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Carrier Screening for Phenylketonuria: Comparison of Two Discriminant Analysis Procedures

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SUMMARY

Absence of a convenient, direct enzyme assay for detecting phenylketonuria (PKU) heterozygotes has resulted in continued effort to develop an accurate and reliable procedure to discriminate the heterozygous individual from the homozygous normal. Our study compares two statistical procedures that combine the semifasting plasma phenylalanine and tyrosine concentrations with the individuals' prior probability of being a heterozygous carrier in order to discriminate carriers from noncarriers. The results of this comparison indicate that the quadratic discriminant function is superior to the linear discriminant function as a method of carrier testing both in theory and in practice. An interactive computer system is described that facilitates the clinical utilization of the quadratic discriminant function.

INTRODUCTION

Phenylketonuria (PKU) is an autosomal recessive disorder. It is a relatively common inborn error of amino acid metabolism, having an incidence in Caucasians of one in 10,000 to one in 20,000 [1]. In the United States, the incidence is reported to be one in 11,000 [2]. Based on an incidence rate of one in 10,000, the calculated gene frequency is .01 and approximately two out of every 100 people are carriers for PKU.

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In classical PKU, the defective enzyme is phenylalanine hydroxylase. A mutation in this enzyme leads to a block in the conversion of phenylalanine to tyrosine. Since the activity of phenylalanine hydroxylase is not easily measured in tissue other than liver, any direct assay of its activity requires a liver biopsy [2]. For this reason, clinically convenient tests for heterozygosity must assess the enzyme's activity indirectly. Since phenylalanine and tyrosine are the enzyme's substrate and product, respectively, and since their blood levels are easily obtained and quantified, they have been used as a means of discriminating between homozygous normals and heterozygous carriers [3–10].

A number of methods and statistical procedures have been proposed and investigated in an attempt to develop an accurate test for assessment of carrier status. Griffin and Elsas [8] and Paul et al. [10] reported success in distinguishing these two populations by using a linear discriminant function (LDF). Griffin and Elsas utilized phenylalanine and tyrosine in their LDF. Paul et al. used tryptophan, in addition to phenylalanine and tyrosine, and reported a better separation between the heterozygous carriers and the homozygous normals. Westwood and Raine [6] proposed using the heterozygote likelihood ratio (HLR), which was the ratio of the probability density functions of the heterozygous and homozygous distribution for the given test result. This value, when corrected for an individual's prior probability, gave the posterior probability of an individual being a carrier. They reported that the best discriminant test was either plasma phenylalanine level determined 4 hrs after a phenylalanine load or the logarithmic transformation of this level. Gold et al. [7] proposed a method of heterozygote identification based on a quadratic discriminant function (QDF) derived from application of Bayes' theorem to probability density functions.

Over the past 4 years, the University of Colorado Health Sciences Center provided PKU carrier screening utilizing an LDF similar to that of Griffin and Elsas [8]. This function was then corrected for the individual's prior probability of being a carrier. This paper presents the results of using this method and compares it to a Quadratic Discriminant method for heterozygote screening. The classification results of the two methods are compared and discussed along with the feasibility of implementing these procedures in a clinical setting.

Our goal in this investigation was to determine which procedure, the LDF or the QDF, would best discriminate between heterozygous carriers and homozygous normals, and then to implement this procedure in our clinical environment.

SUBJECTS AND METHODS

Study Group

The study group consisted of 159 individuals who had been tested for PKU carrier status at the University of Colorado Health Sciences Center. Criteria for acceptance into the study group mandated that the subject's age was greater than 16 years and that none of the females were on oral birth-control medication, or pregnant, or less than 2 months postpartum. This criteria was selected because age, oral birth-control medication, and pregnancy alter phenylalanine and tyrosine metabolism [11–14]. From the study group, a reference group consisting of 122 subjects was used for determination of the reference statistics. This reference group consisted of two separate populations: 63 obligate hetero-

