

## Phenylalanine loading as a diagnostic test for DRD: interpreting the utility of the test

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### Abstract

Phenylalanine loading has been proposed as a diagnostic test for autosomal dominant DRD (dopa-responsive dystonia), and recently, a phenylalanine/tyrosine (phe/tyr) ratio of 7.5 after 4 h was reported as diagnostic of DRD. To test the utility of this test in another sample with DRD, we administered an oral challenge of phenylalanine (100 mg/kg) to 11 individuals with DRD and one non-manifesting gene carrier. Only 6/12 had a 4 h phe/tyr ratio of greater than 7.5, suggesting that additional parameters must be set to avoid missing the diagnosis of DRD, including the need for the plasma phenylalanine to reach a minimum level 600 in order for the test to be valid. We propose that in cases where this minimum plasma phenylalanine level is not reached, plasma tetrahydrobiopterin should be measured or alternatively other symptomatic family members should be screened.

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### Introduction

Dopa-responsive-dystonia (DRD) usually starts in childhood and is characterized by twisting or posturing movements in the legs and trunk, an abnormal gait, and

a dramatic response to levo-dopa [1,2]. Autosomal dominant DRD is a result of mutations in the GTP cyclohydrolase 1 (GTPCH) gene [3–5], and has incomplete penetrance [3,4,6]. GTPCH catalyzes the first and rate-limiting step in the synthesis of tetrahydrobiopterin (BH4) which is an essential cofactor for the conversion of phenylalanine to tyrosine, tyrosine to levo-dopa, and tryptophan to 5-hydroxytryptophan [7]. In contrast to DRD, other inherited defects in BH4 metabolism

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present with baseline hyperphenylalaninemia. Because most GTPCH mutations are unique and up to 40% of patients with DRD do not have identifiable mutations in the coding exons, clinical features, and response to levodopa have remained the diagnostic gold standard for DRD [8]. CSF biopterin (measured after chemical oxidation of BH4) and neopterin are also markers for autosomal dominant DRD, and both are decreased in manifesting and non-manifesting gene carriers [9]. CSF neopterin may distinguish between DRD and juvenile onset Parkinson's disease, two conditions which may present with dopamine responsive dystonia [9]. However, CSF measurements require lumbar puncture.

A phenylalanine loading test was developed as a biochemical diagnostic test for autosomal dominant DRD [10,11]. The test is based on the premise that GTPCH deficiency results not only in decreased CSF BH4, but that it also leads to decreased hepatic BH4 concentrations, which can be assessed by stressing the system with a phenylalanine load [20]. With reduced concentrations of BH4, the activity of phenylalanine hydroxylase is lower resulting in a slower conversion of phenylalanine to tyrosine. The utility of this test has been reported in three series of autosomal dominant DRD, including nine patients with DRD [10], 11 symptomatic and 9 asymptomatic gene carriers [11], and 10 individuals with DRD [12]. These reports consistently found that phe/tyr ratios were predictive of DRD and also concluded that BH4 measurement did not add additional information. However, we identified a patient with a falsely normal phenylalanine loading report [13]. We therefore, proceeded to systematically study the utility of phenylalanine loading in the diagnosis of DRD. We report the results of phenylalanine loading on members of five mutation positive families, and measured phenylalanine, tyrosine and BH4.

## Methods

Five local families with identified GCH1 mutations were selected based on willingness of the probands to undergo phenylalanine loading. The study was approved by the institutional review boards and all participants gave informed consent. Eleven affected family members, and one non-manifesting carrier had phenylalanine loading.

Oral phenylalanine loading with 100 mg/kg of phenylalanine (L-phenylalanine, Ajinomoto, USA) was performed as previously described [10] except that samples were drawn at baseline and 1, 2, and 4 h post-load, but not 6 h post-load. Plasma was separated and frozen, and phenylalanine and tyrosine were assayed. BH4 was measured as total biopterin after acidic oxidation with MnO<sub>2</sub> according to Curtius et al. [14]. For two individuals with a falsely normal phenylalanine loading test, we repeated the test.

