The Genetic Epidemiology of Hyperphenylalaninemia in Québec

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Abstract

The province of Québec screens for autosomal recessive phenylketonuria (PKU) and other forms of hyperphenylalaninemia due to phenylalanine hydroxylase deficiency and tetrahydrobiopterin variants in newborns. A review of the annual reports of the Québec Newborn Screening Program and of the clinical files of individuals with hyperphenylalaninemia born in Québec since 1970 was undertaken. The Newborn Screening Program was evaluated for its ability to detect and identify individuals with hyperphenylalaninemia, to characterize their phenotype, and to continue surveillance. Less than universal participation in the screening (98.6%) and loss to follow-up of individuals not on treatment are causes for concern in the context of maternal hyperphenylalaninemia. Characteristics of individuals with PKU or non-PKU HPA including ethnicity, age at screening test, administrative region of birth, and month of birth were analyzed.

Résumé

La province de Quebec fait le dépistage de la phénylcétonurie autosomal récessive (PKU) et d'autres formes d'hyperphénylalaninémies (HPA) causées par une déficience en phénylalanine hydroxylase et par les variantes de la tétrahydrobioptérine chez les nouveaux-nés. Une révision des rapports annuels du Programme de Dépistage des Nouveaux-nés du Québec et des fichiers cliniques d'individus souffrant d'hyperphénylalaninémie nés depuis 1970 au Québec a été entreprise. Le Programme de Dépistage des Nouveaux-nés a été évalué pour son aptitude à détecter et identifier les individus souffrant d'hyperphénylalaninémie, à charactériser leur phénotype et à maintenir une surveillance. Une participation moins qu'universelle à ce programme de dépistage (98.6%) et un manque de suivi des individus n'étant pas traités sont une source d'inquiétude dans ce contexte d'hyperphénylalaninémie maternelle. Les charactéristiques des ces individus souffrant de PKU ou de non-PKU HPA tel que le groupe ethnique, l'âge au moment du test de dépistage, la région administrative de la naissance et le mois de naissance ont été analysés.

Acknowledgements

Data for this study were collected with the assistance of many people. Information about the Québec Newborn Screening Program and access to its annual reports were provided by Ms. Nicole Belanger, Mr. Pierre Fiset and Dr. Jean Morrisette of the Service Informatique of the Reseau de Médécine Génétique du Québec (RMGQ) located at Centre Hospitalier de l'Université de Laval (CHUL). Collection of data from the Newborn Screening Program annual reports was done by myself and Ms. Robyn McClelland. Additional information about the Newborn Screening Program was obtained from Mr. André Grenier, also of the RMGQ. Data sets held by the RMGQ containing information about newborn screening in the province were retrieved and prepared for my use by Mr. Fiset, who also provided a copy of the registry of individuals with PKU or non-HPA PKU held by the RMGQ.

A review of clinical files of individuals with PKU or non-PKU HPA was performed by myself with the help of the staff at the four metabolic clinical centres. At the Montréal Children's Hospital, patients with HPA were identified for me by Mrs. Carol Clow. After the file review was completed, additional information was obtained from Dr. Charles Scriver and Mrs. Clow. At Centre Hospitalier de l'Université de Sherbrooke (CHUS), data collection sheets were filled out by Mrs. Ginette Plourde to maintain the confidentiality of patient files. The data collection forms from CHUS were then reviewed with Dr. Bernard Lemieux and Mrs. Ginette Plourde. At CHUL, data collection sheets were completed by Mr. Gilles Doucet to maintain confidentiality. At Hôpital Sainte Justine, data collection forms were completed by myself and then reviewed with Dr.

Marie Lambert. Additional information, when not available from the clinical centres, was obtained from Mr. Doucet at CHUL in his capacity as organizer of the registry of affected individuals for the province.

Blood samples for assessment of phenylalanine values of adult volunteers were taken by Mrs. Annie Capua of the Biochemical Genetics Unit in the Department of Medical Genetics at the Montréal Children's Hospital. Column chromatography for phenylalanine measurement was done by Miss Keo Phoumarinh of the Biochemical Genetics unit of the Department of Medical Genetics at the Montréal Children's Hospital.

Information about follow-up of affected individuals at the Montréal Children's Hospital was provided by Mr. John Mitchell of the Division of Biochemical Genetics.

Genotypes of individuals with PKU or non-PKU HPA were obtained from the laboratory of Dr. Scriver of the Division of Biochemical Genetics at the Montréal Children's Hospital, where genotyping of individuals with PKU or non-PKU HPA is done.

Data about the mean monthly humidity recorded at the Dorval weather station was obtained from Mr. Roger Gauthier, of Climate Services at Environment Canada in St Laurent, Québec.

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Chapter 1: Abbreviations and Definitions

Elevation of blood phenylalanine is defined in many ways. Ledley (1991) lists 19 ways to describe an elevation of blood phenylalanine and has not exhausted all possibilities. The following definitions, as defined in Scriver et al. (1994), are used in this thesis, both in the review of the literature and in the thesis work itself.

Hyperphenylalaninemia (HPA): An elevation of blood phenylalanine beyond the normal range, i.e. above 150 µmol/L.

***Phenylalanine hydroxylase* (PAH)**: The enzyme which converts phenylalanine to tyrosine, phenylalanine's main metabolic reaction. Italicized letters *PAH* indicate the phenylalanine hydroxylase gene; capitalized PAH indicates the enzyme (product of the gene).

***Tetrahydrobiopterin* (BH_4)**: A necessary catalytic cofactor for the phenylalanine hydroxylation reaction. It has one pathway for synthesis and another to recycle catalytic amounts for the hydroxylation reaction.

Phenylketonuria (PKU): A persistent elevation of blood phenylalanine above 1,200 µmol/L, caused by deficient activity of phenylalanine hydroxylase enzyme, with a dietary tolerance of phenylalanine of less than 500 mg/day to normalize phenylalanine homeostasis.

Non-PKU hyperphenylalaninemia (non-PKU HPA): A persistent elevation of blood phenylalanine above 150 µmol/L but below 1,200 µmol/L, caused by deficiency of phenylalanine hydroxylase enzyme, with a dietary tolerance for phenylalanine of more than 500 mg/day to normalize phenylalanine homeostasis.

Non-PAH hyperphenylalaninemia (non-PAH HPA): Persistent HPA caused by a deficiency of an enzyme other than phenylalanine hydroxylase. Blood phenylalanine does not respond to simple alteration of dietary phenylalanine, but requires BH_4 replacement.

Transient HPA: HPA in the newborn period which resolves spontaneously over the course of several days to several years.