zygous carriers and 59 homozygous normal controls. Obligate heterozygous carriers were defined as those subjects who had a child with known classical PKU. For the purpose of this project, homozygous normal controls were defined a priori as those subjects who had no clinical symptoms or family history of PKU, excluding those whose plasma phenylalanine values differed significantly (> 2 SD) from the control group, as long as the number of these subjects did not differ from the frequency of expected heterozygotes. Three individuals who were originally thought to be homozygous normal were excluded from the reference group since their phenylalanine values were much greater (> 3 SD) than the homozygous normal reference value. Since 2% of the "normal" population is at risk of being a carrier, 1.24 would be the expected frequency of carriers in a population of 62 individuals with no family history of PKU; this observed carrier frequency does not differ from the expected value of 1.24 according to Fisher's exact test.

Specimen Collection and Sample Preparation

Specimens were obtained from subjects who were instructed to eat a full, protein breakfast sometime between 6 A.M. and 9 A.M. No food or fluid was to be consumed after 9 A.M. except water. Two to 3 ml of venous blood were collected in heparinized tubes (Becton-Dickinson, Rutherford, N.J.) between 11:45 A.M. and 12:15 P.M. of that same day [15]. Specimens were centrifuged to separate plasma from cells within ½ hour after being drawn, and the plasma was frozen immediately. Plasma phenylalanine and tyrosine concentrations were determined by ion-exchange chromatography using a Beckman 121M amino acid analyzer.

Study Design

The Tandem-NS/II computer system was utilized for the collection, storage, and analysis of data on patients referred to the University of Colorado Health Sciences Center Regional Inherited Metabolic Diseases Clinic. The data-flow diagram (fig. 1) describes the interactive system currently in use at the University of Colorado School of Medicine. The design illustrates the processes that are executed for analysis of new subjects and updating the statistics files of the two reference populations. The system includes: data entry and data review, creation of special reports, updating statistical files, and evaluating new subjects' PKU carrier status. Special reporting processes allow for exploratory data analysis, generation of descriptive statistics, and reviewing family pedigree studies.

Laboratory values for subjects who meet the requirements for the heterozygous carriers and the homozygous normal controls are used to update the reference statistics whenever determination of their carrier status is made. The process of classifying new subjects uses these reference statistics as input to the discriminant function. The system allows for both interactive analysis of a single patient or batch processing of many patients at a single request. The design facilitates continuous collection of carrier and noncarrier data for future carrier testing, as well as immediate determination of a subject's calculated carrier status.

Linear Discriminant Procedure

The LDF that maximally differentiated between obligate heterozygotes and homozygous normals for us was determined according to the methods developed by Fisher [16]. This approach assumed that each individual to be evaluated had a prior probability of 50% of being a carrier. The LDF classification criteria was: assign an individual to the carrier group if: $0.154\ P - 0.101\ T > 4.93$, and to the noncarrier group otherwise; where P is the phenylalanine level and T is the tyrosine level. This function was then modified to account for an individual's prior probability of being a carrier [17]. An estimate of the posterior probablity that an individual was a carrier of PKU given the laboratory results was given by

