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Neonatal screening for glutaryl-CoA dehydrogenase deficiency

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Summary: Acute encephalopathic crisis in glutaryl-CoA dehydrogenase deficiency results in an unfavourable disease course and poor outcome, dominated by dystonia, feeding problems, seizures and secondary complications, and quite often leading to early death. The prerequisite for the prevention of irreversible brain damage in this disease is the detection of affected patients and initiation of treatment before the manifestation of such crisis. Apart from macrocephaly there are no signs or symptoms characteristic for this disease in presymptomatic children and, thus, they are usually missed. In some countries, implementation of extended neonatal screening programmes using electrospray ionization tandem mass spectrometry (ESI-MS/MS) allows detection of affected newborns and start of therapy before onset of neurological complications. This article summarizes recent strategies, pitfalls and shortcomings of a mass screening for glutaryl-CoA dehydrogenase deficiency using ESI-MS/MS. Furthermore, an alternative strategy, namely DNA-based neonatal screening for the Oji-Cree variant of this disease, is demonstrated. An optimization of diagnostic as well as therapeutic procedures must be achieved before GCDH deficiency unequivocally fullfils the criteria of a reliable and successful newborn screening programme.

Patients with glutaryl-CoA dehydrogenase (GCDH) deficiency were reported to have elevated blood levels of glutarylcarnitine, and thus acylcarnitine analysis in dried blood spots by fast atom bombardment–tandem mass spectrometry (FAB-MS/MS) and recently by electrospray ionization–tandem mass spectrometry (ESI-MS/MS) has been successfully implemented into some neonatal screening programmes (Chace

et al 2002; Naylor and Chace 1999; Roscher et al 2001; Schulze et al 2003; Wilcken et al 2003; Wiley et al 1999; Zytkovicz et al 2001). However, the diagnostic sensitivity and specificity for the detection of GCDH deficiency by acylcarnitine analysis, especially in patients with atypical biochemical features ('low excretors' according to Baric et al 1999) is uncertain.

We had previously evaluated the sensitivity of acylcarnitine analysis by FAB-MS/MS in the diagnostic process of 38 patients with finally proven GCDH deficiency, including four patients with isolated elevated excretion of 3-hydroxyglutaric acid, and six patients with completely normal urinary organic acids ('nonexcretors') (Zschocke et al 1997). Glutarylcarnitine was found elevated in all patients with a high urinary excretion of glutaric acid but was absent or only marginally elevated in patients with intermittently normal urinary organic acids or isolated elevation of urinary 3-hydroxyglutaric acid (Baric et al 1999; Greenberg et al 2002). This prompted us to perform a detailed evaluation.

The rationale for newborn screening derives from the observation that only early diagnosis and treatment of presymptomatic children can prevent (1) encephalopathic crises and (2) the consequent development of striatal lesions resulting in irreversible neuronal damage. There is growing evidence that the severe neurological impairment seen in affected patients often results from a single encephalopathic crisis in infancy causing acute brain injury. Since GCDH deficiency is considered a treatable condition, the major aim of extended neonatal screening is to detect patients before such crises occur and to initiate appropriate presymptomatic treatment as the most important factor for the prevention of brain injury (Kölker et al 2004a; Mühlhausen et al 2004). Since most crises occur between ages 3 and 24 months and neonatal onset of this disease is a rarity, GCDH deficiency seems to meet essential criteria for neonatal screening.

In the following, we give an overview of the experience with neonatal screening for GCDH deficiency worldwide and describe two different approaches to neonatal screening (in Heidelberg, Germany, and in Winnipeg, Manitoba, Canada).