Chapter 2: Introduction

The context of this thesis is as follows. The province of Québec has screened for phenylketonuria (PKU) and other forms of HPA due to PAH deficiency and BH_4 variants in newborns since 1970. A review was undertaken of the annual reports of the Québec Newborn Screening Program and of the clinic files of individuals with HPA born in Québec since the start of screening in the province. The Newborn Screening Program was evaluated for its ability to detect and identify individuals with HPA, to characterize their phenotype and to continue surveillance of these individuals. Screening test results and demographic characteristics of individuals with HPA were analyzed. Follow-up of identified individuals was evaluated. Less than universal participation in screening and loss to follow-up of individuals not on treatment are causes for concern in the context of maternal HPA.

PKU is a Mendelian (autosomal recessive) metabolic disease characterized by an excess of blood phenylalanine that, if untreated, manifests itself as severe mental retardation and behavioral problems. The mental retardation caused by HPA is preventable if treated by an appropriate, early restriction of dietary phenylalanine. The evidence that PKU patients could benefit from dietary therapy led to the development of newborn screening programs for pre-symptomatic detection and treatment. Widespread screening led to the detection of infants with non-PKU HPA, who did not develop mental retardation in the absence of treatment, and infants with a transient elevation in phenylalanine value (transient HPA) which decreased spontaneously over periods ranging from several days to several years, with no apparent lasting physiological effects.

PKU is considered by some to be a paradigm for treatable genetic disease: an enzyme deficiency is defined, screening detects enzyme-deficient infants in time for successful dietary therapy, and the disease phenotype is prevented by environmental manipulation. The success of such a system depends on two factors: the ability to recognize individuals with PKU and other forms of HPA; and the ability to ensure that these individuals are followed and receive treatment for their condition.

This study examines the epidemiology of HPA in Québec on many levels, from population to molecular.

The Québec Newborn Screening Program serves the entire province of Québec which currently has approximately 90,000 births annually. Analysis, reporting and monitoring of blood phenylalanine of newborn infants is centralized in Sainte-Foy, Québec at the Centre Hospitalier de l'Université de Laval. Since 1973, the screening program has tabulated the number of infants screened and the number of infants with abnormal screening test results, and has kept a sample of records of newborn infants screened containing demographic information and screening test results. This documentation allows investigation of two aspects of the screening process. The percentage of the population which has been screened; and the number and quality of samples received by the Newborn Screening Program.

Records of phenylalanine values of normal screened infants and annual report data were used to examine test results of unaffected infants. The proportion of samples above the threshold by year and by region was analyzed. As well, phenylalanine values of normal infants were analyzed with respect to such demographic variables as age at

screening, gender, and year. Phenylalanine values of individuals with HPA were taken from the registry of individuals with PKU or non-PKU HPA kept by the Newborn Screening Program as well as from clinic file review. Initial (newborn) phenylalanine values for these individuals were analyzed by gender and age at screening.

File review for individuals with PKU or non-PKU HPA was instrumental in examining the demographic characteristics of these individuals. The classification of individuals with PKU or non-PKU HPA is discussed and the two sources of classification data are compared. Incidence of PKU and non-PKU HPA are estimated. The distributions of such demographic variables as gender, year of birth, month of birth, age at screening and ethnicity are analyzed for individuals with PKU or non-PKU HPA. The distributions of age at screening and gender for normal screened infants are examined in comparison to those of affected individuals.

Follow-up of cases is relevant. Pregnant females with persistent (maternal) HPA give birth to offspring with increased incidence of microcephaly and congenital birth defects, including heart defects and growth retardation, corresponding to the blood phenylalanine levels of the mother during pregnancy. Preconception to intrapartum treatment of mothers by restriction of dietary phenylalanine greatly reduces the risk of fetal morbidity. The follow-up of individuals (especially females) with HPA was evaluated for clinics' ability to maintain contact with these individuals until reproductive age. Diagnostic indicators from beyond the newborn period of individuals with HPA were examined by correlation of their original (newborn) phenotype to their phenotype later in life; and correlation of initial phenylalanine phenotype to genotype.

This thesis describes the genetic epidemiology of hyperphenylalaninemia with respect to the five levels outlined above: population, screening, test results, individuals and follow-up; from population to molecule. Methods, results, discussion and the appendices are structured in a similar fashion, to assess screening for hyperphenylalaninemias in the province o 'Québec at all levels.

Chapter 3: Review of the Literature

3.1: Background

While the detection and treatment of PKU and other hyperphenylalaninemias has been a public health concern for close to fifty years, genetics is only now describing the severity and distributions of different forms of HPA. This review of the literature describes the background necessary for understanding PKU and non-PKU HPA in a public health and genetic context, with particular emphasis on screening.

3.1.1: Biochemistry and genetics of hyperphenylalaninemia

The following description of phenylalanine homeostasis was taken from Scriver et al. (1989). HPA is caused by an interruption of the conversion of phenylalanine to tyrosine by hydroxylation by the phenylalanine hydroxylase (PAH) enzyme. If blood phenylalanine concentration is above 500 μmol/L, transamination of phenylalanine occurs to form phenylpyruvate. One to three percent of individuals with HPA have no PAH enzyme defect, but instead have a defect in the production of tetrahydrobiopterin (BH_4). BH_4 deficiency is caused by a deficiency of an enzyme required to synthesize BH_4 (guanosine triphosphate cyclohydrolase (GTPCH)) or 6-pyruvoyl tetrahydropterin synthetase (6-PTPS), or by deficiency of the enzyme required to recycle BH_2 to its active form (BH_4) (dihydropteridine reductase (DHPR) or pterin 4-A-carbinolamine dehydratase (PCD)). Disorders of GTPCH, 6-PTPS and DHPR are severe ("malignant" HPA). Deficiency of pterin 4-A-carbinolamine dehydratase (primapterinuria) is milder and apparently transient (Citron et al., 1993).

The gene for PAH is located in the chromosome region 12q22 to 12q24.1 (Scriver et al., 1989). A search of Online Mendelian Inheritance in Man (McKusick, 1993) shows a total of 53 known mutations at the PAH locus. The PAH Mutation Analysis Consortium database contains over 170 mutations (May, 1994). Different mutations cause different levels of PAH enzyme expression, from no PAH enzyme (null allele) to relatively mild losses of PAH activity. The number of different mutations in the PAH gene results in most individuals with non-PKU HPA being compound heterozygotes at the PAH locus and thus different combinations of enzyme levels show varying phenotypes.

PKU and non-PKU HPA show variation in incidence and mutations among populations. Incidence of PKU is highest in Turkey, as well as Scotland and Ireland (1 / 5,263) and lowest in Finland, Japan, and among Ashkenazi Jews (1 / 200,000) (Scriver et al., 1989). Non-PKU HPA also shows a variation in incidence, but the reported incidences depend greatly on what is defined as non-PKU HPA in each population. Non-PKU HPA is detected only by screening, so estimated incidence of this condition depends upon the screening thresholds which are the levels of blood phenylalanine above which individuals are considered to have non-PKU HPA or PKU.

