Published in final edited form as:

J Inherit Metab Dis. 2016 January 1; 39(1): 115–124. doi:10.1007/s10545-015-9860-6.

Clinical pattern, mutations and *in vitro* residual activity in 33 patients with severe 5, 10 methylenetetrahydrofolate reductase (MTHFR) deficiency

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Abstract

Background—Severe methylenetetrahydrofolate reductase (MTHFR) deficiency is a rare (<200 reported cases) inborn defect of the remethylation of homocysteine to methionine. This retrospective study evaluates clinical, biochemical genetic and *in vitro* enzymatic data in a larger cohort.

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Details of the contributions of individual authors

Brian Fowler, Matthias R. Baumgartner and Martina Huemer conceived the study. Martina Huemer and Regina Mulder-Bleile conducted the study and drafted the manuscript. Matthias R. Baumgartner, D. Sean Froese, Terttu Suormala, Brian Fowler and Patricie Burda contributed the enzymatic and genetic data. Patricie Burda drafted the respective parts of the manuscript. Bruria Ben Zeev, Patrick Chinnery, Carlo Dionisi-Vici, Dries Dobbelaere, Gülden Gökçay, Johannes Häberle, Alexander Lossos, Eugen Mengel, Andrew Morris, Klary E. Niezen-Koning, Barbara Plecko, Rosella Parini, Dariusz Rokicki, Manuel Schiff, Mareike Schimmel, Adrian Sewell, Wolfgang Sperl, Ute Spiekerkötter, Beat Steinmann, Megumi Tsuji, Jürgen-Christoph von Kleist-Retzow, Grazia Tadeucci, Jose Trejo, Friedrich Trefz, María Antònia Vilaseca, Valerie Walker and Jiri Zeman contributed patient data. Brian Fowler, Matthias R. Baumgartner and D. Sean Froese critically revised the manuscript.

Compliance with Ethics Guidelines

Martina Huemer, Regina Mulder-Bleile, Patricie Burda, D. Sean Froese, Terttu Suormala, Bruria Ben Zeev, Patrick Chinnery, Carlo Dionisi-Vici, Dries Dobbelaere, Gülden Gökçay, Johannes Häberle, Alexander Lossos, Eugen Mengel, Andrew Morris, Klary E. Niezen-Koning, Barbara Plecko, Rosella Parini, Dariusz Rokicki, Manuel Schiff, Mareike Schimmel, Adrian Sewell, Wolfgang Sperl, Ute Spiekerkötter, Beat Steinmann, Grazia Tadeucci, Jose Trejo, Megumi Tsuji, Jürgen-Christoph von Kleist-Retzow, Friedrich Trefz, María Antònia Vilaseca, Valerie Walker, Jiri Zeman, Matthias R. Baumgartner, Brian Fowler declare that they have no conflict of interest.

All procedures followed were in accordance with the ethical standards of the responsible local committees on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000. Informed consent was obtained by the reporting physicians from their patients for being included in the study.

Methods—Clinical, biochemical and treatment data was obtained from physicians by using a questionnaire. MTHFR activity was measured in primary fibroblasts; genomic DNA was extracted from cultured fibroblasts.

Results—Thirty-three patients (17 females; mean age at follow-up 11.4 years; 4 deceased; median age at first presentation 5 weeks) were included. Patients with very low (<1.5% control) *in vitro* enzyme activity (n=14) presented earlier and with a pattern of feeding problems, encephalopathy, muscular hypotonia, neurocognitive impairment, apnoea, hydrocephalus, microcephaly, and epilepsy. In comparison, patients with higher (>1.5% control) *in vitro* residual enzyme activity had mainly psychiatric symptoms, mental retardation, myelopathy, ataxia and spasticity. Treatment with various combinations of betaine, folate and cobalamin improved the biochemical and clinical phenotype. During the disease course, patients with very low enzyme activity showed a progression of feeding problems, neurological symptoms, mental retardation, and psychiatric disease while in patients with higher residual enzyme activity frequencies of myelopathy, ataxia and spasticity increased. All other symptoms remained stable or improved in both groups upon treatment as did brain imaging in some cases. No clear genotype-phenotype correlation was obvious.

Discussion—MTHFR deficiency is a severe disease primarily affecting the central nervous system. Age at first presentation and clinical pattern are correlated with low / higher *in vitro* residual enzyme activity. Treatment alleviates biochemical abnormalities and clinical symptoms partially.