DNA from affected individuals was isolated from peripheral leukocytes. Exons 2–6 of the GCH1 gene were amplified using described primers and methods [15]. Exon 1 was amplified using the following primer sets: 5' cgtac cttcctcaggtgactccgg 3' with 5' agctcaggatggacgagtag 3', and 5' ccaggtgcagcaatgggttc 3' with 5' cccgcacgctctagcag 3' using standard PCR conditions. All amplified products were then sequenced directly. In individual 4:1–2, the whole coding region of the phenylalanine hydroxylase (PAH) gene was screened for mutations by denaturing gradient gel electrophoresis as previously described [16].

As we were only assessing sensitivity, we did not perform loading in a control population. However, for descriptive purposes, we also report data from 25 pediatric disease controls (range 1–17 years) who had phenylalanine loading as above. Seventeen had phenylalanine (=T1 or T2) >600 µmol/L and five had phenylalanine <400 µmol/L. All controls were patients in which phenylalanine loading was performed because of suspicion for biopterin defects, but had normal fibroblast GTPCH enzyme activity [19].

## Results

The clinical characteristics, GTPCH mutations, and pedigrees of the five probands and their family members are shown in Table 1 and Fig. 1. All probands were female with childhood onset dystonia starting in the leg with mean age at onset 6.3 years (3–10 years) and all responded to 200 mg or less of levo-dopa.

The phe/tyr ratios, BH4 and phenylalanine/BH4 levels are shown in Figs. 2A–C. The 4-h phe/tyr ratio ranged from 2.4 to 14.74, with intrafamilial variability as well (see Table 1). In 10/12 the phe/tyr ratio was greater than or equal to 5.25, but in two, ratios of <2.5 were found. Their maximum phenylalanine levels were 565 and 502 µmol/L. Both individuals were retested, and one achieved a phenylalanine of >600 µmol/L, and had a phe/tyr ratio greater than 5.25. In contrast, the other had a maximum phenylalanine <600 µmol/L, and again tested negative. Eliminating the two individuals with maximum phenylalanine <600 µmol/L, and using a ratio of 5.25 as indicative of DRD, the test reached 100% sensitivity.

One hour BH4 ranged from 9.6 to 28.9 nmol/l and was ≤19.0 nmol/L in 11/12 DRD subjects, including all probands. Comparison data for pediatric controls is also shown in Fig. 2, which demonstrates differences in biopterin curves depending on whether a phenylalanine level of 600 µmol/L was reached. Therefore, the 1-h BH4 measurement for this level is 92.8% sensitive. The mutation carrier with a normal range BH4 had elevated phe/tyr ratios consistent with DRD. As this could also be explained by heterozygosity for a mutation in the PAH gene, we examined the coding region of that gene through denaturing gradient gel electrophoresis. This method has

Table 1  
Characteristics of DRD family members and phenylalanine loading summary

Pedigree #	Mutation	Age at phe load (years)	Gender	Age-onset (years) D, dystonia P, parkinsonism	phe (4 h) ( $\mu\text{mol/L}$ )	tyr (4 h) ( $\mu\text{mol/L}$ )	phe/tyr (4 h)	BH4 (nmol/l) (1 h)
Family 1								
1:D010	281C > A, T94K	22	F	D = 10	218	19	2.4	18.1
1:B002	281C > A, T94K	76	F	prob P = 76	823	96	8.6	18.9
1:C008	281C > A, T94K	50	M	P = 46	642	60	10.7	15.8
1:B003	281C > A, T94K	62	M	D = 10, P = 60	673	101	6.7	11.7
Family 2								
2:D005	328C > T, Q110X	48	F	D = 6	504	89	5.7	14.3
2:D006	328C > T, Q110X	42	F	Normal carrier	701	89	7.9	9.6
Family 3								
3:D003	646C > T, R216X	32	F	D = 5	219	118	1.9	9.7
3:D008	646C > T, R216X	16	M	D = 8	805	60	13.4	19.0
3:B008	646C > T, R216X	82	F	P = 80	855	58	14.7	28.9
3:C004	646C > T, R216X	59	F	D = 5	805	60	18.2	13.4
Family 4								
4:G002	308A > C, Q103P	41	F	D = 3	488	87	5.6	16.3
Family 5								
5:D003	631/632delAT, Met211X	19	F	D = 9	478	88	15.4	12.9

