to the newborn screening era and leads to significant implications for management and genetic counseling. As opposed to the classic forms of IVA, it is still uncertain whether individuals with the latter type have a disease, a risk of clinical manifestation, or simply express a clinically insignificant biochemical phenotype. While these individuals may have normal leucine homeostasis under physiological conditions, their risk of metabolic decompensation under stress conditions remains to be elucidated.

**Therapy**

There are three goals for therapy of IVA (Table 2). The first is prevention of metabolic crisis by careful clinical observation of the patient regardless of the type of IVA. During times of metabolic stress (including illness and fasting) endogenous leucine from protein catabolism adds significantly to the production of isovaleryl-CoA (Collins et al., 1987; Millington et al., 1987; Pollitt, 1987). Achieving anabolism is the main therapeutic approach. Thus, sick day precautions for patients with IVA should include increased caloric intake in addition to decreased leucine intake. This is most easily accomplished with oral solutions containing simple sugars and leucine free metabolic formulae or powders. IV glucose infusions need to be added if oral intake is interrupted. Leucine intake should be decreased to approximately 50% of the patient’s usual daily minimum, but returned to normal after 24 hr in order to promote protein anabolism. The second goal is long-term reduction of the production of isovaleryl-CoA from leucine catabolism through dietary manipulation (Lott et al., 1972; Levy et al., 1973; Berry et al., 1988; Sweetman and Williams, 2001). Total protein and caloric intake must be adequate to support normal growth in children and maintain an anabolic state, and thus monitoring of weight, length, and head circumference is essential at follow-up. In many cases, it may be sufficient to moderately lower protein intake with natural foods to approximately 1.5 gm/kg per day. In patients with recurrent clinical symptoms, leucine restriction in excess of total natural protein may also be necessary (Sweetman and Williams, 2001). Natural protein necessary to reach the recommended age-appropriate daily requirement must then be provided with leucine-free amino acids. Because of the specific role of leucine in promoting protein synthesis, however, there is a potential for adverse side effects of rigorous leucine restriction including muscle wasting (Harris et al., 2004). Acute episodes of metabolic decompensation can present with emesis, lethargy and signs of overwhelming acidosis. Under these circumstances, immediate hospitalization is required so that IV access can be established and glucose administered. Glucose infusion should be calculated to give at least 8 mg/kg per min with concomitant use of IV insulin if necessary to maintain euglycemia. Reintroduction of oral intake including catabolism-sparing levels of protein (0.5 gm/kg per day) with leucine should occur as soon as it can be tolerated, otherwise parenteral amino acids should be provided. If present, hyperammonemia will reverse with correction of the primary metabolic derangement, alternative ammonia conjugating agents such as sodium benzoate or phenylbutyrate are generally not indicated. The third goal of therapy in patients with IVA is to prevent the accumulation of toxic metabolites by shunting isovaleryl-CoA towards reactions that produce metabolites presumed to be non-toxic and that can readily be excreted. Recognition of isovalerylglycine in urine in the initial patients with IVA first led to the use of glycine to achieve this end (Krieger and Tanaka, 1976; Cohn et al., 1978; Yudkoff et al., 1978; Elsas and Naglak, 1988). Isovaleryl-CoA is enzymatically conjugated to glycine, a reaction that can be augmented by supplementation with exogenous glycine to supra-physiologic levels. Such supplementation prevents or reduces the accumulation of isovaleric acid in blood following a leucine load, and the length and severity of symptoms during intercurrent illnesses (Krieger and Tanaka, 1976; Yudkoff et al., 1978; Shigematsu et al., 1982). Doses of 150–600 mg/kg per day given orally and divided in three or four equal doses of body weight have been proposed, but the optimum dose has not been determined. Patients exhibit a dose sensitive increase in excretion of isovalerylglycine, but at least in one report an increase in the glycine dose from 300 to 600 mg/kg/day of body weight led to a decrease in the excretion of isovalerylglycine, presumably due to inhibition of glycine-N-acylase by glycine (Elsas and Naglak, 1988). Thus, initial dosing in the range of 150–250 mg/kg per day is reasonable in patients with a metabolically severe type of IVA (Table 2). Concern has been raised about the potential for glycine toxicity, though no reports of such an occurrence have been published.

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**Table 2. Recommendations for Therapy in Isovaleric Acidemia**

<table>
<thead>
<tr>
<th>Therapy</th>
<th>Metabolically mild or intermediate</th>
<th>Metabolically severe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prevention of metabolic crisis</td>
<td>Close clinical observation; promote anabolism during illness</td>
<td>None</td>
</tr>
<tr>
<td>Diet</td>
<td>None</td>
<td>Protein restriction</td>
</tr>
<tr>
<td>Medication</td>
<td>Carnitine (30–50 mg/kg per day) if plasma free carnitine concentration is low</td>
<td>Carnitine (100 mg/kg per day)</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>Glycine (150–250 mg/kg per day)</td>
</tr>
</tbody>
</table>

*aMetabolically mild or intermediate: newborn screening blood spot CS acylcarnitine concentration 0.8–6.0 mmol/L; urine isovalerylglycine concentration 15–195 mmol/mol creatinine.

*bMetabolically severe: newborn screening blood spot CS acylcarnitine concentration up to 21.7 mmol/L; urine isovalerylglycine concentration up to 3,300 mmol/mol creatinine.

Values derived from Ensenauer et al., 2004.
The identification of isovalerylcarnitine in blood and urine along with the frequent observation of a secondary deficiency of free carnitine in patients with IVA has prompted treatment with carnitine. A dose of 100 mg/kg body weight per day has generally been used (Table 2) and has been shown to increase the excretion of isovaleryl-carnitine in urine (Mayatepek et al., 1991; Fries et al., 1996). Combined therapy with carnitine and glycine has been shown to maximize the total excretion of isovaleryl-CoA conjugates, but the clinical benefit of combined versus single therapy has not been established through controlled studies (Fries et al., 1996; Itoh et al., 1996). The relative merits of the two therapies either singly or together in patients with more severe presentations including recurrent crises remains a matter of debate.

The necessity of any treatment for individuals diagnosed by newborn screening and carrying the common 932C>T (A282V) mutation is unclear. Specifically, the potential for metabolic decompensation under stress conditions remains to be elucidated. It appears reasonable to observe these individuals clinically, particularly when exposed to metabolic stressors such as febrile illnesses or fasting (e.g., when undergoing surgery). Additional recommendations include low-dose carnitine supplementation if the plasma free carnitine concentration is reduced (Table 2).

Summary
IVA was originally viewed as a relatively rare, life threatening inborn error of metabolism with both acute and chronic manifestations. Recent data from newborn screening studies and additional molecular and cellular laboratory investigations have revealed a far more heterogeneous condition with a potential for normal growth and development. Prospective long-term follow-up of newborns identified with IVA and clinical trials of carnitine and glycine therapy will be critical to optimization of outcome in these patients.

Acknowledgements
This review is dedicated to the memory of Professor Kay Tanaka who originally identified isovaleric acidemia. Dr. Tanaka died on August 21, 2005 at the age of 75. JV was supported in part by NIH grant RO1-DK45482 and the Pennsylvania Tobacco Settlement Fund. RE was supported by the “Hochschul-und Wissenschaftsprogramm” of the Ludwig-Maximilians University Munich, Germany.

Address for correspondence
Jerry Vockley, Department of Pediatrics, University of Pittsburgh School of Medicine, The Children's Hospital of Pittsburgh, 3705 Fifth Avenue, Pittsburgh, PA 15238. Email: gerard.vockley@chp.edu.

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Our integrated approach to study methylmalonic acidemia and related disorders has been to define the natural history observed in patients using clinical research conducted at the National Institutes of Health (NIH) Clinical Research Center and to investigate the disorder in the laboratory using metabolic, genetic and genomic approaches (1). Our long-term objectives are to understand the pathophysiology of the complications in the patients, replicate the disorders in model organisms, and assess new therapeutic approaches in the laboratory and eventually, in patients.

The clinical foundation for understanding the phenotype of methylmalonic acidemia derives from a series of reports published in the late 1960s (2–4). Oberholtzer et al. described two unrelated patients with methylmalonic acidemia (2). The first affected individual was initially suspected to have a renal tubular dysfunction syndrome. This patient had frequent attacks of dehydration and acidosis, and perished during a decompensation at age 21 months. On postmortem examination, fungal pneumonia was noted and a peculiar histopathological appearance of the kidneys was documented. Specifically, the kidneys were shrunken, and the tubules were diminutive and had increased interstitial tissue with a lymphocytic infiltration. The second child displayed a similar phenotype with respect to the acid-base instability. Classical analytical chemical methods were used to demonstrate that she produced large amounts of methylmalonic acid (MMA) in her urine, blood and cerebrospinal fluid (CSF). Of note, she had a CSF:plasma gradient, with concentrations of MMA equal to 1.55 mM in her plasma and 1.575 mM in her CSF. The authors also noted that the metabolic acidosis was only partly explained by the plasma MMA levels. A propensity toward ketosis was demonstrated, with an exquisite sensitivity to oral propionic acid. The child had problems with growth and motor skills in the early years, but when assessed at 5 years of age she had a normal IQ.

The same year Stöckke et al. studied the third child born to a family that had two infants perish in the newborn period with overwhelming acidosis and coma (3). They also demonstrated that the patient produced MMA in enormous amounts. Whole-body metabolism was studied in the index case with C14-valine and H3-MMA. The patient did not respond to parenteral cobalamin but did demonstrate a clinical improvement with simple hyperalimentation consisting of elemental amino acids and glucose given intravenously, and fats and carbohydrates administered by nasogastric feeding, prior to perishing from an intercurrent infection. In the next year, a patient with a similar phenotype of intermittent ketoacidosis and severe methylmalonic aciduria was proven to respond to vitamin B12, firmly establishing a role for the vitamin in human intermediary metabolism (4,5).

The early studies on patients with methylmalonic acidemia generated theories to explain the metabolic perturbations seen in the affected patients, demonstrated fundamental precursor relationships, described the renal lesion seen in the patients, demonstrated that MMA is likely produced de novo or concentrated in the nervous system, showed that the disorder could be treated with precursor restriction and possibly hyperalimentation, localized the biochemical block to the methylmalonyl-CoA mutase step (MCM, also called MUT) and showed that the condition was co-factor responsive in some patients.