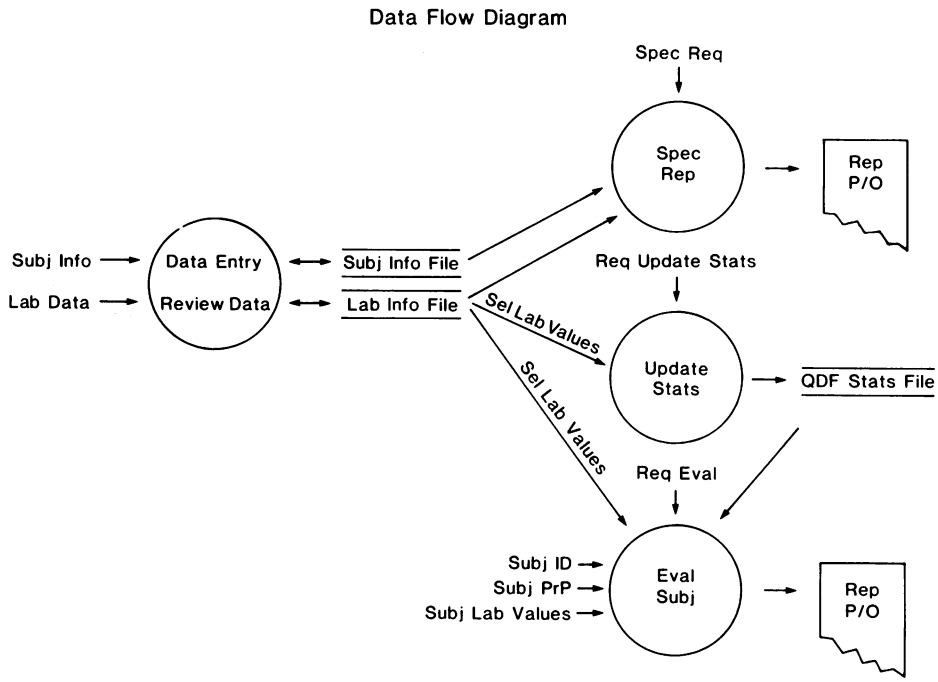

FIG. 1.—Flow diagram representing the interactive system used for PKU carrier screening. *Circles* represent user-initiated computer processes, and *arrows* represent data flow.

$$\Pr(\text{Carrier/P, T}) = \frac{1}{1 + \left(\frac{1-q}{q}\right) e^{20.3-0.633P+0.415T}}$$

where Pr is the posterior probability of being a carrier, q is the prior probability of being a carrier, P is the phenylalanine level, and T is the tyrosine level. Three assumptions are required: (1) the individual's prior probability is accurately determined, (2) the two variables (phenylalanine and tyrosine) have a bivariate normal distribution for each of the two populations, and (3) the two population covariance matrices are equal.

To facilitate implementation of the classification procedure in our clinical setting during the period when the LDF was in use, graphs of phenylalanine vs. tyrosine levels were generated for the four most common prior probabilities: 1.00, .67, .50, and .02. An individual's phenylalanine and tyrosine levels were plotted on the appropriate graph allowing for an approximate posterior probability to be determined.

Quadratic Discriminant Procedure

A quadratic discriminant function (QDF) similar to that outlined by Gold et al. [7] in 1974 was implemented at the University of Colorado Health Sciences Center in the spring of 1982. The specific method used was first introduced by Fisher and is discussed in detail in the text by Morrison [16]. Estimation of an individual's posterior probability of being in each of the two groups was based on an individual's prior probability of being a carrier and on the inherent correlation structure of the phenylalanine and the tyrosine values. The two assumptions required for this approach are: (1) the individual's prior probability

is accurately determined, and (2) the two variables (phenylalanine and tyrosine) have a bivariate normal distribution for each of the two populations.

In the clinical setting, the data are entered into the interactive computer system and a printout and output of the patient's posterior probability are obtained. The individual is then counseled regarding his or her probability of being a carrier for PKU.

Classification Procedure

For the purposes of this report, in order to evaluate and compare the classification results of the two discriminatory procedures, an individual was classified as a heterozygous carrier if the calculated posterior probability of being a heterozygote carrier was greater than the posterior probability of being homozygous normal. In practice, however, an individual is given the calculated posterior probability rather than being defined as a "carrier" or "noncarrier."

RESULTS

Table 1 gives the descriptive statistics of the two populations in the reference group. The mean phenylalanine values of the obligate heterozygotes and the homozygote normals were significantly different ($P < .001$) based on the t-test for independent samples with unequal variances [18]. The mean tyrosine levels of the obligate heterozygotes and the homozygous normals were also significantly different ($P < .02$). Examination of the equality of the two covariance matrices indicated that the matrices were significantly different ($P < .001$) based on the test statistic developed by Box [16].