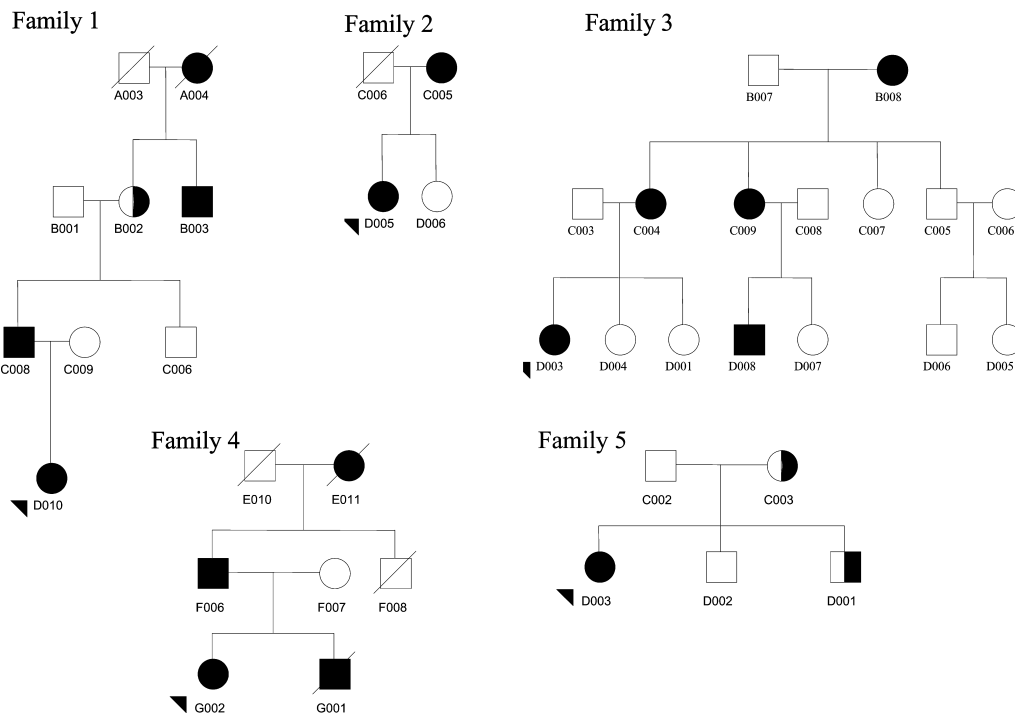


Fig. 1. Partial pedigrees of DRD families: circles denote females, squares designate males, filled symbols are affected with dystonia or parkinsonism, open symbols are unaffected, and arrow indicates proband.

a sensitivity >98% for the identification of disease-causing mutations; no mutation was found in our patient.

**Discussion**

As GTPCH mutation analysis is expensive and detection rate may be only as high as 60%, and varies by

laboratory technique, we further assessed oral phenylalanine loading as a diagnostic test for DRD. Our data generally support the utility of this test in the diagnosis of DRD [10,11], however, false negative test results were obtained. In an effort to explain these false negative results, we examined maximal phe level in this small sample, and found that the test does not appear to identify DRD if the maximal phe level does not reach