The diversity of mutations in different populations and associations of common mutations with particular RFLP haplotypes in different populations has led to the conclusion that PKU and non-HPA PKU had multiple origins in different populations (John et al., 1990).

3.2: Screening for hyperphenylalaninemia

Several criteria are employed for deciding whether a disease is a worthwhile candidate for screening. The following criteria apply to screen a candidate disease (Fox, 1987).

1) The screening test must be inexpensive and easy to perform.

2) Screening must be available to the entire population.

3) Samples for screening must be easy to collect and transport.

4) Sensitivity of screening must be 100%, while maintaining an acceptable specificity (the screening must be valid as a diagnostic test).

5) The screening test must be cost-beneficial. That is, the untreated illness must have a high enough incidence or be expensive enough when undetected so as to exceed the cost of screening and treatment.

6) Services must be available for follow-up, treatment, and management of the disease identified by screening.

7) There must be acceptable, effective and inexpensive treatment or therapy for prevention or reduction of severity of the untreated illness.

Screening for HPA on blood samples meets these criteria and others (NAS, 1975).

3.2.1: The screening test (for HPA)

Testing for PKU was first done through assay of urinary metabolites of phenylalanine. Fölling's original ferric chloride test for detection of phenylpyruvate (a product of the transamination of phenylalanine) in urine (Güttler, 1984) was adapted in the Phenistix test for elevated urine phenylpyruvic acid, paper chromatography test for

urinary o-hydrophenyl-acetic acid and in a microbiological test for urinary phenylalanine (Moncrief et al., 1968). In comparison to procedures which screened blood phenylalanine, urine tests missed cases of PKU, especially if used in the first week of life (Moncrief et al., 1968). Infants were missed by urine screening because it was dependent on transamination, and either the blood phenylalanine was not sufficiently elevated or the enzyme activity was insufficient in the newborn. Evaluation of blood phenylalanine values proved to be a more reliable means of detecting individuals with PKU and other forms of HPA to the extent that only one in seventy infants with PKU are missed by the screening program (Holtzman et al., 1986).

Screening is currently done by microbiologic inhibition assay (Guthrie test), fluorometry or chromatography (Scriver et al., 1989). Both the Guthrie test and fluorometry (the important screening methods) are easily adapted to process large numbers of samples efficiently. Fluorometry measures the phenylalanine value "down to zero", while the Guthrie test only measures phenylalanine above a pre-set threshold (usually 250 μmol/L). Blood samples for phenylalanine analysis by Guthrie, fluorometric or chromatographic methods can be analyzed as a liquid or when dried onto filter paper (Scriver et al., 1989). Blood dried onto filter paper is more frequently used in screening programs because of its stability and ease of handling. Blood phenylalanine values resulting from the screening test are compared to an arbitrarily set threshold value. Infants with phenylalanine values above the threshold level undergo a second blood test to verify the elevation of blood phenylalanine and, if necessary, are followed-up for investigation of HPA.

In Québec, screening is done centrally at the Centre Hospitalier de l'Université de Laval (CHUL) by McCaman - Robins fluorometry (McCaman and Robins, 1962) of blood spotted onto filter paper at the time the infant leaves the hospital. Circles punched from the filter paper cards are analyzed automatically for phenylalanine content at a rate of seventy samples per hour (Grenier and Laberge, 1974). Both computer records and paper outputs of the analysis are produced. Computerized records of phenylalanine and tyrosine data are linked with the corresponding demographic and nominative information which arrives attached to the filter paper. If there is an insufficient amount of blood on the filter paper or if the infant was screened at less than two days of age, another sample of blood is requested. Infants with an elevated phenylalanine value on the screening test are contacted through their hospital of birth for another screening test. If their phenylalanine level is extremely high, they are referred directly to a clinical centre which follows individuals with HPA.

Centre Hospitalier de l'Université de Sherbrooke (CHUS) is the central laboratory testing for aminoacidurias (including phenylalanine) and elevation of uric acid, creatinine and ketones by analysis of urine-containing filter papers (Grenier and Laberge, 1974). The filter papers are sent for analysis by parents when the infant is 21 days and older. Participation in the urine screening program is 98% at 21 days of age (Laberge et al., 1987) and is used primarily to find HPA cases that may have been missed by the blood screening tests.

3.2.2: Screening availability

Screening newborns for HPA, usually in conjunction with screening for other inborn errors of metabolism, is mandatory in some programs and voluntary in others (NAS, 1975). Screening programs which are voluntary usually have a very low (5 / 10,000) parental refusal rate (Holtzman et al., 1983). Informed consent has been proposed as a way of educating mothers about screening while losing very little screening coverage (Holtzman et al., 1983). A policy had been suggested by the American Academy of Pediatrics Committee on Genetics whereby all infants would have a blood sample taken for HPA screening close to the time of departure from hospital (regardless of age) to ensure that all infants born in hospital are screened (Scriver et al., 1982). Samples collected after discharge from hospital of birth require a home visitor.

In Québec, screening is offered voluntarily before infants are discharged from hospital, and has acheived a cooperation rate of greater than 97% (Grenier et al., 1980). Parents leaving the hospital with their newborn infants are given filter papers for urine testing and asked to wet the filter paper with the infant's urine and return the filter papers to CHUS for analysis. Few infants are born outside of hospital (less than 1% of births) and a systematic program to screen them does not currently exist.

3.2.3: Sample collection

In Québec, blood is collected and spotted onto standard filter paper distributed by the central laboratory at CHUL. Batches of blood spotted filter paper are sent from every hospital in the province every few days by regular mail to the central laboratory at CHUL for analysis.

3.2.4: Classification of test results

By definition, an "elevated" phenylalanine value exceeds the threshold value set by the screening program. Maximum sensitivity of the test, with an acceptable rate of false positive tests, is the primary goal of the screening program. In theory, the threshold is set low enough to capture all individuals with HPA. A lower threshold increases sensitivity of the screening test at the expense of specificity. Large variation (six to ten fold) in the number of infants with a second screening test in some programs has led one group of researchers to suggest that a universal screening threshold of 240 μmol/L (for the Guthrie test) may not be low enough to detect all infants with all variants of hyperphenylalaninemia (Morris et al., 1983).

The usefulness the 240 μmol/L screening threshold was challenged by a study which screened infants at 5 days of age using the Guthrie test with a threshold value of 120 μmol/L. Screened infants were recalled for a second test at a rate of 1 / 909 births and the screening test gave a positive predictive value of 9.2% (Clemens et al., 1990). If screening had been done with a threshold of 240 μmol/L or 360 μmol/L, the positive predictive value would have risen to 34% and 50% respectively, but three cases of non-PKU HPA would have been missed. More evidence for the lack of sensitivity of the Guthrie test at standard thresholds is the number of missed cases of PKU due to negative test results in the United Kingdom (Smith et al., 1991). The seven individuals with PKU who were missed on initial screening because of negative test results (1974 - 1988) had all been tested with the Guthrie test, although significantly, only 53% of all infants born in this time period had been tested by the Guthrie test.