Introduction

Severe methylenetetrahydrofolate reductase (MTHFR) deficiency [MIM 607093; *MTHFR*; 1p36.22] is a rare inborn error of metabolism inherited in an autosomal recessive manner. The enzyme MTHFR catalyses the reduction of 5,10 methylenetetrahydrofolate to 5-methyltetrahydrofolate, which serves as a methyl donor for the methylation of homocysteine (Hcy) to methionine (Met). In severe MTHFR deficiency, methylation of Hcy to Met is decreased resulting in highly elevated plasma total Hcy (tHcy) and low plasma Met concentrations (Fattal-Valevski et al 2000; Thomas & Rosenblatt 2005, Burda 2015). Low Met results in a depletion of S-adenosylmethionine, which is the main donor for many methylation reactions involved in e.g. the synthesis of creatine, RNA and DNA (Thomas & Rosenblatt 2005). Elevated tHcy is associated with thromboembolic events and neurodevelopmental disturbances (Naughten et al 1998).

Reports on the long-term course and outcome in patients with severe MTHFR deficiency are scarce and have not been assembled systematically; present knowledge on the natural course of the disease is predominantly derived from case reports or small case series reporting in total on less than 200 individuals. Patients with severe MTHFR deficiency typically present in the neonatal period with feeding problems and failure to thrive, muscular hypotonia, encephalopathy and seizures. However, late-onset forms of the disease with a more variable picture encompassing delayed developmental milestones, cognitive impairment and/or gait abnormalities, psychiatric disorders or thromboembolic events have also been reported (Goyette et al 1995; Thomas & Rosenblatt 2005; Schiff et al 2011; D'Acoet al 2014; Lossos

et al 2014). MRI imaging of the brain often reveals white matter disease and brain atrophy (Thomas & Rosenblatt 2005; Michot et al 2008).

Single reports have described a benefit of treatment with folinic acid (Crushell et al 2012) or methionine supplementation (Abeling et al 1999); however, the mainstay of treatment is betaine (Strauss et al 2007). In a meta-analysis of 36 patients from 15 reports, it has recently been shown that early treatment with 100 mg/kg/day of betaine prevented mortality and resulted in normal psychomotor development in five patients in contrast to 19 patients with delayed treatment (Diekman et al 2014).

Since most of the mutations causing severe MTHFR deficiency are private (Tonetti et al 2003; Burda 2015) and significant interfamilial variation of the clinical phenotype has been described (Haworth et al 1993; Forges et al 2010), no clear genotype-phenotype correlations have been outlined. A relation between *in vitro* enzyme activity and clinical course has occasionally been postulated (Goyette et al 1995; Birnbaum et al 2008; Forges et al 2010) but has not yet been systematically investigated.

The aims of this study are to document clinical symptoms at presentation, symptoms developing during the course of the disease, biochemical parameters and outcome in a larger cohort of patients with severe MTHFR deficiency. Treatment strategies are described and possible correlations between genetic and enzymatic data and clinical findings investigated.

Methods

The local ethics committee (KEK-ZH-No. 2013-0012) approved this retrospective study. A questionnaire (provided in detail as supplementary material) addressing physicians was constructed to obtain information on gender, age at diagnosis, problems during pregnancy and the perinatal period, clinical symptoms and metabolic parameters at initial workup and during follow-up, as well as treatment in patients with severe MTHFR deficiency. The questions were based on signs and symptoms reported in the literature on MTHFR. Open questions left space to report new or unexpected disease characteristics. The World Health Organization (WHO) percentiles were used to evaluate birth weight and head circumference. All physicians who had sent cultured fibroblasts to Switzerland for diagnostic purposes in which severe MTHFR deficiency had been proven were asked to complete the survey after obtaining informed consent from their patient(s) and /or the caregivers. MTHFR activity was measured and DNA extracted and sequenced, from primary fibroblasts derived from skin biopsies obtained for diagnostic purposes (Burda et al 2015). In cell lines with very low MTHFR activity (<1.5% mean control value) only enzymatic activity and FAD-responsiveness were measured. For those with higher residual activity (>1.5% mean control value), further enzymatic characterization was performed (e.g. Km for NADPH; see Burda et al, 2015)."