DIAGNOSTIC SENSITIVITY AND SPECIFICITY

Glutarylcarnitine, a 5-carbon dicarboxylic acylcarnitine (C_5DC) accumulates in GCDH deficiency. The measurement of C_5DC in dried blood spots (DBS) from newborns and the calculation of acylcarnitine ratios (such as C_5DC/acylcarnitines of different chain length) appear to be reliable and accurate approaches to detect patients with GCDH deficiency in the neonatal period. However, some patients may be missed due to a mild biochemical phenotype, demonstrating either no, intermittently increased or borderline elevated C_5DC concentrations. To ensure a high diagnostic sensitivity, any initial elevation of glutarylcarnitine should prompt careful in-depth follow-up evaluation, since a repeat DBS specimen may give a normal, i.e. false-negative, result although the two cases reported in the literature were missed during the establishment of ESI-MS/MS screening and cut-off levels have been adapted meanwhile (Smith et al 2001; Wilcken et al 2003). There are no data or rationales supporting the notion that quantification of acylcarnitines in DBS prior to and after a carnitine load increases the diagnostic sensitivity in these cases—only in patients with severe deficiency of free carnitine will carnitine loading increase C_5DC

concentrations. The confirmation of the diagnosis of GCDH deficiency by organic acid analysis using stable-isotope dilution GC-MS is usually straightforward, but again some patients may also show no biochemical abnormalities in the urine (Baric et al 1999; Busquets et al 2000; Greenberg et al 2002; Hoffmann et al 1996). It needs to be investigated whether the analysis of urinary organic acids or the quantification of 3-hydroxyglutaric acid (3-OH-GA) and glutaric acid by stable-isotope dilution GC-MS is able to further improve the diagnostic sensitivity of a positive newborn screen.

The diagnosis of GCDH deficiency is considered to be very likely when the quantification of characteristic metabolites (glutarylcarnitine in blood, glutaric acid and 3-OH-GA in urine) yields unambiguous abnormal results. This gives sufficient basis to start treatment and to counsel families. It is not recommended to rely solely on the biochemical profile in the urine to establish a diagnosis without confirmation by an independent analytical procedure such as the determination of GCDH activity in fibroblasts or leukocytes or mutation analysis of the *GCDH* gene.

ESTIMATED PREVALENCE OF GCDH DEFICIENCY

Before the implementation of GCDH deficiency to neonatal screening programmes, the estimation of prevalence was quite rough, except for a few small cohorts with a high carrier frequency, such as the Amish or the Oji-Cree Indians. So far, results of newborn screening programmes in various regions and cohorts worldwide suggest that the frequency of GCDH deficiency is lower than previously estimated. Table 1 summarizes the experience from North American, Australian and German neonatal screening programmes, enrolling approximately 2.5 million newborns worldwide with an overall mean frequency of 1:106 900.

STRATEGIES FOR NEONATAL SCREENING—A SEARCH FOR RELIABLE CUT-OFFS AND SECONDARY PARAMETERS

As a deuterium-labelled analytical standard for C_5DC is not yet available, true quantification is impossible at present. However, the signal ratios of acylcarnitines appear to be stable for long periods, allowing reliable relative quantification. The optimal approach seems to relate the signal for C_5DC to the internal standard of octanoylcarnitine (C_8) and to define a ‘normal’ cut-off based on the results of healthy newborns.

Newborn screening centres follow different strategies for defining and updating cut-offs for C_5DC, and they use different secondary parameters (ratios, free carnitine, etc.) to further increase the diagnostic sensitivity and specificity. Mostly, these strategies are experienced-based and the parameters are not robust and are influenced by several factors. At present, there is no universally accepted gold standard for the performance of newborn screening for GCDH deficiency. It is, however, indispensable to first of all carefully establish exact cut-off values for each specific age group. These may differ in different screening programmes and certainly differ from values in older infants and in children investigated in selective screening evaluation by ESI-MS/MS.

Table 1 Estimated prevalence of GCDH deficiency using ESI-MS/MS-based neonatal screening

Screening centre/country	*GCDH*	*Screened newborns*	*Prevalence*	*Source*
Pennsylvania, Ohio, North Carolina, Louisiana, USA	9	687 000	1:76 400	Naylor and Chace (1999)
Pennsylvania, Ohio, North Carolina, Louisiana, USA[a]	13	1 020 000	1:78 500	Chace et al (2002)
New South Wales, Australia	1	137 000	1:137 000	Wiley et al (1999)
New South Wales, Australia[b]	2[c]	362 000	1:181 000	Wilcken et al (2003)
New England, USA	0	164 000		Zytkovicz et al (2001)
Bavaria, Germany	2	308 000	1:154 000	Roscher et al (2001)
Baden-Württemberg, Germany	3	250 000	1:83 300	Schulze et al (2003)
Baden-Württemberg, Germany[d]	6	605 000	1:100 800	Unpublished
Total	23	2 45 9000	1:106 900	