The emotional impact of a repeat screening test is also an important consideration in establishing a threshold for screening. The perception of parents that their child may be ill or abnormal because the infant requires a second screening test is always a concern (Sorenson et al., 1984). Parents may feel less alarmed if the screening process, as well as the need for a repeat test, is explained to them. Discussing an abnormal test result is not more upsetting to parents than declaring that a repeat test was routine or giving no explanation for the repeat test at all (Sorenson et al., 1984). This provides parents with a more realistic perspective.

In Québec, the current screening threshold for a second screening test is 242 µmol/L. Any infant screened at less than two days of age, or who has an insufficient sample, is given a second test. Screening thresholds have changed over the years in response to analysis of the screening data for the previous year (Table 1). No population wide repeat screening is done at any age, so incomplete sensitivity of the screening program would only be detected if there were infants with HPA diagnosed by other means.

3.2.5 Cost effectiveness

The cost of screening programs for HPA have been shown to be outweighed by the economic benefits (Dagenais et al., 1985), especially screening programs which screen for other inborn errors of metabolism at the same time. Screening on a small scale basis (less than 20,000 births per annum) is less cost effective (Smith et al., 1991) and benefits from centralization of several small scale screening programs. The cost to benefit ratio of a screening test increases with a decrease in screening sensitivity.

Table 1. Phenylalanine threshold values used by the Newborn Screening Program for HPA screening

Start date	End date	Threshold for phenylalanine (phe) measurements
January 1, 1969	July 5, 1976	phe > 243 µmol/L
July 6, 1976	May 2, 1977	phe > 213 µmol/L
May 3, 1977	January 1, 1984	≥ 5 days: phe ≥ 242 µmol/L 4 days: phe ≥ 224 µmol/L ≤ 3 days: phe ≥ 212 µmol/L
January 2, 1984	January 27, 1984	≥ 3 days: phe ≥ 242 µmol/L < 3 days: phe ≥ 212 µmol/L
January 28, 1984	November 3, 1987	≥ 3 days: phe ≥ 242 µmol/L 2 days: phe ≥ 212 µmol/L < 2 days: recall for another test
November 4, 1987	present	≥ 2 days: phe ≥ 242µmol/L < 2 days: recall for another test

Source: Réseau de Médécine Génétique du Québec, Secteur Informatique document "Système international - taux de normalité" prepared March 1989 by Nicole Bélanger.

In Québec, a cost-benefit analysis of the Québec Newborn Screening Program was undertaken (Dagenais et al., 1985). Cost was based on the number of PKU patients born from 1969 to 1980. Benefits of HPA screening were: reduction in cost to the state and to parents for the care of a mentally retarded individual, increase in national savings by acquiring an able worker, and increase in well being of parents and of children with PKU. Costs of HPA screening included: cost of treatment, cost of increased incidence of disease (state subsidy of more affected individuals), and cost associated with screening false positives and false negatives. The marginal costs saved from 1969 to 1985, by preventing institutionalization of individuals with PKU through treatment, was estimated to be 17.8 million (1980) dollars (Dagenais, 1985). The Québec Newborn Screening Program has been highly cost effective in its HPA screening component.

3.2.6: Follow-up of identified infants

Careful follow-up of all infants identified by screening as being at risk of being affected with PKU or non-PKU HPA is needed to ensure that they actually receive an accurate diagnosis and appropriate treatment, if necessary. Once an infant is identified as having PKU or non-PKU HPA, many more professional services are required to maintain the health of the child with HPA. In addition to a family doctor and community services, specialist services are necessary: biochemistry, dietetics, nursing, neurology, clinical and molecular genetics, and obstetrics (Anon., 1993a). Most of these services are available within a hospital department or clinic which deals specifically with inherited metabolic diseases. These services are necessary throughout the entire life of the child for phenylalanine value monitoring, dietary regulation, neurological assessment, and family

and reproductive counselling. Current guidelines for follow-up of individuals with non-PKU HPA (with blood phenylalanine above 400 µmol/L) or PKU suggest weekly blood tests for infants under the age of four, every two weeks between the ages of four and ten, and every month thereafter (Anon., 1993b). Such strict follow-up of all individuals with PKU or non-PKU HPA contrasts sharply with earlier practices, where infants with non-PKU HPA were not followed because it was perceived to be a harmless condition until the maternal phenotype became relevant to the patient.

In Québec, follow-up is done through the hospitals of the four universities in Québec which have medical schools and pediatrics departments: Hôpital Sainte-Justine, the Montréal Children's Hospital, CHUL and CHUS (Laberge et al., 1987). Infants with suspected HPA are referred to one of these four hospital centres by the screening laboratory. After confirmatory diagnosis of PKU or non-PKU HPA, the infant is followed by that centre for the rest of his/her life. The hospital centres provide all clinical and molecular genetics, dietetics, and biochemistry services for individuals with HPA. Low-phenylalanine food is provided by the Canadian National Food Distribution Center to the clinical centres which follow individuals with PKU or non-PKU HPA for distribution to individuals on dietary therapy (Clow, 1982). A record of the infant and his disease type is entered into a registry of individuals with PKU or non-PKU HPA kept by the Newborn Screening Program. The registry maintains the records of individuals with PKU or non-PKU HPA who will receive reproductive counselling when they reach puberty (Cartier et al., 1982). After several closely spaced clinic visits to ascertain the severity of the infant's HPA, infants on a low-phenylalanine diet are seen by the clinic for dietary and biochemical monitoring every two weeks to one month, and as older children,

every one to two months. Infants and children not on a low-phenylalanine diet are seen every six months to one year for biochemical evaluation. This contrasts to previous procedure where infants and children not on a low-phenylalanine diet had infrequent or no contact with the clinics because of the perceived benign status of their condition. Children with no regular clinic visits are likely to become lost to follow-up. Over the last few years, the need to follow children with non-PKU HPA, and those with PKU who have terminated diet therapy in the past few years, has been recognized. New recommendations for the management of HPA include treatment and follow-up for life (Anon., 1993b).

3.2.7: Treatment of affected individuals

Treatment for PKU to prevent mental retardation involves dietary restriction of the essential amino acid phenylalanine. HPA caused by cofactor deficiency does not respond to a reduction of dietary phenylalanine alone, but treatment with BH_4 L-DOPA is required. Restricted amounts of dietary phenylalanine cannot be provided simply by a low protein diet. Semisynthetic, commercial products free of phenylalanine plus selected natural diet components provide phenylalanine intakes between 250 to 500 mg/day for children with PKU, and greater than 500 mg/day for children with non-PKU HPA. Normal infants usually ingest several-fold this amount in their diets. Treatment is monitored so that the blood phenylalanine values are maintained below 800 µmol/L and preferably are closer to 300 µmol/L, but not below normal values.