The difference between the mean phenylalanine and tyrosine values of the obligate heterozygotes and the homozygous normals confirmed that it was appropriate to consider the use of these two variables in the discriminant functions. The significant difference between the covariance matrices of the two populations was contrary to an assumption underlying the LDF as a method for classifying individuals and motivated us to investigate the QDF.

Expected heterozygote frequency, misclassification rates, and family pedigrees were used to assess the classification capabilities of the two approaches when applied at our center. Table 2 lists the results of classifying subsets of the entire data set by the two methods. Individuals were grouped according to their prior probability of being a carrier for PKU. Only three groups, with prior probabilities of 1.00, .50, and .02, contained sufficient numbers of individuals to allow for comparison of the two functions. Individuals with prior probability of 1.00 were obligate heterozygotes; thus, all 63 subjects should be classified as heterozygous. The LDF misclassified five individuals, while the QDF correctly classified each

TABLE 1

PLASMA PHENYLALANINE AND TYROSINE VALUES IN THE REFERENCE GROUP

	No. cases	Phenylalanine (nmol/ml)		Tyrosine (nmol/ml)	
		Mean	SD	Mean	SD
Obligate heterozygotes	63	87.5	15.1	61.9	15.2
Homozygous normals	59	54.4	6.2	56.1	10.3

TABLE 2

COMPARISON OF CLASSIFICATION RESULTS IN SUBSETS OF THE ENTIRE DATA SET WHEN USING THE LDF AND THE QDF METHOD

Prior probability	No. subjects	No. heterozygotes classified by LDF	No. heterozygotes classified by QDF	Expected no. heterozygotes
1.00	63	58	63	63
.67	2	2	2	1.34
.50	28	12	16	14
.25	1	1	1	0.25
.13	2	0	0	0.26
.06	1	0	0	0.06
.02	62	0	3	1.24

subject. Of the 28 individuals tested having a prior probability of .50, 14 would be expected to be heterozygotes. The LDF classified 12 as heterozygotes, and the QDF classified 16 as heterozygotes. Of the 62 individuals having a prior probability of .02, 1.24 would be expected to be heterozygotes. With the LDF, no one was classified as a heterozygote; three individuals were classified as heterozygotes by the QDF. None of these differences from the expected numbers were statistically significant using Fisher's exact test.

Misclassification rates are summarized in tables 3 and 4. Table 3 shows the results obtained with the LDF, illustrating that 5/122 individuals were misclassified. Table 4 shows that 0/122 individuals were misclassified by the QDF approach. Sensitivity, the percentage of heterozygous individuals who test positive, and specificity, the percentage of homozygous normal individuals who test negative, were determined to be 92% and 100%, respectively, for the LDF. By the QDF method, both sensitivity and specificity were 100%.

Genotyping of family pedigrees was also used to assess the clinical performance of the two methods. Figure 2 illustrates the results of genotyping by both classification methods for pedigree 1. Individual IV-4, the proband, has been diagnosed as having classical PKU. The LDF method failed to classify this individual's mother, III-6, as a carrier. Classification of the other family members by the LDF followed the expected autosomal recessive inheritance patterns. The QDF, however, classified both paternal grandparents, II-1 and II-2, as heterozygous carriers (using a prior probability of .50). Although this is possible, it is highly

TABLE 3

REFERENCE-GROUP MISCLASSIFICATION RATE OBTAINED WHEN USING THE LDF METHOD

REFERENCE-GROUP CLASSIFICATION	LDF CLASSIFICATION		TOTAL
	Heterozygous carrier	Homozygous normal	
Heterozygous carrier	58	5	63
Homozygous normal	0	59	59
Total	58	64	122

NOTE: Misclassification ratio = 5/122, sensitivity: 58/63 = 92%, specificity: 59/59 = 100%.