Over the past four decades, great progress has been made in understanding and treating this disorder. However, the challenges faced by physicians caring for the early patients, such as the propensity toward metabolic decompensation, growth and feeding problems, renal disease and premature death, still exist (6–9), as current treatment protocol outcomes continue to demonstrate substantial morbidity and mortality in the patient population (10). The larger questions of prognosis, the etiology of the complications, and the development and testing of improved therapies stand as a challenge for translational research endeavors. Along these lines, the use of model systems to study methylmalonic acidemia should help guide the development and testing of newer therapies for these disorders.

Impaired intracellular metabolism of vitamin B12 produces another group of disorders that feature methylmalonic acidemia, as well as (hyper)homocysteinemia. These conditions are named after the corresponding cellular complementation class — cobalamin C (cblC), D (cblD) and F (cblF) — and are also heterogeneous, clinically and biochemically. The spectrum of clinical phenotypes associated with these conditions is incompletely understood and also under investigation in our clinical center. Figure 1 depicts an overview of the steps of intracellular cobalamin metabolism.
observations provide new insights into clinical/imaging correlations and pathways to explore in model organisms. Other ongoing clinical research endeavors include the definition of the natural history of the retinal lesions of cobalamin C (cblC) patients and the larger examination of genotype-phenotype correlations in all patient groups. Future efforts will focus on continued patient characterization, particularly transplant recipients, with the ultimate goal of assembling a large, representative cohort for prospective studies and the testing of newer therapies. An NIH conference on the treatment of isolated methylmalonic acidemia/aciduria is planned and will provide a forum to review current diagnostic evaluations and treatment protocols and to develop consensus guidelines for the future. Understanding the subtleties of the clinical phenotypes seen in the patients is important, because it provides direction for laboratory research and instructs the examination of existing models for findings related to patient care.

Model organism studies of methylmalonic acidemia and related disorders
Methylmalonic acidemia has been difficult to study in model organisms. Earlier efforts have used nutritional (11) and cobalamin-analog treatments (12) to induce methylmalonic aciduria in rats. These treatments are artificial and can produce an incomplete block, especially in the case of nutritional deficiency, causing combined methionine synthase and methylmalonyl-CoA mutase functional impairment. Establishing tractable model organism systems to study methylmalonic acidemia will be required to better understand the symptoms and complications associated with this group of disorders, develop new therapies and further define genes that may play a role in methylmalonyl-CoA and cobalamin metabolism. To date, knock-out murine models of methylmalonyl-CoA mutase have displayed a severe neonatal phenotype with uniform mortality by 24–36 hours of life (1,13). Organisms more amenable to genetic manipulations such as yeast and Drosophila do not possess cobalamin-dependent metabolic enzymes or utilize alternative pathways for propionyl-CoA metabolism, such as the methylcitrate cycle (14).

Table 1. Putative C. elegans homologues of genes involved in propionyl-CoA metabolism

<table>
<thead>
<tr>
<th>Human protein</th>
<th>C. elegans protein gene</th>
<th>Protein % similarity/ e-value</th>
<th>Domains present in human protein</th>
<th>Domain(s) % similarity/ e-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Propionyl-CoA carboxylase alpha subunit (PCCA) NP000273.1</td>
<td>WP:CE04451 F27D9.5 pcca-1</td>
<td>94.6/5.2e-215</td>
<td>Carbamoyl-phosphate synthase Lchain, ATP binding domain, pfam02786</td>
<td>89.8/2.00e-26</td>
</tr>
<tr>
<td>Propionyl-CoA carboxylase beta subunit (PCCB) NP000523.1</td>
<td>WP:CE07269 F52E4.1 pccb-1</td>
<td>95.3/1.4e-212</td>
<td>Biotin caboxylase C-terminal domain pfam02785</td>
<td>100/1.00e-35</td>
</tr>
<tr>
<td>Methylmalonyl-CoA Epimerase (MCE) NP115990.2</td>
<td>WP:CE09082 D2030.5 mce-1</td>
<td>79.6/2.3e-45</td>
<td>Glyoxalase pfam00903</td>
<td>100/7.00e-17</td>
</tr>
<tr>
<td>Methylmalonyl-CoA Mutase (MUT) NP000246.1</td>
<td>WP:CE30404 ZK1058.1 mmcm-1</td>
<td>97.8/7.29e-280</td>
<td>Methylmalonyl-CoA mutase B12 binding domain cd02071</td>
<td>100/5.00e-43</td>
</tr>
<tr>
<td>Methylmalonic aciduria Type A (MMAA) NP758454.1</td>
<td>WP:CE31822 T02G5.13 mmaa-1</td>
<td>82.5/1.2e-89</td>
<td>ArgK protein pfam03308</td>
<td>100/3.00e-105</td>
</tr>
<tr>
<td>Methylmalonic aciduria Type B (MMAB-1) NP443077.1</td>
<td>WP:CE01159 C26E6.11 mmb-1</td>
<td>53.7/5.7e-35</td>
<td>Cobalamin adenosyltransferase pfam01923</td>
<td>97.6/4.00e-50</td>
</tr>
<tr>
<td>MMACHC</td>
<td>WP:CE34461 ZK546.17 cblc-1</td>
<td>52/5e-31</td>
<td>None proven</td>
<td></td>
</tr>
</tbody>
</table>

FIGURE 1. Pathway of cellular processing of cobalamin (CN-cbl, OH-cbl). The class and genes associated with isolated methylmalonic acidemia are cblA (MMAA), cblB (MMAB), cblD variant 2 (unknown gene), cblH (unknown gene) and mut (MCM). Question marks indicate unknown genes or poorly defined cellular processes. The position of the MMAA gene product reflects the probable role that this gene has in protection of the mutase (19). A mitochondrial isoform of the methionine synthase reductase gene is thought to protect or interact with the product of the MMAB gene (20).
Recently, we employed a combination of informatic, genomic, biochemical and metabolic analyses to identify and characterize genes that participate in methylmalonyl-CoA metabolism in the simple worm *Caenorhabditis elegans* (15). In addition to providing direct biochemical evidence for methylmalonyl-CoA mutase activity and adenosylcobalamin synthetic capacity, we utilized genomic approaches to demonstrate that *C. elegans* can be used to define the function of gene products previously suspected to participate in methylmalonyl-CoA metabolism in man, particularly methylmalonyl-CoA epimerase (MCEE).

Informatics was used to identify putative homologues (Table 1) that were further studied using RNAs and metabolite analysis. Biochemical studies on recombinant *C. elegans* MCM and native mitochondrial preparations from the animals provided evidence for a functional mutase reaction (Fig. 2) and further demonstrated that adenosylcobalamin synthetic capacity from hydroxycobalamin was part of the mechanism by which these animals utilized cobalamin. Deletion mutants at the major steps were isolated and characterized and convincingly demonstrated that loss of the MCEE enzyme caused a severe impairment in C<sup>14</sup>-propionate incorporation (Fig. 3). This result, obtained using only model organism genomics and biochemistry, has provided convincing evidence that MCEE is important in the intermediary metabolism of propionyl-CoA and methylmalonyl-CoA. Indeed, two recent reports have described patients with methylmalonic acidemia who have MCEE mutations that are suspected to inactivate the enzyme (16,17). Our *C. elegans* experiments have carefully characterized a number of mutant strains that other investigators might use for future studies on methylmalonic acidemia.

**Murine models of methylmalonic acidemia**

We have generated a targeted deletion of methylmalonyl-CoA mutase (*Mut*) to produce a mouse model of vitamin B12 non-responsive methylmalonic acidemia that displays neonatal lethality, similar to that observed in another murine model (1,13). Using the mice, we have examined the tissue sources of MMA production and used this information to understand the clinical observations of persistent of methylmalonic acidemia in patients who undergo solid organ transplantation. These insights have refined our understanding of the post-transplant physiology in methylmalonic acidemia and suggested new pathways for modulation of metabolism to lower circulating MMA levels.

The creation of murine models to replicate partial deficiency methylmalonic acidemia has been initiated using human patient studies to guide mutation selection. An unusual change in the *MUT* gene was identified in an affected patient enrolled in our clinical protocol. The patient did not respond to high-dose cobalamin therapy *in vivo* but did exhibit a mild increase in cellular enzymatic activity when the patient cells were tested *in vitro*. We determined that the mutant enzyme harbored by this patient exhibits a *K<sub>m</sub>* for adenosylcobalamin 10-fold greater than that of the wild-type enzyme, but has a normal *V<sub>max</sub>*. The homologous mutation has been introduced into the murine gene and used to create a partial deficiency model of methylmalonic acidemia. The resulting animals have methylmalonic acidemia/aciduria but grow and develop normally and are under study as an inducible model of the metabolic changes seen in the more severely affected Mut<sup>−/−</sup> animals.
Gene and cell therapy in murine models of methylmalonic acidemia

We have produced and validated a variety of viral vectors to deliver the Mut gene in cell culture experiments. To demonstrate the efficacy of viral correction, we have successfully corrected primary human methylmalonyl-CoA mutase deficient hepatocytes (18). The extent of correction in the affected human hepatocytes was complete (Fig. 4a, lane 4) and produced a 3-fold increase in propionate metabolism over control hepatocytes after viral correction (Fig. 4b). These studies firmly demonstrate proof of principle for hepatocyte-directed gene therapy strategies in methylmalonic acidemia. Gene delivery and cell therapy experiments will be the subject of future studies.

Conclusions

Integrated, cross disciplinary approaches have led to the development of model organism systems that will be useful to study methylmalonic acidemia using experimental strategies. Patient studies will continue to inform and direct laboratory research.

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Address for correspondence

Charles P. Venditti, Genetic Disease Research Branch, National Human Genome Research Institute, National Institutes of Health, Bethesda, MD 20892, USA. Tel: (301) 496-6213; Email: venditti@mail.nih.gov

References


Propionic acidemia: what we know and don’t know about the pathophysiology

Mendel Tuchman

Children's National Medical Center, the George Washington University, Washington, D.C. 20010

In spite of the identification of many genes responsible for inborn errors of metabolism, their protein products and the relevant biochemical pathways, we still have a poor understanding of how a primary defect in a single biochemical function causes clinical disease. In propionic acidemia, the accumulation of propionyl-CoA within mitochondria is probably responsible for most, if not all of the clinical signs and symptoms of the disease. Yet, the path from this biochemical abnormality to symptoms such as muscle weakness, developmental delay, anorexia, cardiomyopathy and bouts of ketoacidosis is elusive. Here I summarize what is known and unknown about the pathophysiology of propionic acidemia and highlight areas where research in needed in order to better understand this severe disorder and develop better treatments to improve the quality of life and longevity of patients.