Treatment prevents mental retardation if begun early in life and maintained. The sooner the treatment (by 20 days of life), the better the prognosis for normal cognitive

development. The IQ value falls four points for every month delay in treatment, up to the age of four months (Smith et al., 1990b).

Termination of treatment at age four years causes a decrease of four to six IQ points at age eight years for each 300 μmol/L increase in blood phenylalanine since diet relaxation. This loss is due to psycho-neurological dysfunction in the presence of HPA (Smith et al., 1990a). Therapy for life is now recommended for children with PKU (Anon., 1993b).

Revised recommendations for dietary management are more stringent than in the past: *i)* Infants with blood phenylalanine in excess of 400 μmol/L should start dietary therapy no later than 20 days of life and continuing into adolescence or adulthood (earlier onset). *ii)* Phenylalanine values should be routinely maintained below 480 μmol/L (lower level). *iii)* Treatment should continue for life (longer treatment duration) (Anon., 1993b).

Tight control of diet interferes with the lifestyles of the child and family involved. Families with a child with PKU show less family cohesion and adaptability than families with no child with PKU, and children with PKU show less social competence than normal children (Kazak et al., 1988). Parents of children with PKU report difficulty with calculation of daily allowances of foodstuffs; reluctance and inability to strictly monitor their child's diet, and often use punishment, trickery, anger and bribery as ways to enforce their child's dietary control (Awiszuz and Unger, 1990). A parent's fear of developmental delay in their child is balanced by a desire to see them develop a normal personality (Awiszuz and Unger, 1990).

The difficulties of lifelong dietary therapy for individuals with PKU may soon be a thing of the past. Unpalatable low phenylalanine formulas can be made more tolerable

and portable by packaging the formula into gelatin capsules (Kecskemethy et al., 1993). Enzyme therapy with phenylalanine ammonium lyase administered orally in capsule form (permitting phenylalanine to be converted into excretable metabolites) is an attractive but undeveloped alternative (Scriver et al., 1989). More recently, adenovirus mediated gene insertion of PAH in the livers of PAH deficient mice has been shown to successfully correct HPA (Fang et al., 1993), but has yet to be developed further.

In Québec, dietary therapy was not initiated unless an infant's blood phenylalanine exceeded 1,000 μmol/L. Current outcomes will reflect this policy. Practice in future will follow the revised (new) recommendations (see above). Females with PKU or non-PKU HPA who wish to have children restart a low phenylalanine diet before conception and maintain it throughout pregnancy (Lenke and Levy, 1980).

3.3: Factors that affect screening

The success of a screening program depends not only on an acceptable screening threshold, but also on its responsiveness to other factors affecting phenylalanine screening test results. Both environmental causes and screening methodology affect the screening results and influence the number of infants with phenylalanine values above the screening threshold and thus the success with which infants with HPA are detected. Knowledge of these factors allows estimation of their impact on newborn screening phenylalanine values.

3.3.1: Age at screening

The phenylalanine value of infants with PKU has been shown to increase over the first week of life (Holtzman et al., 1974; McCabe et al., 1983; Doherty et al., 1991). McCabe et al. (1983) demonstrated a significant difference in phenylalanine values of infants with PKU at all ages from birth (umbilical cord blood) to 14 days of life. This was shown by taking serial blood phenylalanine measurements of 109 infants with PKU and 114 control infants in the first two weeks of life. With increasing blood phenylalanine over time, a screening threshold of 242 μmol/L would have detected only 20% to 30% of PKU cases on cord blood; 65% to 70% of cases between 0 and 12 hours of life; 86% to 90% of cases under 24 hours of life; and 97.6% of PKU cases screened at 24 to 48 hours of life. Doherty et al. (1991) echo these findings, reporting that 31% of surveyed infants with PKU had phenylalanine of less than 242 μmol/L at under 24 hours of life, with 10% having a blood phenylalanine of less than 120 μmol/L. Infants with less severe HPA would perhaps be more likely to have phenylalanine values below the threshold value in the first week of life.

There is a trend towards early discharge of infants born in hospital from three, four or more days of age to two days of age or less (Jew et al., 1987). Because of the decrease in discharge age and the age dependence of phenylalanine values in individuals with HPA, re-evaluation of screening threshold levels to maintain high screening sensitivity is in progress (McCabe et al., 1983; Doherty et al., 1991).

The question of age dependence of phenylalanine seems to be not "How soon after birth does the blood phenylalanine of a newborn infant increase to levels that will be representative of its phenylalanine metabolism later in life ?" but rather "How soon after

birth does a newborn infant *with a defect in phenylalanine metabolism* acquire enough phenylalanine load to make it phenotypically different from normal newborn infants?".

3.3.2: Gender

Since the start of screening of newborn infants for HPA, an excess of males among infants identified with non-PKU HPA has been noted. In 1970, the Collaborative Study of Children Treated for Phenylketonuria had registered 60 males and 30 females identified by screening, though the gender ratio of individuals with PKU ascertained by the presence of mental retardation was 1 : 1 (Hsia and Dobson, 1970). These authors report significant gender ratio differences in other screening programs of both PKU (91 males to 65 females) and non-PKU HPA (92 males to 65 females). Causes for the gender ratio distortion which were rejected by the authors were: chance, more than one mode of inheritance of PKU and non-HPA PKU, and excessive female fetal loss. The study did not rule out the possibility of ascertainment bias due to a slower increase in phenylalanine value in females, suggesting missed cases of females with PKU or non-PKU HPA (Hsia and Dobson, 1970). The Québec Newborn Screening Program has previously reported (1965 - 1985) an excess of males among individuals with non-PKU HPA (54 males to 23 females) but not among individuals with PKU (28 males to 26 females) (Laberge et al., 1987).

Howell (1970) noted that some males with apparent PKU or non-PKU HPA acquire the ability to metabolize phenylalanine at the age of three months or later and postulated this explanation of the apparent excess of males among screened infants and the equality of genders among those ascertained by mental retardation. Knox and Kang

(1970) suggested re-evaluation of infants with PKU at 15 months would reveal some infants with transient HPA, most of whom would be males and thereby balancing the gender ratio among infants with PKU. Holtzman et al. (1974) found no significant gender ratio difference in individuals with PKU or non-PKU HPA seen in clinics, under the assumption that males were 51.7% of infants screened. However, comparison of the number of males and females detected by age at screening showed variation in the number of each gender detected by day of test, with significantly more males than females being detected when screened at four days of age. To account for this variation, age and gender dependent phenylalanine values were proposed, with females showing a quadratic increase of phenylalanine value in the first week of life and males showing a linear increase in phenylalanine value. By this model, females would have lower phenylalanine values than males at ages two to four days, and thus be less likely to have their PKU detected if screened at this time.