Original values are rounded up or down and are given as even numbers
[a] This study also includes the screening data of Naylor and Chace (1999)
[b] This study also includes the screening data of Wiley et al (1999)
[c] These patients were diagnosed retrospectively. The first patient was born during an early screening period when C_5DC has not been included in the screening panel. The second patient had an elevated C_5DC in the initial sample (1.2 μmol/L) and a C_5DC concentration below the cut-off (0.8 μmol/L) in a repeat sample (0.6 μmol/L)
[d] This study also includes the screening data of Schulze et al (2003)

In the following, the strategy used by the neonatal screening center at Heidelberg (1998–2004) is summarized. Data management was divided into technical interpretation of acquired data and clinical interpretation and decision-making. A cut-off for each analyte was set on the 99.95th centile, based on a control group of 10 000 healthy neonates. The cut-off values were regularly updated by recalculation of centiles from recent samples. Samples crossing the cut-off were automatically flagged, and the analysis was repeated. Data files of samples with two positive tests and with a discrepancy of the two results <30% were transferred to the clinical interpretation and decision-making, performed by an experienced metabolic specialist (Schulze et al 2003). If the discrepancy between initial and repeat sample exceeded 30%, a third analysis was performed. If the flagged sample was judged positive by the metabolic specialist, the parents and referring hospitals were informed immediately and the suspected diagnosis was confirmed or refuted as soon as possible.

From 1998 to 2004, six patients with positive screening for C_5DC have been found in 605 000 screened newborns (Table 1). Diagnosis was confirmed by organic acid analysis in urine (with stable-isotope dilution assay), enzyme and/or mutation analysis. All of these patients could be clearly identified by an elevated C_5DC (Figure 1A). Furthermore, C_5DC/C_8 (Figure 1B) and C_5DC/palmitoylcarnitine (C_{16}; Figure 1C) ratios have been elevated in all patients. Notably, repeated analysis of DBS

