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Classic Phenylketonuria: Heterozygote Detection during Pregnancy

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INTRODUCTION

Classic phenylketonuria (PKU) is an autosomal recessive disease caused by inherited defects in hepatic phenylalanine hydroxylase [1, 2]. Homozygous affected individuals have elevated blood phenylalanine concentrations, relatively normal blood tyrosine concentrations, and excess production of several alternate products of phenylalanine metabolism. Although six variants of hyperphenylalaninemia have been described, we will define the phenotype for "classic PKU" by persistence of these metabolic aberrations as well as the presence of central nervous system impairment if dietary restriction of phenylalanine is not instituted before the third week of life [3].

Several state screening programs detect presymptomatic homozygous affected PKU infants through the bacterial inhibition assay of Guthrie and Susi [4]. In Georgia during 1972, 256 of 70,980 newborns tested had hyperphenylalaninemia (greater than 2 mg/100 ml) and six were ascertained to have classic PKU (L. Warnick, Chief, Child Health Unit, Georgia Department of Human Resources, personal communication). This observation is consistent with other incidence figures and by using the Hardy-Weinberg equation suggests that one in 55 or 1.8% of the general population in Georgia carries the mutant recessive gene for classic PKU [5]. Several benefits would derive from a method to detect heterozygotes in both the general population and in relatives of known PKU patients. Pregestational genetic counseling, identification of high-risk pregnancies, and postgestational differentiation of classic PKU from other forms of hyperphenylalaninemia are some of these benefits.

Previous investigators recognized the need for such a capability [6–16]. Hsia et al. [6] in 1956 used oral phenylalanine loading and measured blood phenylalanine levels by fluorometric methods. Although obligate heterozygotes attained higher blood phenylalanine levels and had slower clearance rates than normal controls, the

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wide range of normal variation, nonspecific quantitative procedures, and problems associated with oral loading made this test difficult to interpret. In 1967, Perry et al. [7] found that a fasting phenylalanine/tyrosine ratio provided better and easier discrimination than either oral or intravenous loading. However, only two-thirds of the obligate heterozygotes were differentiated from controls when relatively nonspecific fluorometric methods were used. Later, Perry et al. [8] and Rosenblatt and Scriver [9] improved discrimination by quantitating fasting plasma phenylalanine and tyrosine ratios using ion-exchange chromatography. These authors demonstrated several variants of hyperphenylalaninemia by plotting phenylalanine/tyrosine ratios versus phenylalanine concentrations. However, application of this graphic analysis to heterozygote detection resulted in 20% of their populations remaining unclassifiable due to overlapping values. Heterogeneity of hyperphenylalaninemia and hormonal effects on phenylalanine hydroxylase activity presumably contributed to this overlap. In 1971 and 1972, Jackson et al. [10] and Yakmyshyn et al. [11] demonstrated that phenylalanine/tyrosine ratios were increased by oral contraceptives and by normal pregnancy to produce an 80% overlap with obligate heterozygotes. These effects were associated with a fall in plasma tyrosine observed during pregnancy [11].

The current investigation quantitates fasting plasma phenylalanine and tyrosine by ion exchange chromatography and compares these values in nonpregnant controls, pregnant controls, and obligate heterozygotes for classic PKU. An empirical examination of the data indicated these values could be used to differentiate obligate heterozygotes from pregnant and nonpregnant controls. Techniques of multivariate analysis were also used to produce linear discrimination functions useful in assessing group differences and in evaluating probable errors of misclassification between obligate heterozygotes and controls. The results of these analyses enabled prenatal genetic counseling in a pregnancy at risk for classic PKU.

MATERIALS AND METHODS

Control Subjects

Single heparinized venous blood specimens were obtained following at least 4 hr of fasting from three cohort groups: 23 controls (seven nonpregnant females and 16 males), 18 pregnant controls, and 10 male and female obligate heterozygotes for classic PKU. No female controls were taking oral contraceptives. Samples were usually obtained at 12:00 noon following a light breakfast at 7:30 A.M. All subjects were between the ages of 18 and 36 years. The pregnant controls were patients on the Voluntary Interruption of Pregnancy Service at Grady Memorial Hospital and were from 8 to 21 weeks gestation.