Amino acids that derive from dietary or endogenous proteins can either be used for synthesis of new proteins or be degraded and oxidized to ammonia carbon dioxide and water while producing energy. Even if an individual consumes very little protein, some of the amino acids from the diet and those generated in the organism by cellular metabolism are destined to degradation. In order for amino acids to be completely degraded, a cascade of several enzymatic reactions needs to
PCoA is a large and highly charged molecule that is unable to readily leave the mitochondria; for this to happen, the CoA molecule has to be removed first, either by enzymatically breaking it off from the propionyl moiety, or by replacing the CoA with carnitine to form propionyl-carnitine, which can readily cross the mitochondrial membrane, leave the cell into the bloodstream, and subsequently be excreted in the urine. One can therefore easily surmise that, when the PCC reaction is blocked, PCoA will accumulate within the mitochondria and this accumulation is likely to be the culprit responsible for the pathophysiology of PA.

By contrast to propionate, the levels of which may be 100-fold higher in the blood of PA patients than in healthy people (Hommes et al., 1968), PCoA is not found in blood or the urine of patients. In addition to the fact that PCoA cannot cross the mitochondrial membrane, CoA derivatives are quite unstable and degrade under physiological conditions. There is, however, indirect evidence for the accumulation of PCoA within the mitochondria.

Urine organic acid analysis in patients with PA reveals the presence of large amounts of methylcitrate (Ando et al., 1972), a compound that is considered synonymous with the diagnosis of PA. Under normal conditions, little methylcitrate is produced, and it is not known what the metabolic role of this compound (if any) might be. In PA, methylcitrate seems to be the result of PCoA condensation with oxaloacetate, a Krebs cycle intermediate (Ando et al., 1972a). This observation tells us that, when PCoA concentration is markedly increased, this molecule can act as substrate of enzymes that normally do not interact with it. Thus, in this case, the Krebs cycle enzyme citrate synthase, which normally uses acetyl-CoA and oxaloacetate to produce citrate, is now flooded with high concentrations of PCoA, which then outcompete acetyl-CoA as substrate, producing methylcitrate instead.

Other biochemical markers of PA are likely a result of similar mechanisms. 3-hydroxypropionic acid, which is excreted in relatively large amounts in the urine of patients with PA, seems to derive from beta-oxidation, a normal oxidation pathway of free fatty acids (Ando et al., 1972b). Thus, the high concentrations of PCoA allow it to inappropriately enter the mitochondrial beta-oxidation pathway generally reserved for fatty acids with an even number of carbons. Propionylglycine is probably the product of the enzyme glycine N-acetylase, which normally produces hippuric acid from benzoyl-CoA and glycine, and now uses PCoA instead (Rasmussen et al., 1972a,b). Similarly, propionylcarnitine is produced in lieu of acetylcarnitine (the most abundant acylcarnitine species) due to the high concentration of PCoA, which displaces some of the acetyl-CoA molecules from the enzyme carnitine acetyltransferase (Roe et al., 1984).

It is also possible to speculate that, because many CoA molecules are “trapped” as PCoA, a relative CoA deficiency could develop within the mitochondria, impinging on other processes that depend on CoA. Similarly, as large amounts of endogenously produced free carnitine are bound with propionic acid (propionylcarnitine) that is lost in the urine of PA patients, a state of free-carnitine deficiency could develop (DiDonato et al., 1984), leading to the dysfunction of systems that use carnitine (e.g. fatty acid metabolism). Indeed, the livers of patients with PA contain excessive fat, a likely consequence of possible interference of the disease with normal fatty acid oxidation. In addition, there is presence of normal long-chain fatty acids with an odd number of carbons that might be derived from enzymatic reactions involving PCoA instead of acetyl-CoA (Hommes et al., 1968).

Other biochemical observations in PA are less readily explicable, but may still be all caused by the accumulation of PCoA. Ketosis and metabolic acidosis can be caused by interference with normal ketone body utilization. Elevated plasma glycine levels seen almost universally in PA patients could result from inhibition of one or more proteins in the glycine degradation pathway (Hillman and Otto, 1974). Hyperammonemia, which is seen frequently during metabolic decompensation, is possibly the result of a secondary effect on a urea cycle component, likely N-acetylglutamate (Coude et al., 1979).

Patients with PA show other biochemical aberrations, including a number abnormally elevated organic acids such as Krebs cycle derivatives or intermediates in the degradation of leucine. These may result from a general mitochondrial dysfunction, indicating that the accumulation of PCoA causes severe disturbances in many metabolic processes.

Notwithstanding the above considerations, there is evidence that the toxic compounds that are generated in PA can be detoxified through cleansing.
of the blood. In the womb, babies with PA appear to develop well and, when they are born, they are indistinguishable from normal newborns. This would seem to indicate that the maternal circulation and metabolism are able to detoxify PA-related toxins. Thus, it is likely that propionic acid and other metabolites that are generated in PA can pass into the bloodstream, provided that the blood is being continuously cleansed to maintain the gradient between blood and tissues. The same can also be accomplished effectively, yet temporarily, by hemodialysis treatment of severe acute decompensations.

From biochemistry to clinical manifestation

The major challenge in medicine is to link biochemical abnormalities seen in various diseases to the clinical manifestation. If we could connect the biochemistry of disease to clinical signs and symptoms, we should be able to develop better therapies to improve the quality of life and survival of patients. Linking genes and proteins on one end with the clinical phenotype on the other has been, and remains the weakest link of medical science. The reason this is so has to do with the complexity of the biological systems and their incalculable components and factors. It is mind-boggling that we were able to find the cause for PA almost 40 years ago, but we still have only a rudimentary understanding of how a single enzyme deficiency can wreck havoc on so many organ systems in the patients.

We do not know whether the biochemical aberrations that are observed in PA exert their effect mainly locally, causing dysfunction of the organ in which they are generated and/or whether toxicity exerted on some organs arrives via the bloodstream. For example, does the toxicity of PA on the brain results from the generation of toxic metabolites within the brain itself, or is it caused by toxic metabolites that are generated in other organs (liver, muscle, kidney, intestine). Indeed, intestinal bacteria produce propionic acid from their metabolism, some of which is absorbed into the blood (Thompson et al., 1990a). This may be a significant source of toxicity for other organs in patients with PA, as temporarily reducing the number of intestinal bacteria with antibiotics has been reported to ameliorate the metabolic crises (Thompson et al., 1990b).

Some insight into the answer to the question of whether the generation of toxic compounds that leads to organ damage occurs locally or distally could be obtained from genetically engineered mouse models of PA, which currently exist (Miyazaki et al., 2001), and from experience of patients with PA who underwent liver transplantation, in whom an affected organ that generates significant amounts of propionic acid has been replaced by a normal liver (Barshes et al., 2006).

Let us consider the main features of the clinical phenotype of PA and explore if and how they can be linked to biochemical mechanism, keeping in mind that all of them result from a single blocked biochemical reaction.

PA manifestations can be arbitrarily divided into acute and chronic. Although some of the acute manifestations could just reflect more extreme aberrations of the same abnormalities that are present in the chronic phase of the disorder, one needs to explain the rapidity and insidiousness of acute attacks. These are sometimes associated with concurrent, and not severe, viral or bacterial infections, but many times no apparent trigger can be identified. It is possible that there exists a pathological threshold that, when crossed, sets in motion a vicious cycle that leads to rapid metabolic decompensation. In an unpublished observation, I have observed that days before acute attacks, the ratio between propionylcarnitine and free carnitine in the blood increases from its already abnormal baseline. When I tried to increase the carnitine supplement dose, it did not seem to make a difference and the patient would still develop an acute ketoacidosis attack within a few days requiring hospitalization and intravenous treatment. Does this tell us that there is a slow phase of decompensation followed by a rapid one? Only well controlled research studies in a sufficiently large number of patients could confirm this observation, allowing us to develop better prevention strategies.

When acute exacerbation occurs, it is usually associated with ketoacidosis, but hyperammonemia does not seem to be a required component of an acute attack. The exact path from the accumulation of P-CoA to acidosis and ketosis, in spite of being intuitive, is not well understood. The acidosis is associated with an increased anion gap, thus pointing to increased blood levels of circulating acids, such as ketone bodies (acetoacetate and 3-hydroxybutyrate) and others. It is possible, even likely, that P-CoA interferes with ketone utilization (Dutra et al., 1991) and, when P-CoA reaches a certain threshold, ketones in the blood rise rapidly leading to significant acidosis.

More research is needed to understand the sequence of these events and the specific enzymes affected, and how to intervene most effectively. Currently, we use what I refer to as a “treatment dogma”, as much of it does not rely on evidence-based medicine or well-controlled studies, but on “medical reasoning” and a trial and error approach. For example, we know that, following treatment with intravenous fluids, large amounts of glucose and carnitine, the acute attacks subside within a few days. Frequently, dialysis is used to treat extreme decompensations, usually in the newborn period. But we have little knowledge of what needs to occur at a mechanistic level for the attack to subside and whether all therapeutic measures that we take are actually useful. Short-term administration of anabolic hormones such as growth hormone (Marsden et al. 1994) or androgens might be useful but, again, their effect needs to be studied in a systematic way.

As acute exacerbations are frequently associated with abnormal signs of the central nervous system, from various degrees of lethargy to full coma, one can assume that each attack causes incremental brain damage. If these attacks could be prevented or minimized, a better long-term outcome should follow. Patients with PA seem to have abnormalities in basal ganglia and the brain’s white matter (Brismar and Ozand, 1994). As the basal ganglia and the brain stem have the highest oxygen consumption in the brain and are affected in disorders of energy metabolism, it is not unreasonable to assume that that energy production is compromised in the brain cells of patients with PA (Chemelli et al., 2000).

Similarly, the hypotonia, muscle weakness and myopathic features observed in patients with PA point to an energy consumption-related mechanism. However, exactly how energy deficits develop in PA and why significant lactic acidosis is not part of the picture while it is commonly observed in disorders of energy metabolism remains unclear. Patients with severe PA tend to develop a hypertrophic cardiomyopathy later in life (Massoud and Leonard, 1993). Although one is tempted to ascribe this sign to mitochondrial dysfunction and perhaps to carnitine deficiency, the reason for its late development in the course of the disease is unclear and requires detailed study.