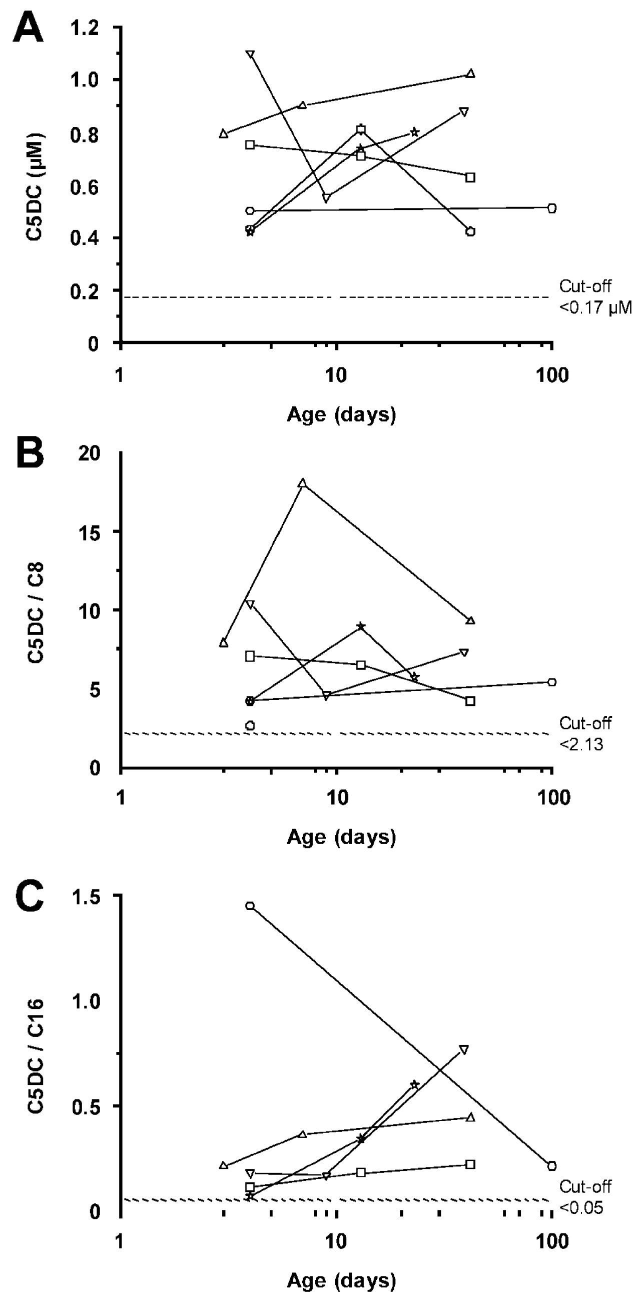

Figure 1 (A) C_5DC, (B) C_5DC/C_8 and (C) C_5DC/C_{16} ratios in six GCDH-deficient patients identified by ESI-MS/MS neonatal screening. C_8, octanoylcarnitine; C_{16}, palmitoylcarnitine. Cut-off values represent the 99.95th centile of 10 000 healthy control individuals

in a second Guthrie card did not show a decrease in any of these parameters below the cut-off value (Figure 1A–C). Furthermore, administration of L-carnitine—usually performed before the investigation of the third sample—did not significantly increase C_5DC or the above-mentioned ratios in our cohort (Figure 1A–C). Obviously, L-carnitine should be administered (for therapeutic and diagnostic reasons) if the concentration of free carnitine is low in a patient.

Since the implementation of newborn screening in Heidelberg, a recall rate of 0.034% (i.e. $n = 206$) has been achieved for C_5DC. An evaluation of the latest 30 recalls (with false-positive C_5DC elevation) showed that C_5DC (0.18–0.26 μmol/L; cut-off 0.17 μmol/L) but not C_5DC/C_8 or C_5DC/C_{16} ratios were elevated in these newborns, whereas all of these parameters were clearly elevated in true-positive patients (Figure 1), including one low excretor. Elevated C_5DC in false-positive individuals was often accompanied by a slight increase in other acylcarnitines, in particular decanoylcarnitine and dodecanoylcarnitine. These false 'C_5DCs' may be C_{10}OH (3-hydroxydecanoylcarnitine; Chace et al 2003). It seems worth considering further increasing the diagnostic specificity by addition of C_5DC/C_8 and C_5DC/C_{16} ratios and by evaluation of parameters indicating secondary changes. In contrast, abnormal C_5DC was also found in patients with multiple acyl-CoA dehydrogenase deficiency; however, this disease could easily be distinguished from GCDH deficiency owing to additional characteristic acylcarnitines.

Fortunately, during the first 6 years of neonatal screening, to our knowledge no false-negatively screened newborn with GCDH deficiency has come to medical attention. However, as an increased glutarylcarnitine is usually found in patients with the high-excretor phenotype (unless free carnitine is low), this subgroup can easily be detected by ESI-MS/MS. The situation is completely different in defined subgroups of the low-excretor phenotype, such as Canadian Oji-Cree aboriginals (Greenberg et al 2002). Notably, ESI-MS/MS in DBS from two of these patients revealed C_5DC clearly below the cut-off (0.08 μmol/L, 0.10 μmol/L; cut-off 0.17 μmol/L) and normal C_5DC/C_8 (1.79, 1.97; cut-off <2.96) and C_5DC/C_{16} (0.03, 0.04; cut-off 0.17) ratios, even after loading with L-carnitine (free carnitine 148 μmol/L; 183 μmol/L). Obviously, in such cohorts alternative strategies for neonatal screening have to be developed.