3.3.3: Diet

The rate at which blood phenylalanine rises after birth is dependent on the feeding practices of the infant. Phenylalanine values are affected by the amount of protein ingested before the time of testing. Mean phenylalanine values of newborn screening programs are lower among infants fed on a cow's milk diet to those on a modified milk (similar to human milk) diet (Walker et al., 1981; Briard et al., 1983), and those who are breast fed (Kleinman et al., 1966). Breast fed infants have a higher level of blood phenylalanine than formula fed infants when the phenylalanine content of the formula (57 mg/dL) is lower than that of the breast milk (105 mg/dL) (Doherty et al., 1991). An

elevated incidence of transient HPA has been noted among preterm infants fed intravenously with a high protein solution (23%) compared to those fed a normal protein solution (1%) (Lucas et al., 1993).

3.3.4: Screening methodology

Screening methodology plays a large part in the interpretation of observed blood phenylalanine values. Screening test results are affected by various variables in the handling of the sample and method of analysis.

A mail delay of seven to eight days, compared to a delay of one day caused an measured increase of 39.9 μmol/L in mean phenylalanine value as tested by fluorometry (Kleinman et al., 1966). The samples which were delayed had a false positive rate that was three times as large as the samples which were received within one day.

Variability in serum absorbency of filter paper among job lots can affect phenylalanine values read by screening (Slazyk et al., 1988). A lot-to-lot variability of 36% was seen between filter papers spotted with whole blood with a true phenylalanine of 370 μmol/L. In one extreme case, filter paper spotted with blood with a phenylalanine concentration of 1,937 μmol/L gave an interpreted phenylalanine value of 1,513 μmol/L on one job lot of filter paper. Mean phenylalanine values for different lots of filter paper differed significantly between lots when identical blood was spotted on each lot. Though the authors of this study did not consider filter paper job lot variability sufficient in itself to cause screening error, they advocated restriction of the number of filter paper lots used by a screening program at any one time to reduce potential screening variation.

Both fluorometric and Guthrie tests for blood phenylalanine measure phenylalanine from blood spotted filter paper by assuming that the volume of blood in a filter paper disk obtained by a punch used in screening is uniform from sample to sample so that volume estimates of phenylalanine can be interpreted as concentration. Deviations from the standard volume of blood on filter paper can occur in two ways. Hematocrit of the blood is related to its spreading on the filter paper so that a low hematocrit causes a larger spread of blood (Hill and Palmer, 1969; Arends, 1987). The thicker the blood spot (the less it has spread), the higher the phenylalanine read by the screening test.

3.3.5: Seasonal variation

Seasonal variation in the distribution of phenylalanine values from filter paper blood spots has been observed (Hill, 1969). Higher values were found in April and October, and lower values in January and July. Hill proposed that values were related inversely to relative atmospheric humidity and demonstrated the relationship with a correlation between climatic changes during the two years of screening and experimentally controlled humidity through change in filter paper absorbency. Morris (1983) also reported variation in monthly phenylalanine means, but failed to mention if the variation was random or if it followed a seasonal pattern.

3.4: Effect of HPA on offspring

3.4.1: Maternal hyperphenylalaninemia

Perry et al. (1973) reported on 104 children of 35 women with PKU. Of the 74 children whose mother's blood phenylalanine was greater than 1,200 µmol/L, 99% were

mentally retarded. Of the remaining 30 infants whose mother's blood phenylalanine values were between 600 µmol/L and 1,200 µmol/L, 91% were mentally retarded. The full spectrum of the effect of maternal HPA was made apparent in a survey of 524 pregnancies affected by HPA. There was an increased risk of spontaneous abortion (25%), mental retardation (95%), microcephaly (73%), intrauterine growth retardation (57%), and congenital abnormalities including cardiac defects (17%) when the mother's blood phenylalanine exceeded 1,200 µmol/L (Lenke and Levy, 1980).

Two factors affect fetal exposure to phenylalanine: *i*) the blood phenylalanine level of the mother; and *ii*) the transplacental gradient of phenylalanine, which concentrates maternal phenylalanine 1.25 to 2.5 times higher in the fetus (Hanley et al., 1987). If the cut-off le el for causation of maternal HPA is 1,200 µmol/L in fetal blood (as for PKU in newborns), any mother who had a placental gradient of at least 2.5 and a blood phenylalanine of 480 µmol/L would be at an increased risk of giving birth to a child with abnormalities caused by maternal HPA. Although it is impossible to predict a transplacental gradient in individual women, women with a blood phenylalanine of 200 - 600 µmol/L are at a higher than normal risk of giving birth to an affected child (Levy, 1985), albeit a lower risk than women with higher blood phenylalanine concentrations (Lenke and Levy, 1980).

The higher the maternal phenylalanine value during pregnancy, the greater the risk to the fetus (Lenke and Levy, 1980). Small surveys and case studies have demonstrated that the earlier a woman with PKU starts on a low phenylalanine diet during pregnancy and the stricter the diet, the better the chance of giving birth to a normal infant (Komrower et al., 1979; Davidson et al., 1989; Hanley et al., 1990).

Prospective studies have more precisely defined the relationship between the blood phenylalanine of the mother during pregnancy and fetal outcome. Drogari et al. (1982) showed that the fetal effects of maternal HPA in early gestation may be dependent on the blood phenylalanine level of the mother at the time of conception. An American collaborative study of pregnancy in women with PKU found that even non-PKU HPA women with a blood phenylalanine of less than 300 µmol/L during pregnancy and women with PKU who maintained a blood phenylalanine of less than 500 µmol/L from their first trimester gave birth to infants that were of a shorter length, lighter weight and smaller head circumference (Koch et al., 1990). Both the collaborative study (where therapeutic success was defined as a maternal blood phenylalanine of less than 360 µmol/L) as well as the United Kingdom PKU registry (Smith et al., 1990) (where therapeutic success was considered to be maternal blood phenylalanine of less than 600 µmol/L) were both able to show an inverse linear relationship of the infants' birth weight and head circumference to the gestational week in which the mother successfully attained dietary control. Most recent findings from the collaborative study (Koch et al., 1993) report satisfactory physical and mental development of infants whose mothers achieved strict dietary control before ten weeks of gestation, though diets started preconceptionally were recommended if possible. With the new knowledge of the strict dietary requirements necessary to minimize the possible effects of maternal HPA, precautions can now be made for follow-up, reinstatement, and maintenance of a low phenylalanine diet in women of reproductive age with either PKU or non-PKU HPA (Anon., 1993b). The high risk of teratogenic effects of phenylalanine being expressed in the offspring of women with uncontrolled

PKU or non-PKU HPA and the access to a dietary solution to this problem motivates the detection and follow-up of all HPA women.