Results

Socio-demographic data

Clinical, biochemical, enzymatic and genetic data was available for 33 (17 females, 16 males; born between 1977 and 2013) unrelated patients with severe MTHFR deficiency. Consanguinity was present in 13 families; in seven families history suggested more than one individual to be affected. Eight patients were of Turkish, six of middle-European (Austria, France, Germany, Switzerland), five of Pakistani, five of South European (Spain, Italy), three of East European (Poland, Russia) and two of North European (UK) decent; two patients originated from the Near East (Israel, Syria) and one from Japan. All patients had been identified by selective, symptom oriented metabolic workup. Twenty-nine patients were alive at the time of data collection (mean age at follow up 11.4 years; median 9.6 years; range 4 months to 37.7 years; mean time of follow-up 9.2 years; median 8.8 years; range 3.8 months to 22.8 years). Four patients had died at ages 5 months, 6 months, 11 months and 20 months, due to apnoea (n=2), apnoea in the circumstance of thrombosis of the pulmonary artery (n=1) or infection (n=1).

Pregnancy and delivery

Non-specific adverse events during pregnancy were reported in five of 33 cases (one intrauterine growth retardation, two episodes of maternal bleeding, and two cases of maternal diabetes mellitus). Mean gestational week at delivery was 39.5 weeks (median 40, range 36 to 41 weeks). Birth weight was within the normal ranges (mean 3285 g; median 3383, range 2270 to 4100 g) in all but two patients who had a birth weight below the 3rd percentile. Mean percentile of head circumference at birth was the 34th (median 25th; range 2nd to 90th). Mean APGAR scores were 8.7 at minute one and 9.7 at minute ten; no major perinatal problems were reported.

Age at onset and time to diagnosis

Information on age at first disease symptoms was available for 30 patients. Mean age at onset of symptoms was 21 months (median 1.25; range 0.1 to 216 months). In 14 patients, first symptoms were observed within the first month of life; in another 11 patients by the sixth month of life. One patient each became symptomatic at the ages of two, five, eleven, thirteen and eighteen years. Patients with very low residual *in vitro* MTHFR activity (< 1.5%; n=14) presented earlier in life (mean 1.5 months, median 1 month; range 0.1 to 6 months) than those with higher *in vitro* MTHFR activity (n=19; mean 36 months, median 2.5 months; range 0.1 months to 216 months).

Information on age at diagnosis was additionally available for 29 patients. Mean time to diagnosis was 16.6 months (median 2.8; range 0 to 108 months). Time between first symptoms and establishment of diagnosis correlated with age at first symptoms ($r=0.82$) and was shorter in patients with early compared to later onset of first disease symptoms.

Clinical presentation and course (n=33)

For the total cohort of 33 patients, main presenting clinical symptoms were muscular hypotonia, feeding problems / failure to thrive, developmental delay / mental retardation and

signs of encephalopathy (including lethargy and confusion). While in particular encephalopathy, lethargy and muscular hypotonia and to a lesser extent apnoea, feeding problems / failure to thrive, confusion, microcephaly and epilepsy improved over time, mental retardation and neurological symptoms such as abnormal gait and spasticity became more frequent over time (Figure 1)

Open questions for “other symptoms” revealed reports of nystagmus in three patients with very low enzyme activity and in a single patient with higher residual enzyme activity; optic nerve atrophy (2 patients with higher residual enzyme activity) and transient macrocytosis or macrocytic anaemia (n=4 with very low enzyme activity). Isolated normo- or microcytic anaemia (n=6) and anaemia with low platelets and leukocytes (n=2) were equally distributed between the subgroups. Venous thrombosis was observed in a single case.

Brain imaging reports (n=30)

Brain MRI reports from local radiologists was available for 30 patients. MRI scans were considered normal in three patients (two with higher residual *in vitro* activity). The most frequently reported pathologies were brain atrophy (n=15) and white matter disease / delayed myelination (n=13). Enlarged ventricles / hydrocephalus were present in nine patients. Interestingly, in some cases MRI images showed stabilisation or even improvement of brain pathology over time. In one child with cerebral atrophy, hypo-myelination and enlarged ventricles at age three months, the follow-up MRI at age three years was considered normal. In another patient white matter hyper-intensities at day 10 had normalized by the age of 1.5 months and remained normal until follow-up at the age of 28 months. Treatment resulted in dramatic improvement of periventricular signal alterations within eight months in another patient and in three patients, leukoencephalopathy remained stable over years under treatment.