Every health professional who has managed patients with PA has likely observed the chronic, severe anorexia
of these patients. While anorexia is not uncommon in metabolic conditions, the disinterest in food manifested by patients with PA is striking, universally requiring enteral feeding. The dependence of the patients’ health on maintaining adequate caloric intake only exacerbates this problem. Yet, we have little understanding of the pathophysiology. Is the anorexia the result of a neurodevelopmental problem? In other words, are brain connections that need to develop early after birth compromised by the abnormal brain biochemical milieu? Or is anorexia the result of never allowing the patients to develop a sense of hunger (and therefore to regulate their own food intake), as their lives from early on depend on constant caloric intake that is often closely monitored? Although some clues to abnormal neurotransmitter physiology have been put forward, this clinical problem remains one of the most frustrating to patients, their families and health professionals. We could not rule out that some of the poorly explained clinical signs we observe in patients with PA are side effects of our management. As we have to resort to treatment based on “medical reasoning”, our common sense might prove incorrect because we do not have an in-depth understanding of the paths that lead from the biochemical abnormality to the clinical manifestation.

Liver transplantation has been tried in a dozen patients with propionic acidemia as a measure to provide normal propionate metabolism (Barshas et al., 2006). As the biochemical defect is present in other tissues, one would not expect complete reversal of the metabolic disorder, as is seen in other genetic conditions that affect the liver almost exclusively. In spite of this, liver transplantation has improved the outcome of patients, reduced the number of acute episodes and improved the metabolic abnormalities. These observations support the role of the liver as having a major role in the pathophysiology of PA and should be a target of future new treatments.

It is known that the circulating white blood cells, especially neutrophils, in patients with PA are frequently reduced in number. It seems that propionic acid in high concentrations can inhibit the growth and maturation of bone marrow cells (Stork et al., 1986). As one of the main roles of white blood cells is to combat bacteria, patients with PA are susceptible to infections. This specifically increases the risk of infections from central venous catheters that need to be frequently used in patients, as vascular access is so difficult in many of them. Again, the mechanism by which bone marrow suppression occurs in PA is unknown.

Concluding remarks
We have important information about the genetic, biochemical and clinical aspects of PA, including the genes and proteins involved, how to diagnose the disorder and how to gauge its clinical severity. The survival of patients with PA has been increasing thanks to more preventive and aggressive treatments, and to the development of specific medical foods. However, we still have a long way to go to prevent the mental retardation and other disabilities that are almost universal in PA.

We have a large gap in knowledge about the path from biochemical abnormality to clinical signs and symptoms, both at a cellular level, the organ level and the whole patient. As effective gene therapy for multiple organs, the ultimate tool for curing PA, remains elusive, we need to focus our efforts on improving our understanding of the factors involved in the pathophysiology and the way they interact. This can only be accomplished by innovative, meticulous research. It would be highly desirable to create a research network for PA, methylmalonic acidemia and other similar organic acid disorders to be able to study larger numbers of patients on identical protocols, as has recently been started for other rare diseases with federal funding.

Acknowledgements
This article is dedicated to the patients with PA and their parents and families from whom I have learned to listen carefully, not to give up, to challenge the dogma, to appreciate my limited understanding of the complexity of the disease, and to realize that the treatments that we currently can offer are grossly insufficient and that we need to do better.

Address for correspondence
Mendel Tuchman, M.D., Children’s National Medical Center, 111 Michigan Avenue NW, Washington, D.C. 20010.
Phone: 202-884-2549; Fax: 202-884-6014; Email: mtuchman@cnmc.org

References


Therapeutic Approaches to Propionic Acidemia
Jan P. Kraus
Department of Pediatrics, University of Colorado School of Medicine, Aurora, CO 80045-0511.

In vitro evidence indicates that the activity of mutant propionyl CoA carboxylase — the enzyme affected in people with propionic acidemia — increases in the presence of protein or chemical chaperones. Propionic acidemia may therefore be a ‘conformational disorder’, opening the door to new therapeutic opportunities.

Four different biotin-dependent carboxylases play a central role in mammalian metabolic pathways, such as oxidation of odd-chain fatty acids, catabolism of branched amino acids, fatty acid synthesis and gluconeogenesis. One of these is propionyl CoA carboxylase (PCC), which catalyzes the conversion of propionyl CoA to α-methylmalonyl CoA in the mitochondrial matrix (Fenton et al., 2001).

This enzyme consists of two nonidentical subunits — α and β — encoded by two different nuclear genes, designated PCCA and PCCB, respectively. Structural studies of the human enzyme have indicated that α PCC is 72 kDa in size while β PCC is 54 kDa. Overall, PCC has an αβαβ structure (Fenton et al., 2001; Haase et al., 1984). Mutations in either gene result in an autosomal recessive disease, propionic acidemia (PA), which usually presents as a life-threatening ketoacidosis in the neonatal period with protein intolerance, vomiting, failure to thrive, lethargy, and profound metabolic acidosis symptoms. This disease can result in mental retardation and can be sufficiently severe to cause neonatal death (Fenton et al., 2001).

The cDNAs for α and β subunits have been cloned and the structure of the genes has been elucidated (Campeau et al., 2001; Lamhonwah et al., 1994; Ohura et al., 1993; Rodriguez-Pombo et al., 1998; Stankovics and Ledley, 1993). With the sequence information available, it has been possible to identify mutations from PA patients (Lamhonwah et al., 1990; Tahara et al., 1993; Ugarte et al., 1999), and many new mutations have been found recently in different ethnic groups (Kim et al., 2002; Perez et al., 2003). Currently, approximately 52 and 53 mutations have been reported in the PCCA and PCCB genes, respectively (for a continuously updated list of all reported PCC mutations see our website at http://www.uchsc.edu/cbs/pcc/pccmain.htm).

Expression of recombinant human PCC in E. coli
We have developed a human PCC expression construct designated pPTH_PCC_AB (Fig. 1). This plasmid has been designed to facilitate the simultaneous and balanced expression of both PCC α and β subunits in E. coli. Subsequent experiments in our laboratory have shown that successful assembly of wild-type PCC in E. coli is greatly aided by the presence of the molecular chaperons GroES and GroEL (Kelson et al., 1996a). There are 54 mutations currently identified in the β subunit of human PCC. To date, we have engineered a total of 21 of these mutations (primarily missense) into our PCC expression construct and studied their expression products in E. coli. The initial analyses of the mutant recombinant enzymes have been all done in semi-purified extracts. Nevertheless, we were able to conclude from the data obtained on all 21 mutants that at least half of them should be amenable to purification and further biochemical and physical characterization at the level of pure proteins.

Currently, we have purified a total of 11 mutants and one polymorphic variant form of PCC (Fig. 2). About one third of these were proteins with significant residual PCC activities (R165W, E168K, P228L, R410W and the polymorphism A497V), while the remainder were inactive (R44P, S106R, G131R, G198D, V205D, T428I, and M442T) (Table 1). The T428I and the R410W are the most prevalent PCC mutations in East Asia (Korea and Japan, in particular). We have further characterized three of the purified PCCs containing the pathogenic mutations (R410W, R165W, and E168K) and one PCCB polymorphism (A497V) in order to elucidate the potential structural and functional effects of these substitutions. The purified R165W and E168K had as much as 42 and 32% of wild-type activity, respectively, while the polymorphic variant, A497V had 138% of wild-type activity. No significant differences have been observed in K_m values for propionyl CoA, oligomeric assembly and secondary structure among these PCCs using PCC assays, size-exclusion chromatography and far ultraviolet circular dichroism. However, the variant PCCs were less thermostable than the wild type. Considering the profound effect of the co-expressed chaperon proteins on PCC folding, assembly, and activity, we believe that the pathogenic nature of these mutations...
is more likely due to lack of assembly rather than any disruption of the catalytic process (Jiang et al., 2005). Taken together with our preliminary results on chemical chaperones (see below), these findings indicate that some PCC mutations exert their pathogenic effect due to an inability to assemble correctly in patient cells and that functional replacement of our GroES/EL chaperone system with chemical chaperones has highly significant potential to restore activity to these mutant forms of PCC in subjects with PA (Chloupkova et al., 2002).

**Is PA a conformational disorder?**

Protein folding is an essential cellular process, which can be impaired by mutations, errors in translation or many environmental factors such as prions, oxidative, osmotic or thermal stress (Soto, 2001). Misfolded proteins are prone to increased ubiquitylation and rapid degradation (Kisselev and Goldberg, 2001), and/or to aggregation with subsequent formation of aggresomes (Johnston et al., 1998). Diseases arising by such a mechanism are collectively named conformational diseases (Carrell and Lomas, 1997; Soto, 2001).

Protein chaperones direct the folding of polypeptides into functional proteins, facilitate developmental signaling and, as heat-shock proteins (HSPs), can be indispensable for survival in unpredictable environments. Chaperones do not alter genotype, but rather the expression of genetic variation as phenotypic variation. Protein folding, maintenance and repair are highly specialized cellular functions (Bukau and Horwich, 1998; Frydman, 2001; Hartl and Hayer-Hartl, 2002). Chaperones act by preventing the formation of promiscuous, but energetically stable, associations in, or between, non-native polypeptides, many use ATP-driven cycles of binding and release to destabilize non-native intermediates. This gives the polypeptides repeated opportunities to reach a stable mature fold (Rutherford, 2003).

Several recent studies have described the use of a group of low-molecular-weight compounds to reverse the mislocalization and/or aggregation of proteins associated with human disease. These compounds, which include polyols such as glycerol, trimethylamines such as trimethylamine-N-oxide (TMAO) and amino acid derivatives, have been called "chemical chaperones". Recent studies have suggested that other compounds, such as 4-phenylbutyric acid (PBA) and membrane-permeable forms of enzyme antagonists, ligands or even substrates, can also act as chemical chaperones for misfolded or mislocalized enzymes. The mechanisms by which chemical chaperones function are not fully understood but are thought to include stabilization of improperly folded proteins, reduction of aggregation, prevention of nonproductive interactions with other resident proteins and alteration of the activity of endogenous chaperones. Chemical chaperones of the glycerol, TMAO and PBA class have general effects on multiple proteins while antagonists, ligands and substrates are thought to affect only the specific proteins with which they interact (Perlmutter, 2002).

With this in mind, ever since we started to express PCC in *E. coli* (Kelson et al., 1996b), we have used a co-expressed GroESL to aid in its assembly. Figure 3 illustrates the response of PCC to GroESL. The activity of the wt and the mutants approximately doubles in the presence of GroESL. The activity of this "chaperoned" PCC persists so that PCC mutants can be purified from crude *E. coli* extracts to homogeneity and the active enzymes do retain very high residual activities (Table 1 and Fig. 2). For all further preliminary studies with chemical chaperones we have concentrated on three βPCC mutants, R165W, E168K, and R410W, that were clearly impaired in folding/assembly rather than in the catalytic steps (Jiang et al., 2005).