3.4.2: Paternal hyperphenylalaninemia

Males with PKU or non-PKU HPA have been examined for effects of paternal PKU on father and offspring. Semen volume and sperm count correlated inversely with blood phenylalanine level in four untreated PKU males (Fisch et al., 1981). Sperm count was abnormally low in two males who had blood phenylalanine levels above 1,700 µmol/L. Brown (1986) reported that 9 males with PKU with blood phenylalanine levels of up to 1,900 µmol/L fathered 33 children. If males with PKU have deviant semen phenotypes, they are not infertile. A survey by Fisch et al. (1991) reported 40 PKU or non-PKU HPA males with offspring. There were 64 children born without congenital malformations. Six PKU males had fathered nine healthy children according to Levy et al. (1991). In no study was the paternity of the offspring verified.

3.5: Remarks

With the exception of the United Kingdom (Smith et al, 1991; Anon., 1993b), few screening programs have provided systematic overviews and assessments of their programs and have given data on the follow-up of individuals detected by screening. This thesis evaluates the Québec Newborn Screening Program by examining the screening program itself, the follow-up of individuals with positive tests, and the phenotype of the affected individuals.

Chapter 4: Materials and Methods

4.1: Demography of the province of Québec

Information about births in the province of Québec from 1970 to 1992 was obtained from publications of the Bureau de la Statistique du Québec (BSQ). Birth rates published by the BSQ are taken from official Québec population registries, and so are the most accurate source of information about the number of infants born per year. The reports of birth rates from the BSQ are based on all live births in the province.

Ethnic origins of the Québec newborn infant population from 1973 to 1990 were estimated from the ethnic breakdown of 0 to 25 year olds for the Canada census data for Québec from 1991. Although this information reflects the current ethnic diversity in the province and not necessarily that of twenty years ago, this was the only detailed published source of ethnic origins from either Statistics Canada or the BSQ.

4.2: Annual reports of the Québec Newborn Screening Program

Annual reports have been compiled by the Newborn Screening Program since 1973 to evaluate the performance of the screening program, and to provide feedback to hospitals which screen newborn infants. Details from the Newborn Screening Program annual reports are described in Appendix A. Reporting periods were from January 1 to December 31 for 1973, 1974 and 1975; and April 1 to March 31 for the years 1976 onwards. The reporting periods are denoted by years. For example, the reporting period April 1, 1976 to March 31, 1977 is denoted 1976-77. A report was also produced for the linking period of January 1 to March 31, 1976.

Data were collected from annual reports for each hospital, for each reporting period. Because of the large volume of data (173 hospitals and 21 reporting periods), only selected variables were used.

For the reporting periods from 1973 to 1987-88, the data collected were as follows. For first screening data: number of samples received, number of insufficient samples and number of samples with abnormal phenylalanine values. The number of first samples actually analyzed was calculated as the difference between the number of samples received and the number of insufficient samples. Also recorded for each hospital was the total number of unique infants who had one or more analyzed samples. For the reporting periods 1988-89 and 1989-90, the variables recorded were: the number of samples received, the number of insufficient samples and the number of samples with abnormal phenylalanine values. For the reporting periods 1990-91 to 1992-93, the variables recorded were: the number of samples received and the number of samples with abnormal phenylalanine values. Data collected for 1988-89 and subsequent reporting periods have not yet been verified for accuracy by the Newborn Screening Program. The variation in the variables collected reflects changes over the years in the information available from the annual reports.

The annual reports are oriented towards providing the individual hospitals with information they may find useful. The emphasis is thus often placed on the number of *samples* which were received, analyzed or insufficient, rather than the number of infants being screened.

Data for each hospital were combined to produce regional totals, and regional totals were combined to give province-wide totals for each reporting period.

4.3: Registry of affected individuals

A registry of individuals diagnosed as having either PKU or non-PKU HPA is kept by the Newborn Screening Program. This is not a static registry, but is updated and modified as new information is received by the Newborn Screening Program. Records of individuals with HPA in the registry may, over time, have more test results added, may be modified with a change in diagnosis, or may be deleted if the individual moves from the province or their phenylalanine value becomes normal. A copy of selected information in the registry dated July 9, 1992, was obtained for this project. The registry includes a record of all results of blood phenylalanine tests done by the Newborn Screening Program, but not the results of tests performed by other centres. Names and addresses of individuals with PKU or non-PKU HPA were removed from the copy of registry information by the Newborn Screening Program, so that only the identification numbers of individuals remained as unique identifiers. The initial diagnosis of either PKU or non-PKU HPA and gender were also recorded.

The registry information was used as a starting point for the collection of data about individuals with PKU or non-PKU HPA. Although a clinical file review provided most of the information, registry information provided the system of ID numbers to link these individuals to population records, and data from the registry was used when clinical files could not be found.

4.4: File review

I reviewed clinical files at the hospital centres of the Réseau de Médécine Génétique du Québec (RMGQ): the Montréal Children's Hospital, Hôpital Sainte-Justine,

Centre Hospitalier de l'Université de Laval and Centre Hospitalier de l'Université de Sherbrooke. Data were collected under the supervision of the clinic Director.

4.4.1: The data collection form

After reviewing several clinic files at the Montreal Children's Hospital to assess file content, I designed a form to collect data (Figure 1). I also translated this form into French for centres which were predominantly French speaking.

Variables on the form were as follows. ***File number***, ***initials***, and ***father's initials*** were used to identify the patient. The ***file number*** of the Newborn Screening Program is used because the file numbering system of each clinic was different from that of the provincial registry, so did not correspond to the registry's identification of infants. ***Initials*** and ***father's initials*** were recorded so that the file could be retrieved to collect additional information about a patient or to confirm existing information at a later date from clinics' alphabetical filing systems, if necessary.

Centre was the clinical centre at which the patient was followed. This information, as well as the gender and date of birth, was used to uniquely identify individuals with PKU or non-PKU HPA. It was necessary to use all of the above variables in order to distinguish between individuals born on the same day, individuals with the same initials and individuals who were followed at several centres.

Ethnicity was self-identified in the hospital file or as listed on interview: *French Canadian* (Québecois, whose families have lived in Québec for several generations); *English Canadian* ; *Mediterranean* (Southern European ancestry); *Other European* (Northern, Eastern or Northwestern European ancestry, including persons whose parents

File # _____ Center _____
Initials _____ Father's initials _____

Sex 0. Male 1. Female

Ethnicity
1. French Canadian
2. English Canadian
3. Other European
4. Mediterranean
5. Oriental
6. Black
7. Jewish
8. French & English
9. French & Mediterranean
10. French & European
11. English & Other
12. Other

Place of Birth _____

Premature 0. No 1. Yes
Current File 0. No 1. Yes 2. Yes but uncooperative 3. Moved or died

Phenylalanine Status
0. Transient hyperphenylalaninemia
1. Hyperphenylalaninemia [phe] < 1000
2. Hyperphenylalaninemia 1000 < [phe] < 1200
3. PKU [phe] > 1200

On Diet 0. No 1. Yes
Date of Birth _____

	test date (MMDDYY)	[phe] mg%	[phe] mmol/L
1.			
2.			
3.			
4.			
5.			
7.			
8.			
9.			
10.			
11.			
12.			
13.			
14			
15.			
16.			
17.			
18.			
19			
20.			
21.			
22.			
23.			
24.			
25.			