Family Study

A 26-day-old white male was referred to the Henrietta Egleston Hospital for Children for evaluation and dietary regulation of phenylketonuria. A bacterial inhibition assay performed on the eleventh day of life demonstrated more than 20 mg/100 ml of blood phenylalanine [4]. Fluorometric studies on the twenty-second day of life showed 56 mg/100 ml blood phenylalanine and 3.1 mg/100 ml blood tyrosine. Urinary ferric chloride reactions were persistently positive. Dietary restriction of phenylalanine to 40 mg per

pound was initiated on the twenty-sixth day of life and urinary ferric chloride became negative within 24 hr. Repeat blood phenylalanine by bacterial inhibition assay after 7 days of this diet was 2 mg/100 ml. Dietary restriction between 20 and 30 mg phenylalanine per pound for 6 months maintained his blood phenylalanine between 2 and 8 mg/100 ml. Higher phenylalanine intake produced corresponding increases in blood phenylalanine. There were no other siblings (see fig. 2). The maternal aunt (I-4) was married to the paternal uncle (I-1) and was 8 weeks pregnant at the time they presented to the genetic counseling clinic requesting information concerning their risks for having a child with PKU. The patient's father, mother, paternal uncle, and maternal aunt were studied.

Quantitation of Phenylalanine and Tyrosine

Plasma was separated from whole blood by centrifugation immediately after venipuncture, refrigerated for no more than 8 hr, deproteinized by previously described techniques [17], and frozen until analyzed. Plasma phenylalanine and tyrosine were quantitated using the medium-sized column (23 × 0.9 cm) of a Beckman model 120C amino acid analyzer packed with PA 35 resin. Tyrosine and phenylalanine were eluted with type D buffer (*p*H 5.28) at 20 and 25 min, respectively. Micromolar concentrations were quantitated by hand using the area under the curve.

Discriminant Analysis

Discriminant analysis permits the classification of individuals into two or more groups (in this case, genotypes) in terms of several variables (in this case, phenylalanine and tyrosine concentrations). The purpose of such an analysis is to find a discriminant function, that is, a linear combination of the phenylalanine (P) and tyrosine (T) values, $a_1(P) + a_2(T)$, that will maximally differentiate between obligate heterozygotes and controls. The values for a_1 and a_2 are estimated from the data. The resulting discriminant function can then be used to classify new individuals as obligate heterozygotes or controls on the basis of their phenylalanine and tyrosine values. The analyses were performed with the aid of the UCLA biomedical computer program BMD04M [18].

RESULTS

Phenylalanine and Tyrosine Determination: Empiric Comparison

Table 1 summarizes the means and standard deviations of phenylalanine (P) and tyrosine (T) values and their ratios P/T and P^2/T for the three groups of patients. Obligate heterozygotes had higher mean phenylalanine values ($P < .001$) and lower mean tyrosine values ($P < .05$) than nonpregnant controls. There was no difference between male and female controls. Despite the significantly different mean values, there was substantial overlap of the distribution of phenylalanine and tyrosine in these two groups. Thus, neither parameter alone was a useful discriminant. However, the distribution of the ratios of phenylalanine and tyrosine values (P/T) among obligate heterozygotes and nonpregnant controls did not overlap. Hence, the ratio P/T could be used to differentiate obligate heterozygotes and nonpregnant controls.

Mean phenylalanine and tyrosine concentrations for pregnant controls were significantly lower ($P < .001$) than for nonpregnant controls. However, the greater relative change in tyrosine concentrations produced higher P/T ratios for pregnant controls and reduced the differential utility of the P/T ratio in separating pregnant

TABLE 1

ARITHMETIC MEANS AND STANDARD DEVIATIONS OF PHENYLALANINE (P) AND TYROSINE (T) AND THEIR RATIOS P/T AND P^2/T FOR NONPREGNANT AND PREGNANT CONTROLS AND OBLIGATE HETEROZYGOTES

COHORT GROUP	PHENYLALANINE (μmoles/liter)		TYROSINE (μmoles/liter)		P/T		P^2/T	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Nonpregnant controls	60.0	10.2	62.3	14.8	0.99	0.13	57.5	10.2
Pregnant controls	46.0	6.8	41.1	8.4	1.22	0.14	57.7	9.8
Obligate heterozygotes	80.4	13.9	47.8	13.1	1.72	0.24	138	26