TABLE 4

REFERENCE-GROUP MISCLASSIFICATION RATE OBTAINED WHEN USING THE QDF METHOD

REFERENCE-GROUP CLASSIFICATION	QDF CLASSIFICATION		TOTAL
	Heterozygous carrier	Homozygous normal	
Heterozygous carrier	63	0	63
Homozygous normal	0	59	59
Total	63	59	122

NOTE: Misclassification ratio = 0/122, sensitivity: 63/63 = 100%, specificity: 59/59 = 100%.

unlikely. Recalculation of these two individuals' status using the QDF with a prior probability of .02 resulted in classification of the paternal grandfather, II-1, as homozygous normal. This is in agreement with the results obtained using the LDF. The other individual of interest, IV-3, was classified as homozygous normal by the LDF, but as heterozygous by the QDF. This individual was less than 16 years old at the time of testing, and, therefore, differs from the reference group.

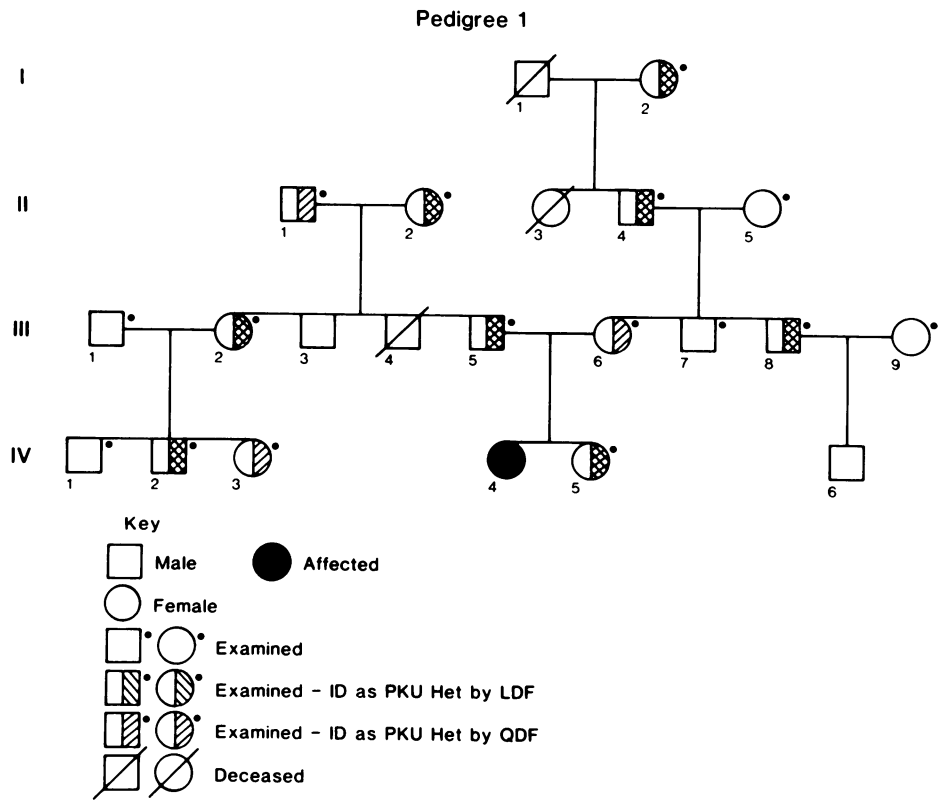

FIG. 2.—Comparison of LDF and QDF for genotyping pedigree 1

Figure 3 illustrates the results of the LDF and QDF classification in pedigree 2. Both classification results followed an autosomal recessive pattern, and the LDF and the QDF classifications agree except for individual II-8. No additional information concerning this individual's PKU carrier status has been obtained. Both individuals II-4 and II-7 were pregnant at the time of carrier testing; thus, they differ from the reference group.

DISCUSSION

A study group consisting of 159 individuals was selected from those individuals who sought or volunteered for PKU carrier testing at the University of Colorado Health Sciences Center from 1978 to 1982. Selection criteria required the subjects to be greater than 16 years old and none of the females to be on oral birth-control medication, or pregnant, or less than 2 months postpartum. From this study group, 63 obligate heterozygotes and 59 homozygous normals were used as a reference group for determination of the population parameters for the discriminatory functions.