ALTERNATIVE STRATEGIES IN BIOCHEMICALLY MILD PHENOTYPES—DNA-BASED NEONATAL SCREENING

The First Nations of Oji-Cree descent from the remote Island Lake communities in northeastern Manitoba and northwestern Ontario have a high prevalence of GCDH deficiency, with an estimated carrier frequency of ~1/15. The Oji-Cree variant of GCDH deficiency is caused by a splicing mutation in intron 1 (IVS1+5g>t) of the *GCDH* gene and is biochemically characterized by mostly normal concentrations of glutarylcarnitine in DBS and a low or even normal urinary excretion of glutaric acid and 3-OH-GA (Greenberg et al 2002). Twenty-eight patients with this disorder have been identified in Manitoba, 26 of whom are Oji-Cree with the identical homozygous IVS1+5g>t splicing mutation. Since the mild biochemical phenotype in this

particular subgroup of affected patients does not allow a reliable detection by neonatal screening based on detection of C_5DC by ESI-MS/MS or of glutaric acid and 3-OH-GA by GC-MS, an alternative strategy has been developed.

DNA-based newborn screening for GCDH deficiency targeted to all newborns of Oji-Cree descent began in Manitoba in May 1998. There are ~400 births annually from this high-risk community. A high-risk sample was flagged by a special 'GCDH deficiency at risk' sticker affixed to the filter paper newborn card after consent was obtained. After the routine newborn screening tests are performed by the provincial laboratory, any filter paper card marked with the 'GCDH deficiency at risk' sticker is subjected to the supplemental DNA-based test. Mutation testing is performed weekly by an allele-specific polymerase chain reaction (PCR) method as previously described (Greenberg et al 2002). Each run contains a PCR no-template control and samples from a known homozygous affected, a heterozygous and a homozygous normal individual co-amplified with the weekly run. If a baby is identified to be homozygous for the *GCDH* IVS+5g>t mutation, this reaction is immediately repeated on a sample punched from another blood spot from this baby. If the second result confirms the first, the metabolic specialist is immediately notified. The baby is assessed clinically, and repeat samples for DNA testing, acylcarnitine profiling and urinary organic acid analysis are obtained. Treatment is generally instituted as soon as confirmatory results are positive.

As of June 2003, a total of 2200 babies have been screened and 7 affected babies (5 females and 2 males) have been identified (estimated prevalence 1 in 300 newborns). All parents and siblings have been tested and to date no asymptomatic homozygous affected parent or sibling has been identified by extended family testing. To date there have not been any known false-positive or false-negative DNA-based newborn screening results for GCDH deficiency. Despite early diagnosis and standardized treatment with protein restriction, carnitine supplementation, riboflavin and emergency treatment during intercurrent illness, those Oji-Cree patients detected by DNA-based screening still have had a poor outcome (Greenberg et al 2002; C.R. Greenberg, personal communication). This supports the notion that early diagnosis *per se* is the first prerequisite for the prevention of neurological disease in these children, with the establishment and continuous optimization of reliable treatment strategies and therapy monitoring schedules being the second.

In conclusion, ESI-MS/MS for mass neonatal screening or DNA-based neonatal screening for high-risk groups with a single or limited number of mutations seem reliable approaches to detecting GCDH-deficient patients before onset of neurological deterioration. Using ESI-MS/MS-based screening it has been demonstrated in different centres that the majority of affected newborns will be identified by this method. However, single reports of false-negative screening results have been published, induced by mistakes in technical (cut-off) or medical (false interpretation) validation (Smith et al 2001; Wilcken et al 2003). Thus, optimization of newborn screening for GCDH deficiency is still necessary. Various attempts are under investigation to increase the diagnostic sensitivity and specificity, ranging from calculation of different C_5DC ratios, through administration of L-carnitine to the baby before obtaining a second analysis, to inclusion of follow-up urinary organic acid analyses

by stable-isotope dilution GC-MS for borderline C_5DC levels. In addition, outcome studies in presymptomatic GCDH-deficient patients have highlighted that there are still shortcomings in the recent therapeutic management of affected patients. Continuation and extension of outcome studies of children who are diagnosed early and treated presymptomatically are needed and will allow the estimation of the long-term benefit of newborn screening programmes (Schulze et al 2003; Kölker et al 2004b). Optimization of diagnostic as well as therapeutic procedures must be achieved before it may be hoped that GCDH deficiency will soon unequivocally fulfil all the criteria for a reliable and successful newborn screening programme (Superti-Furga 2004).

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