Biochemical parameters (n=33)

As expected, plasma tHcy, plasma free homocystine and urine homocystine concentrations were higher and Met concentrations were lower at diagnosis / before treatment compared to measurements at follow-up (Table 1). tHcy and Met levels at first presentation varied widely and were not correlated with residual *in vitro* enzyme activity, type / location of mutation or age at first symptoms.

Outcome (n=33)

Physicians were asked to name what was, in their consideration, the most burdensome symptom for their patient. Neurocognitive impairment (retardation or developmental delay, learning problems, and impaired speech) was mentioned for 19 cases, neurological symptoms (seizures, peripheral neuropathy, paraparesis and spasticity) were considered the most burdensome for four cases, and eye disease (poor fixation, visual impairment) for three cases.

Treatment (n=33)

Treatment approaches were very heterogeneous regarding dosage, choice and combination of drugs. Betaine was applied to 29 patients (dose range 200 to 24 000 mg/d), in 19 patients

in combination with folate / folinate (dose range 3 to 100 mg per day). Cobalamin preparations were used in 23 patients (n=19 OH-cobalamin: dose range from 1 mg/d IM/ PO to 1 mg IM per month; n=4 cyanocobalamin: dose range from 0.5 mg every month - 1 mg/d). Twelve patients received vitamin B6 (dose range from 10 to 900 mg/d); eleven were supplemented with methionine (dose range from 100 to 1500 mg/d. Riboflavin (n=4; from 9 to 20 mg/d) and carnitine (n=4; from 1000 to 1500 mg/d) were rarely used. None of the patients treated with riboflavin carried FAD-responsive mutations.

Genetic and enzymatic data (n=33)

Mutations, residual enzyme activity and age at first symptoms are listed in Table 2. The majority of mutations are private and patients with very low *in vitro* MTHFR activity often had mutations located in the catalytic domain. However, neither type nor location of mutation correlates with age at onset and pattern of clinical symptoms in this cohort.

Clinical presentation and course in patients with very low (n=14) compared to higher *in vitro* residual enzyme activity (n=19)

Figure 2 depicts the different symptom patterns at presentation as solid columns and during the course (striped columns) in individuals with very low (black columns) compared to those with higher residual activity (grey columns). Patients with higher enzyme activity showed predominantly psychiatric symptoms, confusion, mental retardation and neurological symptoms such as myelopathy, ataxia and spasticity. In patients with low / absent enzyme activity, apnoea, hydrocephalus or microcephaly and feeding problems occurred more frequently.

In addition, figure 2 allows the comparison between the frequencies of specific symptoms over time in both groups by comparing the respective solid and striped columns. Patients with very low enzyme activity more frequently developed failure to thrive, ataxia, spasticity, mental retardation, confusion, and psychiatric disease during the course of their disease. However, all other symptoms remained stable or improved over time, in particular encephalopathy and muscular hypotonia. In patients with higher *in vitro* enzyme activity, frequencies of myelopathy, ataxia and spasticity slightly increased while all other symptoms remained stable or even improved over time.

Discussion

This large cohort of 33 patients illustrates the spectrum of clinical manifestations of severe MTHFR deficiency and provides follow-up data over a long period of time (mean time of follow-up 9.2 years). However, it has to be emphasised that this study has clear limitations owing to its retrospective, proxy-reported design and the biased manner of recruitment of physicians from the senders of diagnostic material to a single diagnostic laboratory. Additionally, the cut-off for the separation of patient groups into “very low” and “higher” residual *in vitro* MTHFR deficiency groups is arbitrary in terms of possible clinical relevance but is practically relevant since it relates to the minimum level of activity needed to perform kinetic measurements in cells.

Analysis of data from the complete cohort reveals that disease onset in severe MTHFR deficiency occurs predominantly early in life following an uneventful pregnancy, delivery and perinatal adaptation. Patients with very low enzyme activity in fibroblasts present at a younger age. Although low Met and high tHcy are the biochemical hallmarks of the disease, their concentrations are not correlated with age at onset, genotype, residual enzyme activity or severity / pattern of clinical symptoms, which is consistent with previous reports (Tonetti et al 2003; Thomas & Rosenblatt 2005; Forges et al 2010; Lawrance et al 2011).