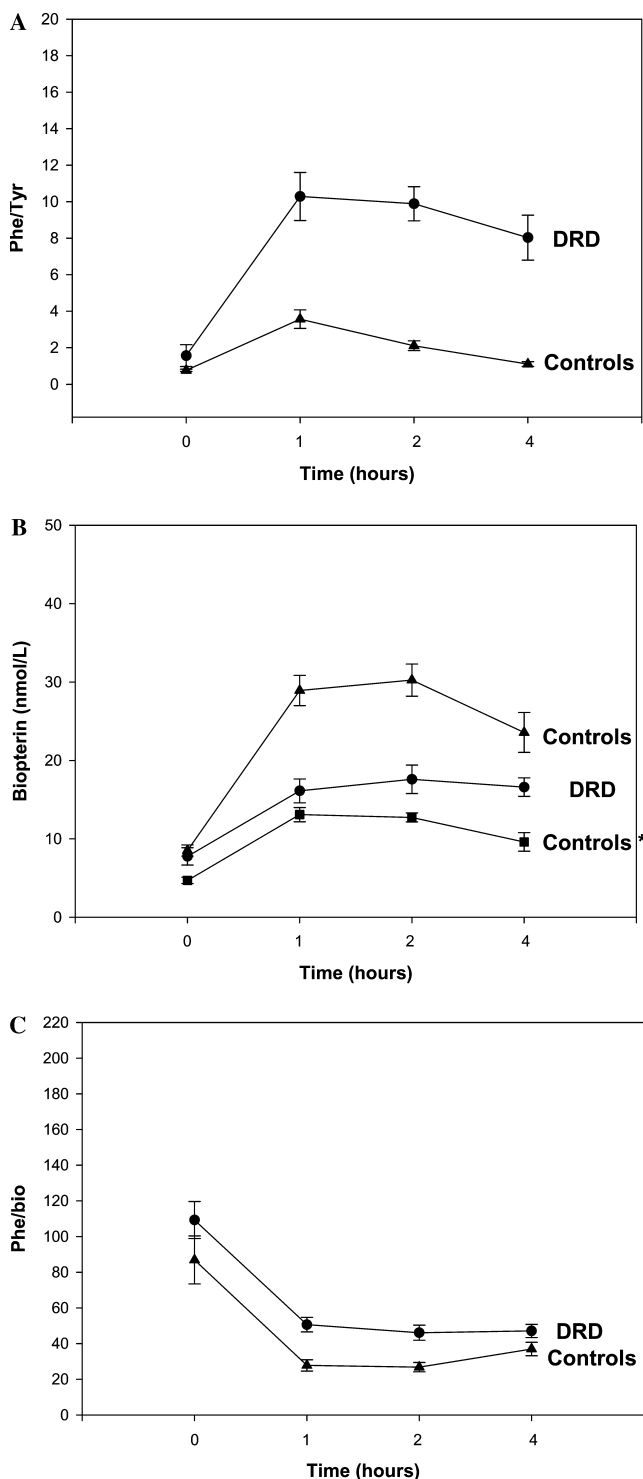


Fig. 2. (A) Plasma phenylalanine/tyrosine ratio in response to oral phenylalanine load as a function of time for mutation carriers and controls. (B) Plasma biopterin (nmol/L) in response to oral phenylalanine load as a function of time, control indicates those “controls” with phenylalanine (= T1 or T2) > 600  $\mu\text{mol/L}$  and those “\*controls” with phenylalanine <400  $\mu\text{mol/L}$ , and (C) phenylalanine/biopterin ratio.

600  $\mu\text{mol/L}$ . This is supported by control data showing different phe loading curves with >600  $\mu\text{mol/L}$  or <400  $\mu\text{mol/L}$ . Second, in our sample, the proposed ratio

of 7.5 [12] was too high to detect all other cases. We noted that sensitivity of the test improved when using a lower cut-off of 4 h phe/tyr ratio of 5.25, however, our sample was too small to make a recommendation about new cut-offs. Additional controls, including disease controls with juvenile Parkinson’s disease need to be assessed to fully develop the best cut-off levels. Further, as we report controls from other studies, which are not age matched, we cannot report on the specificity of this test.

BH4 is difficult to measure outside of specialized laboratories. Furthermore, the initial phenylalanine loading report of Hyland et al. [10] showed that plasma biopterin levels were consistent with phe/tyr interpretations in adult DRD patients, and did not provide additional diagnostic information. Therefore, the results of the phenylalanine loading test have been subsequently reported based on the phe/tyr ratios alone [11]. We note that measuring total plasma BH4 levels supplemented the phe/tyr ratio data, overcoming our false negative results and leading to increased test sensitivity.