**Looking for chemical chaperones for PCC**

An initial screen of several candidate chemicals was performed using wild-type recombinant human PCC expressed in *E. coli* without GroESL but with the individual chemicals in the growth medium. As can be seen in Table 2, the presence of 5% glycerol in the medium had clearly a negative impact on PCC activity in cell extracts. While DMSO and TMAO had no influence on PCC activity, betaine and taurine had a clearly favourable influence. By far, the most pronounced effects were seen with 4-phenylbutyrate (PBA) and valproic acid (VPA). The ~2.5-fold increases in activity roughly paralleled those observed with GroESL. We then performed a screen of the three selected mutants with four of the most promising chemicals from the

### Table 1. Activities of purified mutant PCCs

<table>
<thead>
<tr>
<th>Mutations</th>
<th>SA (pmol/min/mg protein) × 10^4</th>
<th>% of WT Mean ±SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>23.35 ±1.11</td>
<td>100.00</td>
</tr>
<tr>
<td>R44P</td>
<td>9.93 ±0.37</td>
<td>42.50</td>
</tr>
<tr>
<td>S106R</td>
<td>7.54 ±0.09</td>
<td>32.30</td>
</tr>
<tr>
<td>G131R</td>
<td>9.43 ±0.06</td>
<td>40.41</td>
</tr>
<tr>
<td>G198D</td>
<td>0.59 ±0.10</td>
<td>2.55</td>
</tr>
<tr>
<td>V205D</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T428I</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M442T</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>R165W</td>
<td>9.32 ±0.09</td>
<td></td>
</tr>
<tr>
<td>E168K</td>
<td>7.54 ±0.09</td>
<td></td>
</tr>
<tr>
<td>R410W</td>
<td>9.43 ±0.06</td>
<td></td>
</tr>
<tr>
<td>P228L</td>
<td>0.59 ±0.10</td>
<td></td>
</tr>
</tbody>
</table>

**Figure 3. Expression of PCC with and without GroESL. The values represent the mean and SE derived from 3–19 independent experiments. The significant levels of differences between experiments with and without GroESL are shown as ** and **** for P<0.01 and P<0.0001, respectively.**

**Figure 4. The impact of four chemical chaperones on three mutant PCCs. Control signifies no added chaperone. The values represent percentages of WT activity in the absence of GroESL and are shown as a mean ± SE derived from 3–8 independent experiments. The significant levels of differences between no treatment and PBA or VPA treatments yielded P<0.0001.
Table 2. Impact of chemicals on wild-type PCC activity

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Specific activity (pmol/min/mg protein) × 10⁻³</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>296.6 ±44.0</td>
<td>100.0 ±14.8</td>
</tr>
<tr>
<td>Betaine</td>
<td>375.7 ±93.0</td>
<td>126.7 ±31.3</td>
</tr>
<tr>
<td>Taurine</td>
<td>422.1 ±122.0</td>
<td>142.3 ±41.2</td>
</tr>
<tr>
<td>Glycerol</td>
<td>170.5 ±42.2</td>
<td>57.5 ±14.3</td>
</tr>
<tr>
<td>DMSO</td>
<td>294.1 ±78.1</td>
<td>102.9 ±26.3</td>
</tr>
<tr>
<td>TMAO</td>
<td>305.4 ±78.0</td>
<td>99.2 ±26.4</td>
</tr>
<tr>
<td>PBA</td>
<td>701.5 ±71.9</td>
<td>236.5 ±24.3</td>
</tr>
<tr>
<td>VPA</td>
<td>750.3 ±124.2</td>
<td>252.9 ±41.9</td>
</tr>
</tbody>
</table>

The preliminary wild-type PCC testing.

The response of the mutant PCCs was more pronounced that the one observed for the wild-type enzyme. Figure 4 shows that PBA and VPA yielded activities that approached 60 and 80% of wild-type activity, respectively.

Conclusion and outlook

We have found that some misfolded PCC mutants respond robustly to chaperones. The most potent response so far has been observed with PBA or VPA and in combinations of the molecular chaperone GroESL and the chemical chaperone VPA. The combined treatment of the R165W mutant increased the activity ~9-fold reaching >90% of the untreated wild-type enzyme activity. In addition to characterizing the enzyme in crude extracts, we have purified the wt and R165W enzymes following four different treatments. The results with the purified enzymes showed that the changes in the PCC dodecamer conformation are permanent (results not shown). The experiments with the purified enzymes have provided evidence that the chaperoned enzymes are expressed in increased amounts, and that they are catalytically more efficient and conformationally more stable to heat treatments.

Among the chemical chaperones we used in our preliminary trials, three of them are already in clinical use for different conditions. Betaine has been used extensively in pyridoxine nonresponsive homocystinuria as a donor of a methyl group for remethylation of homocysteine to methionine by betaine homocysteine methyltransferase. PBA (4-phenylbutyric acid) is already in routine use for ameliorating hyperammonemia in subjects with PA and urea cycle disorders. It is also in clinical trials for treatment of solid tumors, for cystic fibrosis and for spinal muscular atrophy. VPA is the treatment of choice for some forms of epilepsy. It has also been used for treatment of spinal muscular atrophy.

Future studies

We will continue to study any responses of recombinant mutant PCC enzymes to treatments with chaperones in E. coli. We will measure the amounts of PCCA and PCCB mRNAs in our skin fibroblast lines to determine which ones should be tested for a treatment with chemical chaperones. We have recently obtained Arimoclomol, a co-inducer of heat shock proteins that is being tested for the treatment of amyotrophic lateral sclerosis and has been shown to have cytoprotective effects including a murine model of ischemia and wound healing in the diabetic rat. We are in the process of testing this compound in our previously characterized constructs and in skin fibroblast cultures of propionic acidemia patients.

It is our hope that one or more of the compounds that we are currently testing to patients suffering from these diseases.

Address for correspondence

Jan P Kraus, Ph.D., Professor of Pediatrics, UCHSC at Fitzsimons, Mental Retardation & Developmental Disabilities Research Center, Mail Stop 8313, PO Box 6511, Aurora, CO 80045-0511. Email: jan.kraus@uchsc.edu.

References


Gene Therapy for Propionic Acidemia

Sean Hofherr¹ and Michael A. Barry²

¹Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, TX, USA
²Department of Internal Medicine, Department of Immunology, Division of Infectious Diseases, Translational Immunovirology Program, Molecular Medicine Program, Mayo Clinic, Rochester, MN 55902

Propionic acidemia (PA) is a metabolic genetic disease that occurs in ~1 in 35,000 live births in the United States and up to 1 in 3,000 births in Saudi Arabia. This disease causes metabolic acidosis, ketosis, vomiting, lethargy, mental retardation and death. This metabolic decomposition occurs in response to the consumption of normal levels of proteins in the diet, but can also be precipitated by other stimuli. PA is caused by deficiency in either the α or β subunits of the mitochondrial enzyme propionyl CoA carboxylase (PCC), encoded by the PCCA and PCCB genes. Since there is currently no cure for PA, we discuss the feasibility and pilot testing of gene therapy to correct this disease in mice deficient for PCCA.

Propionyl CoA carboxylase deficiency

Propionic acidemia (PA) is an autosomal recessive inborn error of metabolism estimated to occur in ~1 in 35,000 live births in United States and perhaps up to 1 in 3,000 incidence in Saudi Arabia (reviewed in Ugarte et al., 1999). The disease is heterogeneous in clinical manifestation, presenting within the first week of life or after 6 weeks. Clinical features include metabolic acidosis, ketosis, vomiting and lethargy. Mental retardation and median survival of 3 years is observed in early-onset patients, whereas late-onset patients may present with movement disorders and dystonias.

PA is caused by deficiency in the mitochondrial enzyme propionyl CoA carboxylase (PCC). PCC is involved in the metabolism of branched chain amino acids, odd-numbered chain length fatty acids, cholesterol and other metabolites (Fig. 1). In the absence of PCC activity, metabolites build up in patient cells and in their circulation causing metabolic ketoacidosis (Fig. 1a). This ketoacidosis is complicated by hyperammonemia that is produced by the inhibition of N-acetylglutamate synthetase by excess propionyl-CoA (Coude et al., 1979). The presence of byproducts in the blood or urine including propionyl-carnitine, 3-hydroxypropionate and methylcitrate are also indicators of failure of this enzyme (Fig. 1a). Because of its role in amino acid metabolism, consumption of normal protein levels in the diet can exacerbate the symptoms of PA.

PCC is a biotin-dependent enzyme consisting of six α and six β subunits (Lamhonwah et al., 1986). The PCCA protein is covalently biotinylated on lysine 569 near its carboxy terminus by the biotin ligase holocarboxylase synthetase (Saunders et al., 1982). Once conjugated to PCCA, biotin mediates the transfer of carbon dioxide from bicarbonate to propionyl-CoA (Fig. 1a and Chapman-Smith and Cronan, 1999). In cells deficient for the α subunit, the β subunit is unstable (Lam Hon Wah et al., 1983). The α subunit is encoded by the PCCA gene on human chromosome 13 and the β subunit is encoded by the PCCB gene on human chromosome 3. Most mutations in the PCCA and PCCB in patients are thought to be point mutations or small insertions or deletions (Desviat et al., 2004). However, splicing mutations have been more recently identified that skip whole exons (Desviat et al., 2006), suggesting that larger regions of the proteins could be lacking in some PA patients.

A mouse model of PCC deficiency

Dr. Toru Miyazaki’s group developed a mouse model of PA by deletion of the α subunit of propionyl CoA carboxylase (PCCA) (Miyazaki et al., 2001). Mice null for PCCA have exceptionally high concentrations of propionic metabolites including serum propionyl-carnitine,

There is currently no cure for PA. Patients are currently managed with intensive alkali therapy and protein restriction, as normal protein consumption can drive severe metabolic ketoacidosis. Administration of excess carnitine can attenuate symptoms, as carnitine can act as a carrier to remove propionyl-CoA from cells (Roe et al., 1984). In addition, N-carbamyl-glutamate administration can reduce hyperammonemia by bypassing propionyl-CoA inhibition of N-acetylglutamate synthetase (O’Connor et al., 1989). In two cases, children with PA who suffered from frequent metabolic decompensation have been treated by liver transplantation (Yorifuji et al., 2000, Kayler et al., 2002). Although liver transplantation had only minimal effects on the levels of propionyl-CoA metabolites in the blood, hyperammonemia was corrected and metabolic decompensation was controlled (Yorifuji et al., 2000). More importantly, the growth rate and mental development of these children also improved significantly after transplantation.