The central muscular hypotonia and acute encephalopathy seen in our cohort are common features in early-onset inborn errors of metabolism. However, the severe feeding problems as observed in this cohort are consistently reported in MTHFR deficiency and other remethylation disorders (Fischer et al 2014) as well as in nutritional vitamin B12 deficiency (Roschitz et al 2005) and seem to be a more specific finding. Thus, when such problems are identified in undiagnosed patients they should focus the clinicians' attention towards a disease from this group. Apnoea was particularly frequent in patients with severe MTHFR deficiency, but the underlying pathophysiological mechanisms are not yet understood.

The predominance of neurological symptoms, cognitive impairment, white matter disease and brain atrophy in the individuals reported here underscores the fact that MTHFR deficiency is a severe disease affecting primarily the central nervous system (CNS). Brain disease is mainly attributed to the cerebral deficiency of S-adenosylmethionine due to impaired synthesis from methionine, resulting in defective myelinisation (Surtees 1991, Kishi et al 1994; Strauss et al 2007). Course and outcome of severe MTHFR deficiency are dominated by neurocognitive impairment in almost all patients and neurological sequelae such as ataxia, spasticity and seizures in many patients. These findings are in line with other reports (Thomas & Rosenblatt 2005; Fattal-Valevski et al 2000; Forges et al 2010; Strauss et al 2007; Diekman et al 2014).

Surprisingly, although retinopathy has been shown in a mouse model (Lawrance et al 2011) and eye involvement is frequent in other methylation disorders (e.g. the cblC, cblE or cblG defect), eye disease has rarely been reported in severe MTHFR deficiency (Ronge et al 1996; Lossos et al 2014). This study indicates that patients with severe MTHFR deficiency should regularly be monitored for eye disease since nystagmus, visual impairment and optic atrophy were present in this cohort in 18% of cases and symptoms were considered burdensome in 50%. Folate deficiency, which is often associated with severe MTHFR deficiency, excessive oxidative stress induced by hyperhomocysteinemia or direct Hcy toxicity has been suggested to cause eye disease (Lawrance 2011). However, as in other remethylation disorders, the patho-mechanisms have not yet been completely elucidated and in many patients eye disease cannot substantially be alleviated by treatment (Weisfeld-Adams et al 2013). Macrocytosis and macrocytic anaemia, which are generally not considered part of the clinical spectrum of severe MTHFR deficiency (Watkins & Rosenblatt 2012), were reported in four patients and it remains unclear whether this finding is correlated with the underlying metabolic disorder or caused by other factors.

When stratifying the reported cohort according to *in vitro* residual enzyme activity (very low and residual), two distinct patterns of disease presentation evolve. First patients with very

low enzyme activity that predominantly experience early-onset disease with severe neonatal encephalopathy often accompanied by apnoea. Second later-onset disease in patients with higher residual activity so that MTHFR deficiency must be kept in mind in the differential diagnosis of mental retardation and psychiatric disease in children, adolescents and adults. This psychiatric type of the disease has occasionally been described before (Haworth 1993; Goyette et al 1995; Birnbaum et al 2008; Lossos et al. JAMA Neurol 2014) and has been responsive to treatment in some cases (Birnbaum et al 2008; Michot et al 2008), which is consistent with our findings.

Although a clinical difference between very low and higher *in vitro* residual MTHFR activity was present, we found no linear relationship between absolute values of residual activity, age at onset of first symptoms and disease course. Therefore it must be kept in mind that these *in vitro* studies are not to be used as prognostic markers in individual patients or families.

It is of note that patients with very low MTHFR activity often have mutations in the N-terminal catalytic domain, which seems to cause almost complete loss of catalytic activity. However, genotype-phenotype correlations could not be delineated for the 33 patients reported here. The idea of genetic heterogeneity is also supported by the observation that siblings with an identical genotype may present with a completely different clinically course (Haworth et al 1993).

In this cohort, treatment was found to normalise plasma methionine, decrease tHcy concentrations and considerably improve or at least stabilise the clinical course. The response of brain disease detected by MRI (especially white matter damage) in response to treatment in the presented cohort is an important finding and in line with observations from single cases (Engelbrecht et al 1997; Al—Essa et al 1999). Similar resolution of white matter changes has been shown in treated patients with nutritional vitamin B12 deficiency (Ertan et al 2002) or the cblG defect (Outteryck et al 2012).

Generally, patients with higher residual activity seem to respond better to treatment. None of them died from the disease and the overall disease load did not increase as significantly over time as in the very low activity group; this finding corresponds with the observations in the late-onset cblC defect (Huemer et al 2014).