It is unclear why 2 of the 12 affected individuals had low 4 h phe/tyr ratios in the normal range whereas those previously reported did not [10–12]. We postulate that maximal phenylalanine levels may play a role in the false negative tests. While BH4 inhibits its own biosynthesis via the GTPCH feed-back regulatory protein (GFRP), phenylalanine stimulates BH4 production through the GFRP–GTPCH complex [7]. Both individuals were petite therefore the per kilogram dosing may have led to a different level of phenylalanine stimulation and auto-feedback than the equivalent dose in larger subjects. Alternately, individual differences in intestinal absorption or metabolism may explain differences in maximal phenylalanine levels (<600  $\mu\text{mol/L}$ ) in these two. Although we repeated the phe load in both, one individual reached maximum phenylalanine level >600  $\mu\text{mol/L}$  and had an abnormal (i.e., consistent with GTPCH deficiency) phe/tyr ratio, but the other still had a sub threshold phenylalanine level and did not have an abnormal phe/tyr ratio. This suggests that body weight alone is insufficient to explain the false negative result, and that it may be a combination of factors.

Further, the false negative test results may be due to factors unrelated to the maximal level of phenylalanine reached. The discrepancy between studies due to methodological differences is a less likely explanation, as the protocol did not vary, and analysis was performed in the same laboratory as the first two reports [10,11]. The effect of different GTPCH mutations is also unlikely as other family members had abnormal phe/tyr ratios, and both individuals were severely affected prior to treatment with levo-dopa. Neither individual was taking a medication that should affect liver metabolism.

We cannot explain the normal plasma BH4 and abnormal phe/tyr ratio in patient 3:I-2, our oldest

affected participant. Peripheral BH4 should be reduced in the setting of GTPCH deficiency unless there is abnormal feedback in the hepatic synthesis of BH4, secondary to phenylketonuria, for example. However, this individual did not have an identifiable PAH mutation to suggest PKU heterozygosity, nor was there known liver disease to suggest problems in phenylalanine to tyrosine conversion.

In addition, we report GTPCH mutations in these families. Two Ashkenazi Jewish families had different mutations, arguing against a founder mutation for DRD in this population. One of the mutations (family 2) has been reported only in the setting of autosomal recessive GTPCH deficiency that caused hyperphenylalaninemia [17] reinforcing that GCH1 mutations may cause both autosomal dominant DRD as well as autosomal recessive hyperphenylalaninemia [18]. The mutations in family 1 and family 3 were previously described in other families [17] supporting that GTPCH mutations might not be as unique as once believed.

We therefore, conclude that the phenylalanine loading test may be a useful test in the diagnosis of DRD, however false negative tests occur. In our sample, the sensitivity improved when criteria for maximum plasma phenylalanine levels were achieved, as well as when used in combination with BH4 measurements. Alternately, phenylalanine loading in an affected relative or carrier may also allow for diagnosis. We are particularly concerned about the potential for a false negative load in a child with DRD as shown above, our results are divergent from prior studies. Excluding the diagnosis of DRD (and hence not administering a trial of levo-dopa) to a DRD patient could have poor (serious) consequences, therefore, we recommend that a larger population of DRD subjects of different age groups, particularly children, be studied. This would confirm the utility of the test, including determining the necessity of screening for plasma BH4 and help set more generalizable parameters for an abnormal test. Until such time, measurement of CSF BH4, neopterin, and dopamine metabolites provides an excellent method to discriminate autosomal dominant DRD from juvenile parkinsonism [9]. Evaluation of neopterin and biopterin production and of GTPCH activity in cytokine-stimulated fibroblasts may be another extremely useful laboratory tool in the diagnosis of DRD [19]. Fibroblast GTPCH activity was found to be significantly reduced in some patients without mutations in the GCH1 gene [20]. Because DRD is a highly treatable disorder, empiric treatment with levo-dopa should be considered in any individual with possible DRD.

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