Despite the cell autonomous nature of PA, these data suggest that liver correction of PCC can attenuate the systemic and at least some of the most problematic neurologic symptoms of PA. But while liver transplantation can attenuate PA, the danger and immunosuppression intrinsic to this approach mandate that alternate therapies be developed to correct the PA metabolic defect.

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PCCA gene delivery can further reduce the low stringency model in which genetic function by liver-directed gene therapy to the potential to replace missing PCCA to near wild-type levels within 3 weeks. Through 8 days after birth, but then rises remains at 10–20% of normal levels decreases blood propionyl-carnitine levels expression of PCCA at 10–20% of wild-type correction that will likely be required in provides guidance on the level of genetic ketonuria and dehydration. This strain urinary methylcitrate upon birth and die urinary 3-OH-propionate (3-HP) and urinary methylcitrate upon birth and die within 24 hours of birth due to acidosis and poor feeding. These PCCA deficient mice provide a stringent model for PCCA gene therapy where genetic correction must occur before birth or within 36 hours of birth.

Dr. Miyazaki’s group generated two additional mouse strains by transgenesis in which the PCCA cDNA is expressed in a liver-specific fashion from the serum amyloid P component (SAP) promoter (Miyazaki et al., 2001). In one PCCA−/− SAP strain, liver-specific PCCA expression remains at 10–20% of normal levels through 8 days after birth, but then rises to near wild-type levels within 3 weeks. This increase in liver-specific PCCA activity decreases blood propionyl-carnitine levels from 17 to 11 μM, but these levels remain substantially higher than the 0.9 μM level in PCCA−/− mice (Miyazaki et al., 2001). These data provide proof of principle for the potential to replace missing PCCA function by liver-directed gene therapy to attenuate the phenotypes associated with PCCA deficiency. This work also provides a low stringency model in which genetic modification is not needed to rescue the animals, but that can be used to test if PCCA gene delivery can further reduce the systemic levels of propionyl-carnitine.

A second strain of PCCA−/− SAP mice also expressed PCCA in a liver-restricted fashion but, unlike the high strain, this expression never rose above 10–20% of wild-type levels (Miyazaki et al., 2001). As a result, these mice survive to weaning, but die before 3 weeks of age due to severe ketonuria and dehydration. This strain provides guidance on the level of genetic correction that will likely be required in any therapy for PA. In this case, long-term expression of PCCA at 10–20% of wild-type levels was insufficient to rescue the animals. These data suggest that any therapy for PA must establish greater than 20% of wild-type expression to rescue the deficiency.

### Gene therapy for PA

Dr. Miyazaki’s work and past liver transplantations in patients provide excellent proof of principle for the possibility of performing gene therapy in the liver to at least mitigate the symptoms of PA. This is fortuitous, as most gene therapy vectors preferentially deliver genes into this organ. The liver is a logical target for gene therapy for PA, because the liver is thought to be the ‘sink’ for much of the metabolism in the propionic pathway. While a number of useful gene therapy vectors are being developed for liver gene therapy, very few can express the massive levels of transgene that will likely be needed to correct PA deficiency even in a mouse. While non-viral vectors may be potentially safer gene delivery vehicles than viral gene therapy vectors, few or none currently can drive the high level correction that will be needed for PA. Given this, we will discuss two promising viral vectors for PA gene therapy and look forward to the development of safer and more effective non-viral and viral vectors for PA treatment.

### Adenoviral vectors for liver-directed gene therapy

Adenoviral (Ad) vectors are arguably the most robust vectors for mediating high level gene expression in the liver. Several studies have shown that intravenous injection of Ad vectors into mice and primates results in 70% of total gene delivery occurring in the liver (Tang et al., 1994; Huard et al., 1995). The high level of gene delivery occurs predominantly by transduction of hepatocytes (Hegenbarth et al., 2000). Ad vectors are able to transduce a higher fraction of liver hepatocytes with levels of modification occurring in nearly 100% of hepatocytes (Yang et al., 1996a; Morral et al., 1997). Few other vectors have been shown to mediate the same robust level of liver transgene expression as Ad vectors. For example, liver transduction by Ad vectors expressing α1-antitrypsin produces supraphysiologic levels of 6 mg/ml of α1-antitrypsin in the blood of mice (Morrall et al., 1998). Or in other words, modification of the liver with Ad vectors was able to produce this transgene product at such high levels that α1-antitrypsin became the second most abundant protein in the blood of the animals. Similarly, Ad vector delivery of the apolipoprotein A-1 gene to the liver also produces supraphysiologic levels of this transgene in Apo-E-deficient mice (Pastore et al., 2004).

From these observations, it is possible to argue that the supraphysiologic expression ability of Ad vectors may produce sufficient PCCA protein in the liver to further reduce propionyl-carnitine levels in PCCA−/− SAP high mice. Likewise, Ad vectors should be able to produce the greater than 20% of wild-type PCCA expression needed to correct the PA phenotype in PCCA−/− mice. While Ad vectors are potent for mediating gene delivery, they also have the reputation for being one of the most immunogenic vectors for gene therapy (Fields et al., 2000). Both innate and adaptive immune responses can be elicited Ad capsid proteins and against the transgene protein itself to attenuate gene delivery and expression. Innate immune responses occur in a dose-dependent fashion in mice, non-human primates and humans after intravenous injection of Ad vectors. Uptake of Ad virions by immune and non-immune cells precipitates the release of massive amounts of inflammatory cytokines including interleukin (IL)-6 and tumor necrosis factor (TNF)-α within 3 to 24 hours of intravenous injection (reviewed in Liu and Muruve, 2003). These events produced by intravenous administration of large doses (e.g. 1013 virus particles/kg) can lead to lethal events in non-human primates (Brunetti-Pierri et al., 2004).

Innate responses to Ad vectors also likely played a significant role in the unfortunate death of Jessie Gelsinger in the ornithine transcarbamylase (OTC) gene therapy trial (Marshall, 1999).
Reducing the toxicity of Ad vectors using polyethylene glycol

Polyethylene glycol (PEG) is a clinically-approved conjugation agent used to improve the pharmacokinetics of a variety of protein therapeutics. In these applications, the hydrophilic PEG molecule is cross-linked to the therapeutic agent to ‘shield’ or reduce interactions of it with proteins and cells that would normally decrease the therapeutic’s interactions with its target. PEG has also been applied to improve the pharmacology of Ad vector. PEG molecules bearing reactive groups are chemically conjugated to free amine groups on the virion surface. By this approach, as many as 15,000 PEG molecules can be added to the virion surface to reduce its interactions with a variety of biomolecules and cells. Previous work has shown the utility of this approach to protect Ad vectors pre-existing neutralizing antibodies to allow multiple administration into immune recipients and has also demonstrated that PEGylation reduces the production of new antibody and cellular immune responses against Ad proteins (Cwole et al., 2001).

Based on PEGs shielding functions, we have tested its ability to reduce in vivo non-specific interactions that are involved with provoking the problematic responses related to this vector. First-generation Ad (FG-Ad) and HD-Ad vectors were PEGylated with approximately 15,000 PEG molecules coated each virion. Notably, PEGylation reduced innate immune responses directed against Ad vectors, as evidenced by 75% reduction in IL-6 responses at 6 hours and a 90% reduction in cumulative IL-6 over 48 hours (Mok and Barry, 2005). While PEG itself did not reduce liver damage after 5 days for FG-Ad vectors, PEGylated HD-Ad vectors produced no liver damage. The effects of PEGylation on reducing innate immune responses appears to be due in part to the ability of this shielding agent to reduce non-specific uptake of the vector by antigen-presenting cells like macrophages and Kupffer cells (Mok and Barry, 2005). Our more recent work has shown that PEGylation also blocks binding of Ad to platelets, red blood cells, and endothelial cells, and also blunts Ad-induced thrombocytopenia, disseminated intravascular coagulation (DIC), and splenomegaly (unpublished observations). These data suggest that this simple vector modification may have utility to improve the safety of these robust Ad vectors for PA gene therapy.

Adeno-associated virus vectors

Adeno-associated virus (AAV) vectors also hold promise for liver gene therapy of PA. AAV vectors can deliver genes episomally to cells, but also integrate genes into host chromosomes to a low degree. So, AAV vectors have the benefits of both non-integrating Ad vectors and integrating retroviral vectors, but also perhaps their types of problems—loss of transgene during cell division by episomal vector and potential for oncogenic activation for integrating vector. AAV-2 vectors have demonstrated successful and sustained liver gene therapy of factor IX deficiency in mouse models (Snyder et al., 1997; Wang et al., 1999). One primary advantage that AAV vectors have over Ad vectors is that they appear to be substantially less immunogenic and cause lower side effects in animal models (Jooss et al., 1998; Fields et al., 2000). AAV-2 vectors pseudotyped with AAV-8 capsid (AAV-2/8) have recently been shown to transduce 80 times higher transgene expression from the liver than AAV-2 vectors and 10-fold higher expression than vectors pseudotyped with the capsids from AAV-5 and AAV-7 (Gao et al., 2002). When applied in LDL-receptor-deficient mice, AAV-2/8 mediated near complete normalization of serum lipids and protected from atherosclerosis (Lebherz et al., 2004). These data suggest that AAV vectors may provide sufficient genetic correction in the liver to produce the greater than 20% of wild-type PCCA expression needed to rescue PCCA−/− mice from lethal PA.

Although these data suggest the potential of AAV, this vector integrates to a low level into the genome, increasing the possibility of oncogenesis (Hacein-Bey-Abina et al., 2003a; Hacein-Bey-Abina et al., 2003b) and chromosome rearrangements (Miller et al., 2002). In addition, recent human trials have observed immune-mediated rejection of vector-modified cells similar to that observed with FG-Ad vectors (Manno et al., 2006). These data suggest AAV may confront problems with pre-existing or vector-induced immunity in a manner similar to that confronted by Ad vectors. Nonetheless, AAV does not generate the same innate immune responses that Ad vectors provoke and may ultimately be a safer gene therapy vector for PA than Ad vectors.

Where should gene therapy for PA be delivered?