Betaine was applied to almost all, folate / folinate and hydroxycobalamin to the majority and methionine to many patients reported here. Since the patients were born between 1977 and 2013, exact dosages and time points of treatment initiation were often not exactly documented. However, since early betaine treatment has been recently shown to prevent neurocognitive decline in patients with severe MTHFR deficiency (Strauss et al 2007, Diekman et al 2014) we assume that most probably betaine and maybe methionine as an add-on treatment (Abeling et al 1999) have been the effective therapeutic agents in this cohort. In the present cohort, riboflavin has not been applied to any of the patients with *in vitro* FAD responsiveness and this may in the future be considered as an add-on trial.

Folate concentrations in serum and red blood cells as well as 5- methyltetrahydrofolate concentrations in cerebrospinal fluid (CSF) are generally low in severe MTHFR deficiency

(Crushell 2012; Schiff & Blom 2012). Application of CH₃-tetrahydrofolate was neither able to normalise neurotransmitters (Schiff & Blom 2012) nor alleviate the clinical course (Clayton et al 1986). Some authors argued in favour of a therapeutic effect for folinic acid on neurological symptoms (Crushell et al 2012) while others identified no clinical benefit (Holme et al 1989; Diekman 2014). In summary, the impact of folinic acid on clinical manifestations has not been convincing (Diekman 2014).

As in other inborn errors of remethylation, the older the patient at first symptoms, the longer the time until the correct diagnosis can be established. The pattern of an acutely ill neonate / infant with signs of neurological involvement seems likely to prompt timely metabolic workup, while an underlying inborn error of metabolism is not generally considered early in the diagnostic process in a child or adolescent with a more variable, non-specific psychiatric or neurological presentation (Huemer et al 2014). Clinicians should be encouraged to actively seek severe MTHFR deficiency in later presenting cases as well in order to shorten delay to diagnosis and allow timely initiation of treatment.

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Figure 1. Clinical symptoms at onset and during the course of the disease for the complete cohort (n=33)

Figure 2. Percentage of individuals presenting with specific symptoms at diagnosis and during the course by very low (<1.5% ; n=14 patients) and higher (n=19 patients) *in vitro* residual enzyme activity

Table 1
tHcy, homocystine and Met concentrations at diagnosis and under treatment

Parameter units and reference ranges	At diagnosis			Under treatment		
	Median (range)	Mean	n	Median (range)	Mean	n
Plasma tHcy (5-15 $\mu\text{mol/L}$)	170 (30-316)	163	28	71(23-127)	73	29
Free homocystine ($\mu\text{mol/L}$) Normally undetectable	29 (9-70)	32	14	3.1(0.6-14)	5.7	8
Urine homocystine ($\mu\text{mol}/\text{mmol creatinine}$) Normally undetectable)	29 (4-627)	113	13	-	-	3
Plasma methionine (10-39 $\mu\text{mol/L}$)	9 (0-29)	9	30	27 (11-89)	35	23

Table 2
Genotype and *in vitro* MTHFR activity characteristics in 33 patients with severe MTHFR deficiency (adapted from Burda et al 2015)