NOTE.—Patients studied, preparation, and quantitation of plasma phenylalanine and tyrosine are described in Methods section. The number of nonpregnant and pregnant controls and obligate heterozygotes studied was 23, 18, and 10, respectively.

controls and obligate heterozygotes. In order to compensate for this relative effect on the P/T ratio, all individual ratios were multiplied by their respective phenylalanine concentrations yielding P^2/T values. No overlap existed between pregnant controls and obligate heterozygotes using P^2/T values. Furthermore, virtually identical means and standard deviations were obtained for P^2/T values in the nonpregnant and pregnant controls. As seen in figure 1, the distribution of P^2/T values was similar in nonpregnant and pregnant controls. More importantly, P^2/T values for the combined control groups did not overlap with those for the heterozygotes. The highest control P^2/T value was 85 μmoles/liter whereas the lowest value for the obligate heterozygote was 93 μmoles/liter.

Phenylalanine and Tyrosine Determinations: Discriminant Analysis

In order to further characterize genotypes, the phenylalanine and tyrosine values were analyzed using the standard techniques of discriminant analysis (table 2). For discrimination between obligate heterozygotes and nonpregnant controls, the discriminant function was

$$\hat{Z} = 0.024(\mathrm{P}) - 0.017(\mathrm{T}), \qquad (1)$$

where P and T are the phenylalanine and tyrosine concentrations for an individual, 0.024 and −0.017 are estimates of a_1 and a_2, respectively, and $\hat{Z}$ is the value of the discriminant function for an individual. The arithmetic mean and standard deviation of the $\hat{Z}$ values for nonpregnant controls were 0.303 and 0.141, respectively, and for obligate heterozygotes, 1.050 and 0.186, respectively. Based on this discriminant function, individuals with $\hat{Z}$ values greater than 0.529 would be classified as obligate heterozygotes, whereas individuals with $\hat{Z}$ values less than 0.529 would be classified as nonpregnant controls. For the data in this study, the discrim-

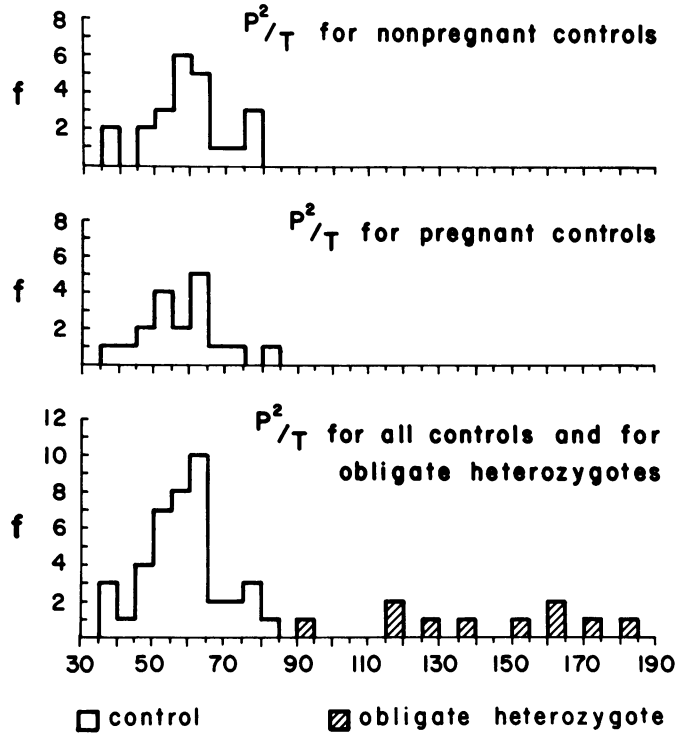

FIG. 1.—Distribution of P^2/T ratio for controls and obligate heterozygotes. The P^2/T is quantitated in μmoles/liter; *f* represents number of individuals.

inant function correctly classified all individuals in these two groups.