The liver is a logical target for gene therapy interventions given its pivotal role in metabolism. However, it is unclear if it is the only site of genetic intervention that can mitigate the symptoms of PA. While much is known about the genetics and disease symptoms of PA, little data can be found in the literature regarding the distribution of PCCA protein in different tissues. We thought this was an important question, as the level of protein may explain some of the tissue damage and symptoms due to loss of PCCA. Likewise, knowing where PCCA is expressed might better guide how transplantation and gene therapies need to be applied. For example, one might predict that the liver expresses the highest level of PCCA given its role in metabolizing excess amino acids and fatty acids. Conversely, one might predict that the basal ganglia might express lower amounts of PCCA, as many of the symptoms of the disease are manifested in those brain structures.

Given these issues, we generated antibodies against both mouse and human PCCA and performed a screen for PCCA protein in mouse and human tissue. While we expected PCCA to be either ubiquitously expressed or expressed at highest levels in the liver, to our surprise we observed a marked variation in amount of PCCA in different tissues. In both mouse and human tissues, the kidney appeared to have the highest level of PCCA protein, higher than in the liver (unpublished observation). In contrast, in the brain, PCCA was undetected in mouse (but not necessarily zero), and was detectable, but at low levels in the human samples. These data suggest that PCCA is not ubiquitously expressed at high levels and that the kidney may play a significant role in elimination. While the kidney had higher PCCA levels (even if normalized to tissue protein content), it should be noted that the liver likely still plays a more important role than the kidney in the disease, given this organ’s substantially higher mass and other metabolic flux.

Prospects

Dr. Miyazaki’s work has provided excellent proof of principle for the possibility of performing liver gene therapy to at least mitigate the symptoms of PA. While promising, his group also pointed out that this approach does not genetically correct all of the cells of the body. So, if there is cell autonomous toxicity within each cell, liver gene therapy cannot correct every aspect of the disease. Our work with the PA null mice has revealed some unreported phenotypic changes in the mice that might speak to this issue. While surviving PCCA−/− SAP mice are reported to be essentially normal, we have found that these mice have some growth delays and breed poorly (unpublished observations). This suggests that the level of liver genetic correction achieved in these mice may be insufficient to fully counter the systemic effects of the disease. Indeed, even 50% of normal PCCA expression may be somewhat limiting, as mice heterozygous for
While genetic correction in a growing mouse or human presents a more challenging environment to gene therapy, intervening as early as possible in a PA patient may also have some immunological benefits. If PA children have mutations that affect the immunogenicity of the protein or that delete sections of the protein, this may make later therapy more difficult. In these cases, if the correct gene is successfully delivered, expression of the previously missing protein sequences in PA may provoke the immune system to consider them as foreign, and these cells will be killed by the immune system. If observed, one may have to apply immunosuppression similar to that used in liver transplantation itself. Conversely, if the PA gene (or protein) is delivered to children in the neonatal period, this may have the benefit to tolerate their immune system to the protein. This could provide a future window of opportunity for subsequent, more effective, gene or stem cell therapies to be performed without the danger of immune attack. This will likely not be necessary in patients with single point mutations, but may be needed if additional research finds splicing mutations that delete sections of the protein (Desviat et al., 2006). While many issues still challenge gene therapy for PA, an increasing number of laboratories are working to take these first steps towards providing treatments to reduce the symptoms of the disease. Preliminary data from our laboratory indicates that delivery of Ad vectors expressing PCCA into newborn PCCA null mice within hours of birth can extend their lifespan beyond their normal death at 24 hours (unpublished observation). These data provide hope that gene therapy can be used to treat PA. Work is underway to validate these results and optimize these approaches to provide safe and persistent genetic correction.

Address for correspondence
Michael A. Barry, PhD., Mayo Clinic, 200 First Street SW, Rochester, MN, USA. Tel: 507-266-9090; Fax: 507-255-2811; Email: mab@mayo.edu

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FUNCTIONAL ANALYSIS OF SPLICING MUTATIONS CAUSING ORGANIC ACIDEMIAS

Lourdes R. Desviat, Belén Pérez and Magdalena Ugarte.

Centro de Biología Molecular Severo Ochoa, Universidad Autónoma de Madrid. 28049. Madrid. Spain

Mutations affecting the splicing of pre-mRNA are one of the most common causes of disease and may have different functional consequences depending on the nucleotides affected and the local sequence context. We have investigated the transcriptional profile of different splicing mutations identified in patients with propionic acidemia, isolated methylmalonic acidemia or methylcrotonylglycinuria with the aim of clarifying their functional effect and involvement in the disease phenotype and to serve as basis for the development of novel pharmacological or molecular therapies aimed at the correction of erroneous splicing. In some cases, we identified detectable levels of normally spliced transcripts, which may be increased using different compounds that act as transcriptional activators such as aclarubicin, valproic acid or kinetin. In addition, we have identified aberrant inclusions of pseudoxonic sequences, which may be targeted with antisense oligonucleotides to correct the splicing process.

In the past few years, the field of genetic diseases has given special attention to research on mutations affecting splicing, which generally account for 10–30% of the total mutant alleles (Krawczak et al., 2000). In eukaryotes, protein-coding genes have large non-coding sequences (introns) separating the sequences that code for the protein (exons), all of which are transcribed into the pre-mRNA. The introns are removed in a highly specific manner during the splicing process. To correctly identify and join together the exons, there are conserved motifs in or near the intron-exon boundaries that act as splicing signals: the 5′ and 3′ splice sites, a polypyrimidine tract and the branch site. In practically all cases, the 5′ splice site includes a guanine/thymidine (GT), guanine/uracil (GU) in the mRNA dinucleotide, whereas the 3′ splice site has an adenine/guanine (AG) dinucleotide. In addition to these conserved sequences, there are regulatory elements (splicing enhancers or silencers) located in exons or introns needed to allow normal splicing of some exonic sequences (Fig. 1). Mutations in all these sequences may disrupt splicing (Blencowe, 2000). The two most common consequences of a splicing mutation are exon skipping or cryptic splice site activation, resulting in the insertion or deletion of nucleotides in the mature mRNA (Faustino and Cooper, 2003). The mechanism by which one or other is chosen for a given mutation in a specific gene context is not yet fully elucidated, but clearly depends on the local sequence, which makes it practically impossible to predict the effect of a given splicing mutation. It is therefore necessary to analyze the transcriptional profile associated with each splicing mutation, either in patients’ cells or by the use of minigenes that contain the genomic sequences to be analyzed in vectors specifically designed to allow exon splicing. These vectors with normal or mutant sequences are transfected into established cell lines to characterize the pathological effect of each mutation (Cooper, 2005). The analyses require RT-PCR and qRT-PCR (quantitative RT-PCR by real-time PCR methodologies) using specific primers designed to amplify the corresponding transcripts, and cloning to rescue minor transcripts.
Most of the splicing mutations result in a frameshift in the coding sequence and the introduction of a premature termination codon (PTC) and are therefore substrates of nonsense-mediated decay (NMD). NMD actively degrades PTC-containing mRNAs, preventing the generation of truncated proteins potentially toxic to cells (Maquat, 2004).

Some splicing mutations completely abolish exon recognition and result in complete absence of correctly spliced transcripts. Others generate both aberrantly and correctly spliced transcripts, the level of which may vary among patients and tissues. Moreover, the levels of aberrant and normal transcripts may be associated with specific disease phenotypes; the higher the levels of normal transcripts, the milder the phenotypes (Clavero et al., 2004, Lualdi et al., 2006). These observations have raised the possibility that splicing modulation may be a modifier of disease severity in patients carrying splicing mutations that generate normal transcripts (Nissim-Rafinia et al., 2004).

Splicing modulation can be achieved by overexpression of specific splicing factors or by use of drugs such as aclarubicin, sodium butyrate, kentin or valproic acid, which have been proven useful in the reversion of aberrant splicing (Andreassi et al., 2001, Nissim-Rafinia et al., 2004). These compounds act as transcriptional activators due to their ability to inhibit histone deacetylase, and can increase the levels of correctly spliced transcripts, improving the prognosis of disease caused by splicing mutations.

Another promising splicing therapy, already in clinical trials, is the use of antisense oligonucleotides (AONS) directed at the prevention of the inclusion of aberrant sequences in the mature mRNA, such as intronic sequences (pseudoexons), which are recognized as exons due to mutations activating cryptic splice sites. AONS can modulate the splicing pattern by steric hindrance of the binding of the splicing apparatus to the selected sequences, thus forcing the machinery to use the natural sites. Antisense therapy modulating splicing has been used successfully in cystic fibrosis and Duchenne muscular dystrophy. In the latter case, it is already in use in patients (Takeshima et al., 2006).

Our group is involved in the diagnosis of and research on different inborn errors of metabolism (IEM), in particular, three organic acidurias: propionic acidemia (PA), methylmalonic acidemia (MMA) and methylcrotonylglycinuria (MCG). PA is caused by defects in the PCCA or PCCB gene, which code for the two subunits of the enzyme propionyl-CoA carboxylase. MMA is linked to mutations in the genes MUT, MMAA or MMAB, which code for the enzyme methylmalonyl-CoA mutase and for enzymes involved in the synthesis of the active form of the cofactor adenosylcobalamin, respectively. MCG results from mutations in the genes MCCA or MCCB, which code for both subunits of the enzyme methylmalonyl-CoA carboxylase. Our research in these disorders has covered different aspects, from gene identification and characterization, mutation analysis and functional expression of variant alleles, to the molecular basis of the pathophysiology and investigation on novel pharmacological and genetic mutation-specific therapies.

In this work, we review our results on the functional analysis of splicing mutations in organic acidemias. We summarise the results of the analysis of the transcriptional profile of mutations affecting splice sites by RT-PCR and qRT-PCR using patients’ fibroblasts and minigenes. We have characterized some mutations with detectable levels of correctly spliced mRNA, which may be candidates for splicing therapeutics such as those described above. In addition, we have recently identified several aberrant inclusions of pseudoexonic sequences, candidates to antisense therapeutics.