Patients with very low <i>in vitro</i> enzyme activity (<1.5%)								
ID	Age at presentation (months)	Mutation allele 1	Predicted amino acid change	exon / intron	Mutation allele 2	Predicted amino acid change	Residual <i>in vitro</i> enzyme activity + α FAD ^a	exon / intron
20	0.25	c.188G>C	p.Trp59Ser	exon 1	c.188G>C	p.Trp59Ser	< 1.5%	exon 1
82	0.2	c.349G>A	p.Ala113Thr	exon 2	c.792+1G>T	splice site	< 1.5%	Intron 4
39	0.75	c.391C>T	p.His127Tyr	exon 2	c.655_657del	p.Lys215del	< 1.5%	exon 4
36	2	c.452A>C	p.Gln147Pro	exon 2	c.452A>C	p.Gln147Pro	< 1.5%	exon 2
41	4	c.452A>C	p.Gln147Pro	exon 2	c.452A>C	p.Gln147Pro	< 1.5%	exon 2
60	1	c.458_459delinsTT	p.Gly149Val	exon 2	c.458_459delinsTT	p.Gly149Val	< 1.5%	exon 2
42	0.1	c.559C>T	p.Arg183*	exon 3	c.559C>T	p.Arg183*	< 1.5%	exon 3
44	0.5	c.779T>A	p.Ile256Asn	exon 4	c.1025T>C	p.Met338Thr	< 1.5%	exon 5
40	0.5	c.1027T>G	p.Trp339Gly	exon 5	c.1027T>G	p.Trp339Gly	< 1.5%	exon 5
48	6	c.1027T>G	p.Trp339Gly	exon 5	c.1027T>G	p.Trp339Gly	< 1.5%	exon 5
21	1	c.1179-2delA	p.Trp389Trpfs*1	intron 6	c.1179-2delA	p.Trp389Trpfs*1	< 1.5%	intron 6
25	-	c.1420G>T	p.Glu470*	exon 8	c.1420G>T	p.Glu470*	< 1.5%	exon 8
22	2.5	c.1542G>A	(p.Lys510=)/splicing	exon 8	c.1542G>A	(p.Lys510=)/splicing	< 1.5%	exon 8
51	1	c.1542G>A	(p.Lys510=)/splicing	exon 8	c.1542G>A	(p.Lys510=)/splicing	< 1.5%	exon 8
Patients with <i>in vitro</i> enzyme activity >1.5%								
Cell lines with <i>in vitro</i> FAD responsiveness								
32	3.4	c.482G>A	p.Arg157Gln	exon 2	c.482G>A	p.Arg157Gln	10.4%	exon 2
55	0.3	c.535G>A	p.Ala175Thr	exon 3	c.1178G>A	(p.Trp389*) /splicing	1.7%	exon 6
33	216	c.596C>T	p.Ala195Val	exon 3	c.596C>T	p.Ala195Val	17%	exon 3
Cell lines with reduced affinity for ^b NADPH ^b NADPH								
30	132	c.-40_-41delTC	-	5' UTR	c.1727C>T	p.Pro572Leu	9.9%	exon 10
29	2	c.276_314dup	p.Leu89_Pro101dup	exon 2	c.1528T>G	p.Tyr506Asp	8.1%	exon 8
52	0.1	c.1126A>G	p.Lys372Glu	exon 6	c.1542+2T>C	p.Tyr512Trpfs*3	6.3%	exon 8
54	0.75	c.1141C>T	p.Arg377Cys	exon 6	c.1359+1G>A	splice site	7.4%	intron 7

Patients with very low <i>in vitro</i> enzyme activity (<1.5%)								
ID	Age at presentation (months)	Mutation allele 1	Predicted amino acid change	exon / intron	Mutation allele 2	Predicted amino acid change	Residual <i>in vitro</i> enzyme activity + <i>a</i> FAD ^a	exon / intron
31	60	c.1142G>A	p.Arg377His	exon 6	c.1142G>A	p.Arg377His	34.8%	exon 6
10	-	c.1274G>C	p.Trp421Ser	exon 7	c.1420G>T	p.Glu470*	3.8%	exon 8
37	2.5	c.1644+2T>G	splice site	intron 9	c.1644+2T>G	splice site	1.8%	intron 9
12	1.25	c.1764+1G>T	splice site	intron 10	c.1764+1G>T	splice site	3.7%	intron 10
14	-	c.1764+1G>T	splice site	intron 10	c.1764+1G>T	splice site	3.0%	intron 10
13	1.25	c.1765-18G>A	p.Asp585Glyfs*14	intron 10	c.1765-18G>A	p.Asp585Glyfs*14	2.5%	intron 10
26	24	c.1765-18G>A	p.Asp585Glyfs*14	intron 10	c.1765-18G>A	p.Asp585Glyfs*14	3.5%	intron 10
49	1	c.1780delC	p.Leu590Cysfs*72	exon 11	c.1780delC	p.Leu590Cysfs*72	2.0%	exon 11
38	1	c.1805T>C	p.Leu598Pro	exon 11	c.1805T>C	p.Leu598Pro	3.1%	exon 11
Cell lines with normal affinity for <i>b</i> NADPH ^b								
4	156	c.148C>T	p.Arg46Trp	exon 1	c.167G>A	p.Arg52Gln	9.7%	exon 1
35	6	c.148C>T	p.Arg46Trp	exon 1	c.1982G>C	p.*657Serext*50	2.3%	exon 11
72	4	c.1332G>C	p.Ser440-/splicing	exon 7	c.1644+2T>G	splice site	2.2%	intron 9

^aFAD flavin adenine dinucleotide^bNicotinamide adenine dinucleotide phosphate