For discrimination between obligate heterozygotes and pregnant controls, correct classification was also obtained with the discriminant function

$$\mathcal{Z} = 0.024(P) - 0.014(T), \tag{2}$$

where the terms are defined as in equation (1) above. The mean and standard deviation of the $\mathcal{Z}$ values for the pregnant controls were 0.523 and 0.144, respectively, and for the oblgiate heterozygotes, 1.283 and 0.213, respectively. Individuals were classified as pregnant controls or obligate heterozygotes on the basis of their individual $\mathcal{Z}$ values being less than or greater than 0.794, respectively.

Application in Prenatal Counseling

To test these methods, the parents were first studied. Both techniques of discrimination were then applied to analyze the pedigree (see fig. 2 and table 3). As a result of this analysis, the parents were reassured that they had virtually no risk of having a child affected with classic PKU. Subsequently, a son was born to them who had normal blood phenylalanine values as measured by the Guthrie bacterial inhibition assay [4].

TABLE 2

DIFFERENTIATION BY DISCRIMINANT FUNCTION OF NONPREGNANT CONTROLS AND PREGNANT CONTROLS FROM OBLIGATE HETEROZYGOTES FOR CLASSIC PKU

COHORT GROUP	$\hat{Z}$ VALUE		P
	Mean	SD	
Nonpregnant controls	0.303	0.141	<.001
Obligate heterozygotes	1.050	0.186	
Pregnant controls	0.523	0.144	<.001
Obligate heterozygotes	1.283	0.213	

NOTE.—$\hat{Z}$ values were obtained by comparing two cohort groups computed from the function $\hat{Z} = a_1(P) + a_2(T)$. See equations (1) and (2) in text. *P* values were obtained using Student's *t* test. The number of nonpregnant and pregnant control and obligate heterozygotes studied was 23, 18, and 10, respectively.

DISCUSSION

Our observations indicate that concentrations of plasma tyrosine and phenylalanine in pregnant controls during the eighth to twenty-first weeks of gestation, a critical period for prenatal counseling, are lower than in nonpregnant controls. Both plasma tyrosine and phenylalanine concentrations are lower but tyrosine decreases relatively more. This nonproportional difference elevated the phenylalanine/tyrosine ratio for the pregnant controls causing their distribution to overlap with that for heterozygotes for classic PKU, thus eliminating this simple ratio as a genotypic determinant during pregnancy. Yakmyshyn et al. [11] reached a similar conclusion in their study of 48 pregnant controls during the thirty-sixth to fortieth weeks of gestation. They reported a mean and standard deviation for tyrosine of 40 and 12 μmoles/liter, respectively, in pregnant controls as compared with our mean and standard deviation of 41.1 and 8.4 μmoles/liter (table 1). They also reported a mean and standard deviation for phenylalanine concentrations of 57 and 17 μmoles/liter at term which are similar to our values for nonpregnant controls. Our value of 46.0 μmoles/liter during the early second trimester and those of Cockburn et al. [19] of 37 μmoles/liter at term suggest that pregnancy lowers fasting plasma phenylalanine concentrations. Although the biochemical and physiological mechanisms producing these changes are unknown, the observed fluctuations in plasma phenylalanine and tyrosine during pregnancy indicate that precise control groups of similar gestational age should be used when prenatal heterozygote detection is attempted.

Why was the P^2/T ratio chosen to differentiate pregnant and nonpregnant controls from obligate heterozygotes? Our observations that plasma phenylalanine and tyrosine were lower during pregnancy and that tyrosine fluctuated more relative to phenylalanine suggested that an exponential ratio of phenylalanine and tyrosine would provide a more stable parameter of normality. This empiric approach was supported by the observation of a consistent inverse relationship between the P/T

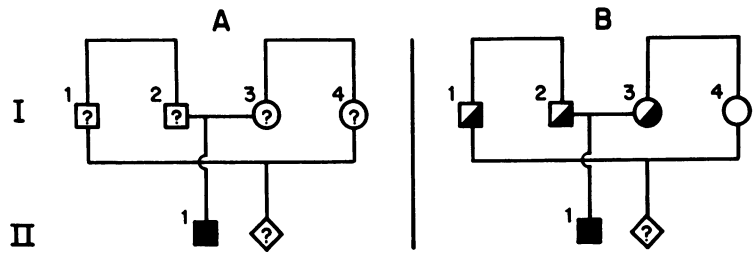

FIG. 2.—Attenuated pedigrees of family Rus before (*A*) and after (*B*) genotyping. See table 3.