The two populations in the reference group, the obligate heterozygotes and homozygous normals, were shown to have unequal mean phenylalanine and tyrosine values. The two populations were also shown to have unequal covariance matrices. The statistically significant differences between the mean phenylalanine and tyrosine values of these two groups established the usefulness of these two variables in the discriminant function. The statistically significant differences between the covariance matrices of the two populations suggested that the LDF should not

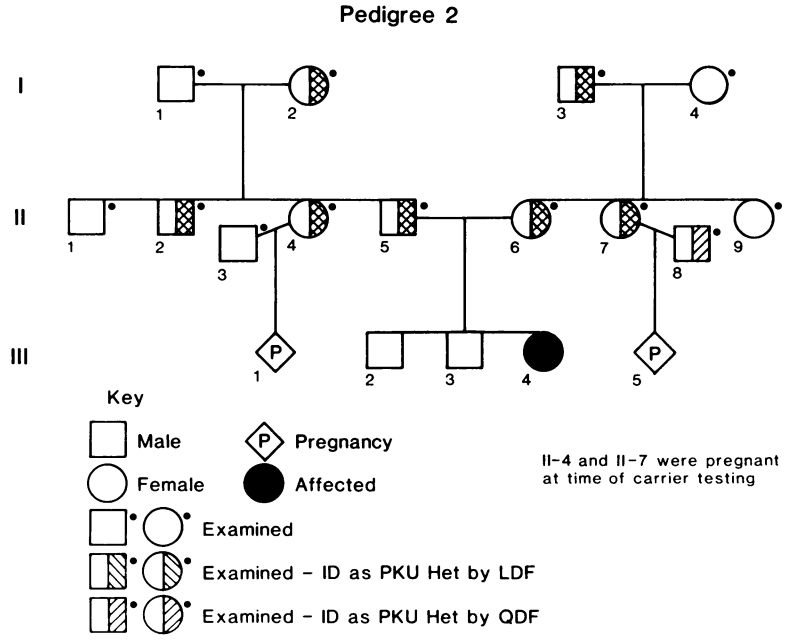

FIG. 3.—Comparison of LDF and QDF for genotyping pedigree 2

be used as the method for determining posterior probabilities, but that the QDF should be used. The LDF was statistically inappropriate in that an assumption of equal covariance matrices was required for its determination. This assumption was not required for the determination of the QDF.

Comparison of the classification results for the two methods provided empirical evidence supporting the appropriateness of the QDF method. The LDF tended to underestimate the number of expected heterozygotes when the entire data set was evaluated. It had a reference-group misclassification ratio of 5/122, a sensitivity of 92%, and a specificity of 100%. In pedigree 1, the LDF misclassified the proband's mother as homozygous normal.

The QDF, on the other hand, was more conservative. The QDF tended to overestimate the number of expected heterozygotes when the entire data set was evaluated. It had a reference-group misclassification ratio of 0/100 and a sensitivity and specificity of 100%. While it is probable that the QDF misclassified the proband's paternal grandfather in pedigree 1, the error (a false positive) is more tolerable than a false negative.

These results suggest that the QDF procedure classifies carriers and noncarriers more proficiently than does the LDF procedure. Clinically, the QDF procedure is a better method of carrier screening because of its superiority in identifying heterozygous individuals without creating a substantial increase in false positives.

Previously, use of the QDF was not feasible because of the limited availability of computers required to facilitate its application [10]. Use of the LDF procedure provided an alternative method—simple tables or graphs could be generated. These tables or graphs were readily implemented in the clinical setting, allowing for easy classification. Today, however, our approach is not limited by computer availability. Use of the LDF without meeting the requirements of equal covariance matrices results in loss of discriminatory power, reducing the usefulness of the carrier-screening procedure.