<p>| Table 1. Intronic sequence changes affecting mRNA splicing found in genes defective in organic acidemias |
|-------------------------------------------------|-------------------------------------------------|-----------------------------|-----------------------------|-------------------------------------------------------------------|</p>
<table>
<thead>
<tr>
<th>Mutation</th>
<th>Gene</th>
<th>Disease</th>
<th>Splice analysis methods</th>
<th>Normal transcripts</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>IVS2-2A&gt;G</td>
<td>MCCA</td>
<td>MCG</td>
<td>Minigenes</td>
<td>Yes</td>
<td>(Rincon et al., 2007a)</td>
</tr>
<tr>
<td>IVS13+3A&gt;G</td>
<td>PCCA</td>
<td>PA</td>
<td>qRT-PCR (fibroblasts)</td>
<td>2–3%</td>
<td>(Desviat et al., 2006)</td>
</tr>
<tr>
<td>IVS21+3del4</td>
<td>PCCA</td>
<td>PA</td>
<td>qRT-PCR (fibroblasts)</td>
<td>6.3%</td>
<td>(Clavero et al., 2004)</td>
</tr>
<tr>
<td>IVS22-2A&gt;g</td>
<td>PCCA</td>
<td>PA</td>
<td>qRT-PCR (fibroblasts, minigenes)</td>
<td>6–16%</td>
<td>(Clavero et al., 2004)</td>
</tr>
<tr>
<td>IVS1+2T&gt;C</td>
<td>PCCB</td>
<td>PA</td>
<td>RT &amp; real time PCR and melting probe analysis (fibroblasts)</td>
<td>Not detected</td>
<td>(Desviat et al., 2006)</td>
</tr>
<tr>
<td>IVS10-11del6</td>
<td>PCCB</td>
<td>PA</td>
<td>RT &amp; real time PCR and melting probe analysis (fibroblasts)</td>
<td>Yes</td>
<td>(Clavero et al., 2004)</td>
</tr>
<tr>
<td>IVS14+2T&gt;C</td>
<td>PCCB</td>
<td>PA</td>
<td>qRT-PCR (fibroblasts)</td>
<td>0.1%</td>
<td>(Desviat et al., 2006)</td>
</tr>
<tr>
<td>c.291-1G&gt;A</td>
<td>MMAB</td>
<td>MMA</td>
<td>qRT-PCR (fibroblasts)</td>
<td>Not detected</td>
<td>(Rincon et al., 2005)</td>
</tr>
</tbody>
</table>
Mutation IVS21+3del4 (c.1899+3del4) in the PCCA gene lowers the splicing score of the 5' splice donor site of exon results in exon 21 skipping, corresponding to an in-frame deletion of 54 base pairs (bp, 18 amino acids), which are associated with a total absence of enzyme activity and an accelerated turnover of the mutant protein in mitochondria. These results contrast sharply with the mild phenotypic expression of the disease (late onset and near-normal development) in a homozygous patient. To test if some normally spliced transcript was present in the patient’s cells, which could explain the mild phenotype, we performed RT-PCR analysis with exon-specific primers, which would selectively amplify exon 21 containing transcripts even if they were present in very low abundance. Some amount of normally spliced transcript, as confirmed by sequence analysis, was indeed detectable in these conditions by ethidium bromide staining. The exact levels of normal transcript were quantified by real time PCR and found to be ~3% (Clavero et al., 2004).

Mutation IVS22-2A>G (c.2041-2A>G) in the PCCA gene affects the invariant AG dinucleotide at the 3' acceptor splice site in exon 23 of the PCCA gene, causing exon 23 skipping. Exon 23 contains the conserved A-M-K-M motif responsible for biotin binding, and absence of this sequence results in a non-functional protein, but the phenotype of a homozygous patient was moderate. Using specific primers, normal transcript was quantified in patients’ fibroblasts corresponding to 16 ± 4% compared to the amount obtained in normal fibroblasts (Fig. 2). This result was reproduced using minigene constructs (Clavero et al., 2004).

Mutation IVS13+3A>G (c.1209+3A>G) in the PCCA gene is a transition affecting the 5' donor splice site of exon 13. At the cDNA level, the skipping of exon 13 is observed, which predictably gives rise to a protein with an internal deletion of 48 aminoacids. This change has been detected in several patients of common geographical origin, one of them so far asymptomatic after being detected in a neonatal screening program. We investigated the possibility of the presence of some normal transcript in patients’ fibroblasts. For that purpose, we isolated RNA from fibroblasts of patients homozygous for this change and quantified the presence of exon 13-containing (i.e. normal) transcripts by real-time PCR using two methodologies: i) amplification using an oligonucleotide hybridizing to exon 13 and the double helix-specific dye SYBR Green 1; ii) amplification and detection with a Taqman probe specific for the cDNA region corresponding to the exon 13-exon 14 junction. Using the SYBR Green 1 methodology, we could not detect any normal transcript compared with control samples. Using a Taqman probe specific for exon13-exon14 containing sequences, we could detect 1.8%-2.7% of normal transcripts in fibroblasts (Desviat et al., 2006).

**Exonic splicing defects affecting U1snRNP binding**

The last exonic nucleotides are included in the 5' donor splice site (Fig. 1) and mutations affecting them may be classified erroneously as missense mutations, if their effect on splicing is not analyzed. This effect depends on the sequence context and the strength of base pairing with the RNA component of U1snRNP, which binds to this site during splicing. We have investigated the transcriptional profile of different base substitutions in the last exonic nucleotides identified in different genes to characterize precisely their effect (Perez et al., 2003; Martinez et al., 2005). The base pairing of the mutant sequences with U1snRNP is shown in Table 2. In some cases, complementation with modified U1snRNAs can provide clues to a specific therapeutic strategy to correct splicing defects at the 5’ splice site (Susani et al., 2004).

The c.653A>G mutation in the PCCB gene was initially classified as missense, causing the change K218R which was expressed in vitro and found to be associated with high (70%) residual activity (Perez-Cerdà et al., 2003). However, subsequent mRNA analysis revealed that the mutation, located in the last codon of exon 6 of the PCCB gene, causes aberrant splicing, activating a cryptic splice acceptor site within exon 6. The effect is an in-frame deletion of six nucleotides in the mRNA, which would result in a protein with two asparagine acids deleted (V217–K218) with null activity. This mutation is present in heterozygous fashion in a patient carrying...
Table 2. Exonic sequences changes found in MMA and PA genes affecting U1snRNP binding

<table>
<thead>
<tr>
<th>Mutation (gene)</th>
<th>5’ splice site score wt→mutant</th>
<th>U1snRNP binding†</th>
<th>Major effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>c.763G&gt;A (PCCB)</td>
<td>0.95→0.63</td>
<td>AGuaguagg</td>
<td>100% correctly spliced with missense change G255S</td>
<td>–</td>
</tr>
<tr>
<td>c.653A&gt;G (PCCB)</td>
<td>1→0.99</td>
<td>Guaguagg</td>
<td>Cryptic splice site activation; 6 nt deletion</td>
<td>(Clavero et al., (delV217-K218) 2004)</td>
</tr>
<tr>
<td>c.1808G&gt;A (MUT)</td>
<td>0.96→0.53</td>
<td>AAguagg</td>
<td>Cryptic splice site activation, 62nt deletion</td>
<td>(Martinez et al., (V583fs) 2005)</td>
</tr>
<tr>
<td>c.562G&gt;C (MMAA)</td>
<td>1→0.98</td>
<td>ACguagucu</td>
<td>100% correctly spliced with missense change</td>
<td>(Rincon et al., (G188R) 2007a)</td>
</tr>
<tr>
<td>c.733G&gt;A (MMAA)</td>
<td>0.96→0.53</td>
<td>UAguaggu</td>
<td>Exon 4 skipping and minor levels with</td>
<td>(Rincon et al., (G245S) 2007a)</td>
</tr>
<tr>
<td>c.584G&gt;A (MAMB)</td>
<td>0.99→0.6</td>
<td>CAguaggu</td>
<td>Exon 7 skipping.</td>
<td>(Rincon et al., (G188R) 2007a)</td>
</tr>
</tbody>
</table>

†BDGP software (www.fruitfly.org/seq_tools/splice.html)
‡Underlined bases represent base pair matches with U1snRNA. U1 consensus sequence AGuaguagg

R165Q on the other allele, a mutation that also has null residual activity in expression analysis (Perez-Cerda et al., 2003). The patient has a mild phenotype with normal development.

In view of this, we decided to test the possibility that the c.653A>G mutation also produced some amount of normally spliced product, which would result in a protein with the K218R missense change with partial residual activity that would account for the mild phenotype in the patient. The mutation was tested in an in vitro splicing assay using minigenes and was found to result in the 6-nucleotide deletion in the mRNA through activation of the cryptic splice site in exon 6, as observed in patient cells.

As we could not discard the presence of normal spliced product in very low amounts not detectable by conventional RT-PCR and sequence analysis, we designed allele-specific hybridization probes to use with real-time fluorescent PCR methodology analysing patient's cells transcripts. The results confirmed the absence of normally spliced transcript in the patient's cells indicating that the mutation completely abolishes the recognition of the mutant 5’ splice donor site (Clavero et al., 2004).

In contrast, the c.562G>C mutation in the MMAA gene and the c.763G>A mutation in the PCCB gene produce mainly molecules correctly processed bearing the missense changes G188R and G255S respectively, an observation that correlates with the very slight decrease in splicing score.

The two other mutations tested, c.733G>A in the MMAA gene and c.584G>A in the MAMB gene cause skipping of the corresponding exon generating a frameshift and a premature termination codon in the coding sequence. In the first case, we could detect cDNA molecules of complete length bearing the missense mutation G245S by subcloning the fragments obtained by RT-PCR in patient's fibroblasts and sequencing. In the latter case, minor but detectable amounts of correctly spliced transcripts were obtained in minigenes transfected in some cell types (HEK or COS) and not in others (hepatoma), indicating tissue specific difference in splicing factors that may modify the phenotype (Rincon et al., 2007a).

Pseudoexon splicing defects

Recently, we have found deep intronic mutations leading to the insertion of a pseudoexon or cryptic exon in the mRNA of the genes defective in MMA and PA. Exonization of intronic sequences has been described in other genetic diseases such as β-thalassemia/HbE disorder (Suwanmanee et al., 2002), cystic fibrosis (Friedman et al., 1999), Duchenne muscular dystrophy (Aartsma-Rus et al., 2003), ocular albinism and skeletalennonomous cases.

Concluding remarks

These results reflect the different consequences derived from splicing mutations, which, in turn, can be influenced by complex networks of splicing factors differentially expressed in each tissue and/or individual, thus resulting in phenotypic variability. Investigation of the functional consequences of splicing mutations using different approaches is essential for the development of novel pharmacological or molecular therapies aimed at the correction of erroneous splicing. The blocking of aberrant splicing with antisense oligonucleotides and use of drugs or splicing factors that modulate splicing is the aim of our present research.

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Address for correspondence

Magdalena Ugarte, Centro de Biología Molecular “Severo Ochoa” CSIC-UAM, Universidad Autónoma de Madrid. Cantoblanco, 28049 Madrid, Spain. Tel: 34-914974867, Fax: 34-917347797; Email: mugarte@cbm.uam.es
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