TABLE 3

RESULTS OF GENOTYPING

PATIENT	P^2/T (μmoles/liter)	$\hat{z}$	PROPOSED GENOTYPE FOR CLASSIC PKU	PROBABILITY OF MISCLASSIFICATION	
				P^2/T	$\hat{z}$
Uncle (I-1)	143	1.071	Heterozygote	<.001	<.001
Father (I-2)	154	1.147	Heterozygote	<.001	<.001
Mother (I-3)	116	0.880	Heterozygote	<.001	<.001
Aunt (I-4)	62	0.614	Homozygous normal	<.004	<.001

NOTE.—The P^2/T ratio and discriminant function $\hat{z}$ are described in the text. Probability of misclassification was obtained by comparing data from the individual pedigree member (fig. 2) to the appropriate control groups described in tables 1 and 2.

ratio and P. That is, as P/T decreased, P increased. Although other exponents of P, such as P^3 or P^4, could be used, P^2/T proved effective. Normal distributions around identical mean values were found in both pregnant and nonpregnant controls without overlap in the obligate heterozygote group.

A second method of heterozygote detection utilized discriminant analysis. Only two variables, plasma phenylalanine and tyrosine, the precursor and product of the genetically controlled reaction, were used to determine whether heterozygotes would express partial impairment. This method could have included other variables such as age, sex, or prior probability of being a carrier. Since complete discrimination was obtained when either pregnant controls or nonpregnant controls were compared with obligate heterozygotes, the use of these other variables was deemed unnecessary. Why were we able to obtain complete discrimination using only two variables in a linear discriminant analysis when Gold et al. [20] have suggested the need for quadratic functions with multiple variables including prior probability? There are two reasons, both of which reveal the limitations of our method in broader application. First, our subjects were carefully selected so as to obtain obligate heterozygotes from a homogeneous group of pedigrees in which homozygotes expressed the classic PKU phenotype. Therefore, application of our simplified methods of discriminant

analysis ($\mathcal{Z}$) and P^2/T at present may be restricted to heterozygote detection within families of classic PKU patients. Second, the method cannot be used to identify heterozygotes for all forms of hyperphenylalaninemia which may produce mental retardation in the general population, since these disorders are heterogeneous and noncomplementing mutant alleles for variants of hyperphenylalaninemia are probable. Similarly, a variant hyperphenylalaninemia may or may not express a bimodal distribution for $\mathcal{Z}$ and P^2/T. However, in clearly defined pedigrees in which probands have classic PKU and parental P^2/T and $\mathcal{Z}$ values are contained within the normal distribution of heterozygotes for classic PKU, pregnant and nonpregnant relatives can be genotyped and counseled with respect to their risks for having children with classic PKU.

SUMMARY

Methods were developed to enable prenatal counseling of high-risk pregnancies for "classic PKU" by accurately genotyping pregnant and nonpregnant parents. Fasting plasma phenylalanine and tyrosine were quantitated by ion exchange chromatography in three cohort groups: nonpregnant controls, pregnant controls, and obligate heterozygotes. Phenylalanine and tyrosine values were lower in pregnant controls compared with nonpregnant controls. Phenylalanine/tyrosine (P/T) ratios from pregnant controls overlapped with values from obligate heterozygotes. This eliminated use of the P/T ratio as a means of genotyping the pregnant female. However, use of P^2/T values resolved this overlap. Nonpregnant controls, pregnant controls, and obligate heterozygotes had P^2/T values of 57.5, 57.7, and 138 μmoles/liter, respectively. Further classification of genotypes was accomplished by multivariate analysis techniques and the use of a linear discriminant function ($\mathcal{Z}$) relating fasting plasma phenylalanine and tyrosine concentrations. Both approaches were then applied to a counseling problem involving a paternal uncle and maternal aunt of a child with hyperphenylalaninemia who presented for genetic counseling during her eighth week of gestation. Both parents of the proband were classified as heterozygotes for classic PKU by P^2/T and $\mathcal{Z}$ values. The uncle was classified as a heterozygote for classic PKU and the pregnant aunt was classified as a normal. The aunt and uncle were counseled that they had virtually no risk for having a child affected by classic PKU and subsequently gave birth to a normal child with normal neonatal plasma phenylalanine levels. We conclude that both the empiric approach using P^2/T and the discriminant technique provide reliable indications of genotype for counseling pregnant and nonpregnant parents within high-risk pedigrees for classic PKU.