As illustrated in the flow diagram (fig. 1), an individual's name, phenylalanine and tyrosine levels, and prior probability are entered into the computer. The individual's posterior probability is determined by the QDF procedure based on the reference-group statistics. The data can then be entered into the data set to be stored and used for further studies and to update the reference-group statistics if the individual meets the reference-group requirements. The accessibility of computers makes implementation of the QDF method available to other clinics and investigators. It is recommended, however, that each individual clinical setting develop its own reference-group statistics [8, 10].

In our clinical application, the results are not provided in a binary fashion (carrier vs. noncarrier), but a probability counseling approach is employed. The individual is given the calculated posterior probability value. Explanation of this value is provided in an attempt to make the value more meaningful to the individual. The consequences of having an affected child are discussed in an attempt to provide the individual with a better perception of issues involved. It is hoped that in doing this individuals will be able to use this information in formulating their subsequent reproductive decisions.

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REFERENCES

1. NORA JJ, FRASER FC: *Medical Genetics: Principles, and Practice*. Philadelphia, Lea & Febiger, 1981
2. STANBURY JB, WYNGAARDEN JB, FREDRICKSON DS, GOLDSTEIN JL, BROWN MS, EDS: *The Metabolic Basis of Inherited Disease*, 6th ed. New York, McGraw-Hill, 1983
3. PERRY TL, TISCHLER B, HANSEN S, MACDOUGALL L: A simple test for heterozygosity for phenylketonuria. *Clin Chim Acta* 15:47–55, 1967
4. ROSENBLATT D, SCRIVER CR: Heterogeneity in genetic control on phenylalanine metabolism in man. *Nature* 218:677–678, 1968
5. JACKSON SH, HANLEY WB, GERO T, GOSSE GD: Detection of phenylketonuric heterozygotes. *Clin Chem* 17:538–543, 1971
6. WESTWOOD A, RAINE DN: Heterozygote detection in phenylketonuria: measurement of discriminatory abililty and interpretation of the phenylalanine loading test by determination of the heterozygote likelihood ratio. *J Med Genet* 25:646–654, 1973
7. GOLD RJM, MAAG UR, NEAL JL, SCRIVER CR: The use of biochemical data in screening for mutant alleles and in genetic counselling. *Ann Hum Genet* 37:315–326, 1974
8. GRIFFIN RF, ELSAS LJ: Classic phenylketonuria: diagnosis through heterozygote detection. *J Pediatr* 86:512–517, 1975
9. PERRY TL, HANSEN S, TISCHLER B, BUNTING T: Determination of heterozygosity for phenylketonuria on the amino acid analyzer. *Clin Chim Acta* 18:51–56, 1976
10. PAUL TD, BRANDT IK, ELSAS LJ, ET AL.: Phenylketonuria heterozygote detection in families with affected children. *Am J Hum Genet* 30:293–301, 1978
11. CABALSKA B, NOWACZEWSKA I, DUCZYNSKA N: Plasma amino acid patterns in phenylketonuric heterozygotes during pregnancy. *Biochem Med* 27:269–277, 1982
12. CUNNINGHAM GC, DAY RW, BERMAN JL, DAVID YH: Phenylalanine tolerance tests in families with phenylketonuria and hyperphenylalaninemia. *Am J Dis Child* 117:626–635, 1969
13. ROSE DP, CRAMP DG: Reduction of plasma tyrosine by oral contraceptives and oestrogens: a possible consequence of tyrosine aminotransferase induction. *Clin Chim Acta* 29:49–53, 1970
14. YAKYMYSHYN LY, REID DWJ, CAMPBELL DJ: Problems in biochemical detection of heterozygotes for phenylketonuria. *Clin Biochem* 5:73–81, 1972
15. SCRIVER CR, ROSENBERG LE: *Amino Acid Metabolism and Its Disorders*. Philadelphia, W. B. Saunders, 1973
16. MORRISON DF: *Multivariate Statistical Methods*. New York, McGraw-Hill, 1967
17. AFIFI AA, AZEN SP: *Statistical Analysis, A Computer Oriented Approach*. New York, Academic Press, 1977
18. ROSNER B: *Fundamentals of Biostatistics*. Boston, Duxbury Press, 1982