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REFERENCES

1. Følling A: Über Ausscheidung von Phenylbrenztraubensäure in den Harn als Stoffwechsel Anomalie in Verbindung mit Imbezillität. *Z Physiol Chem* 227:169–176, 1934
2. Jervis GA: The genetics of phenylpyruvic oligophrenia. *J Ment Sci* 85:719–762, 1939
3. Rosenberg LE, Scriver CR: Disorders of amino acid metabolism, in *Duncan's Diseases of Metabolism,* 6th ed, edited by Bondy PK, Rosenberg LE, Philadelphia, Saunders, 1969, vol 1, pp 366–515
4. Guthrie R, Susi A: A simple phenylalanine method for detecting phenylketonuria in large populations of newborn infants. *Pediatrics* 32:338–343, 1963
5. Hardy GH: Mendelian proportions in a mixed population. *Science* 28:49–50, 1908
6. Hsia DY, Driscoll KW, Troll W, Knox WE: Detection by phenylalanine tolerance tests of heterozygous carriers of phenylketonuria. *Nature* (Lond) 178:1239–1240, 1956
7. Perry TL, Tischler B, Hansen S, MacDougall L: A simple test for heterozygosity for phenylketonuria. *Clin Chim Acta* 15:47–55, 1967
8. Perry TL, Hansen S, Tischler B, Bunting T: Determination of heterozygosity for phenylketonuria on the amino acid analyzer. *Clin Chim Acta* 18:51–56, 1967
9. Rosenblatt D, Scriver CR: Heterogeneity in genetic control of phenylalanine metabolism in man. *Nature* (Lond) 218:677–678, 1968
10. Jackson SH, Hanley WB, Gero T, Gosse GD: Detection of phenylketonuric heterozygotes. *Clin Chem* 17:538–543, 1971
11. Yakmyshyn LY, Reid DWJ, Campbell DJ: Problems in the biochemical detection of heterozygotes for phenylketonuria. *Clin Biochem* 5:73–81, 1972
12. Berry H, Sutherland BS, Guest GM: Phenylalanine tolerance tests on relatives of phenylketonuric children. *Am J Hum Genet* 9:310–316, 1957
13. Knox WE, Messinger EC: The detection in the heterozygote of the metabolic effect of the recessive gene for phenylketonuria. *Am J Hum Genet* 10:53–60, 1958
14. Anderson JA, Gravem H, Ertel R, Fisch R: Identification of heterozygotes with phenylketonuria on the basis of blood tyrosine responses. *J Pediatr* 61:603–609, 1962
15. Cunningham GC, Day RW, Berman JL, Hsia D: Phenylalanine tolerance tests in families with phenylketonuria and hyperphenylalaninemia. *Am J Dis Child* 117: 626–635, 1969
16. Rampini S, Anders PW, Curtiiuss HC, Marthaler TH: Detection of heterozygotes for phenylketonuria by column chromatography and discriminatory analysis. *Pediatr Res* 3:287–297, 1968
17. Rosenberg LE, Durant JL, Elsas LJ: Familial iminoglycinuria: an inborn error of renal tubular transport. *New Eng J Med* 278:1407–1414, 1968
18. Dixon WJ (ed): *University of California Publications in Automatic Computation, no.2: BMD Biomedical Computer Programs.* Berkeley, Univ. California Press, 1967
19. Cockburn F, Robins SP, Forfar JO: Free amino-acid concentrations in fetal fluids. *Br Med J* 3:747–750, 1970
20. Gold RJM, Maag U, Neal J, Scriver CR: Analysis of biochemical data for genetic screening. *Am J Hum Genet* 24:24a, 1972