Figure 1. Data collection form for clinical file review

are from France, but who are not included in the French Canadian category); *Oriental*; *Black* (African or African - American); *Jewish* (any Jewish origin). The categories which are combinations of different ethnic groups reflect some of the admixtures seen in the population of Québec. Information on ethnicity, if missing from the hospital files, was obtained from the clinical centre personnel.

Place of birth was initially recorded as town or city of birth, then recoded according to the administrative region of the province.

Premature was coded as "Yes" only when the file stated that the infant had been born prematurely, otherwise it was coded as "No".

Current file described the follow-up of the patient in the past 2 years. Patients who had been seen in the clinic in the past two years or had a date set for a clinic appointment were coded "Yes". "No" was used to describe individuals who had not been seen at the clinic in the past two years "Uncooperative" was used for patients who had been contacted in the previous 2 years, but had refused to attend the clinic. "Moved" was used for patients who had notes in their files indicating that they had emigrated from the province and would be followed by a clinic elsewhere.

Phenylalanine status was determined from information in the file or, where ambiguous, from discussions with the centre doctors or nurses. This variable was used to determine the revised disease status of an individual with PKU or non-PKU HPA based on the follow-up and long term evaluation of an individual by the centre. If a blood phenylalanine test result without dietary restriction was unavailable, dietary tolerance of phenylalanine was used to determine whether an individual was hyperphenylalaninemic or phenylketonuric.

The diagnosis of PKU was defined by a blood phenylalanine of greater than 1,200 µmol/L without a phenylalanine restricted diet, or by a dietary tolerance of less than 500 mg of phenylalanine per day. Individuals with non-PKU HPA had a dietary tolerance for phenylalanine of greater than 500 mg per day. The term transient HPA was applied to infants who had elevated phenylalanine values for several tests, but then had their levels return to normal. Transient HPA is a arbitrary classification made by the clinician that varies among clinics. The classification of transient HPA was also used on the follow-up sheet from the Newborn Screening Program that was completed by centre personnel to confirm a diagnosis of PKU or non-PKU HPA with the Newborn Screening Program registry.

On diet was coded "Yes" if the patient was ever on a restricted phenylalanine diet, otherwise it was coded "No".

The ***test date*** and ***phenylalanine value*** were recorded for a maximum of 25 phenylalanine tests for each individual. No record was made of any test result when the individual was on dietary therapy. The unit "mg %" was converted to "µmol/L" by multiplying by 60.54.

The data form for clinical file review was completed for every individual in the province who had a centre file and who was followed for transient HPA, PKU or non-PKU HPA. Files were ascertained by reviewing all patient files that dealt with phenylalanine and checking for eligibility criteria.

4.4.2: Data collection

Once all the patient files were reviewed, the data forms were linked to the corresponding record in the program's registry list by matching on gender, date of birth and date of the initial screening test. ID numbers of the individuals listed by the Newborn Screening Program for whom no clinic files were found were sent to the registry at CHUL for identification. The names of the people identified by CHUL were sent to the clinic at which they would have been followed. The clinics then provided information about as many of these individuals as possible. The screening program registry ID number was used for identification of the data.

Two pieces of information were added to the information collected at the centres, based on the linkage of the registry and clinic information. Comparison of the clinic information with the Newborn Screening Program indicated whether an individual had a record with the screening program registry and, if so, whether the individual's classification (PKU or non-PKU HPA) in the screening program registry matched the diagnosis in the clinic file. The amount by which each individual's initial (i.e. newborn) phenylalanine value exceeded the applicable newborn screening threshold was calculated from the screening thresholds shown in Table 1.

The individuals considered for analysis had a clinical diagnosis of PKU or non-PKU HPA not attributable to a cofactor defect and were born in the province since the start of the Newborn Screening Program (1970). Individuals were included if they had a Screening Program registry record and/or a clinic file. The file review provided the majority of the information, and data not found in files was supplemented by registry data. Registry information alone was used for individuals with no clinic file.

4.5: PAH mutations of individuals with PKU or non-PKU HPA

Identification of both of an individual's phenylalanine hydroxylase mutations was available for 20 individuals with PKU or non-PKU HPA born since the start of the Québec Newborn Screening Program. This information was linked to these individuals' file-review information by matching as previously described. Enzyme activity was determined from published articles (John et al., 1992; Ramus et al., 1993; Svensson, 1993; Weinstein et al., 1993; Scriver et al., in press) to provide a continuous variable to describe the genotype of individuals with PKU or non-PKU HPA.

4.6: Statistical methods

Statistical analysis was done for this project using SAS for UNIX, version 6.07, on Sun SPARCstations 1 and 10. Data were managed in Reflex 2.0 for DOS and SAS for Unix, version 6.04.

Parametric statistics were used when the data were approximately normally distributed. Statistics included χ^2 and t tests; discriminant analysis; and F-tests for linear and multiple regression. Chi-squared tests with small numbers were performed by the procedure outlined by Smith (1986). Normality of data distributions was tested using the Shapiro-Wilk test statistic for less than 2,000 observations, or the Kolmogorov-Smirnov goodness of fit test statistic for more than 2,000 observations. Non-parametric statistics used included the Wilcoxon rank sum test and Friedman two-way analysis of variance by ranks. Twin study and heritability estimates were calculated as described by Sofaer (1990).

Chapter 5: Results and Interpretation

The procedure for screening for hyperphenylalaninemias and the structure of the decision process are abstracted in Figure 2.

Phenylalanine concentration is measured by volume, approximated to estimate concentration, and thus is sensitive to the thickness of blood spotted onto the filter paper. Thicker blood spots give higher estimates of blood phenylalanine values. Blood samples which have phenylalanine values above the screening threshold are evaluated for blood spot thickness. Thick blood spots with phenylalanine values below 265 μmol/L are considered to be normal on the assumption that the estimated phenylalanine value is inflated because of the excess blood in the spot tested. The determination of blood spot thickness is made by inspection by laboratory personnel. Although thick blood spots do occur on filter papers of samples with phenylalanine values below the threshold, the thickness is never evaluated because it is of no concern.

Where the phenylalanine value measured by the test is abnormal, further decisions are made. If the phenylalanine value exceeds 363 μmol/L, a metabolic clinic is contacted directly to initiate the evaluation and follow-up of the infant. Otherwise, if the blood spot was thick, or if the phenylalanine value was above the threshold but was less than 363 μmol/L, the hospital at which the infant was born is contacted and asked to send another blood spotted filter paper. If the phenylalanine value on this repeated test again exceeds the threshold, the infant is considered to have an abnormality of phenylalanine metabolism and a metabolic clinic is contacted to follow the infant. When an infant's name is given to a metabolic clinic, information about the infant is also entered into the

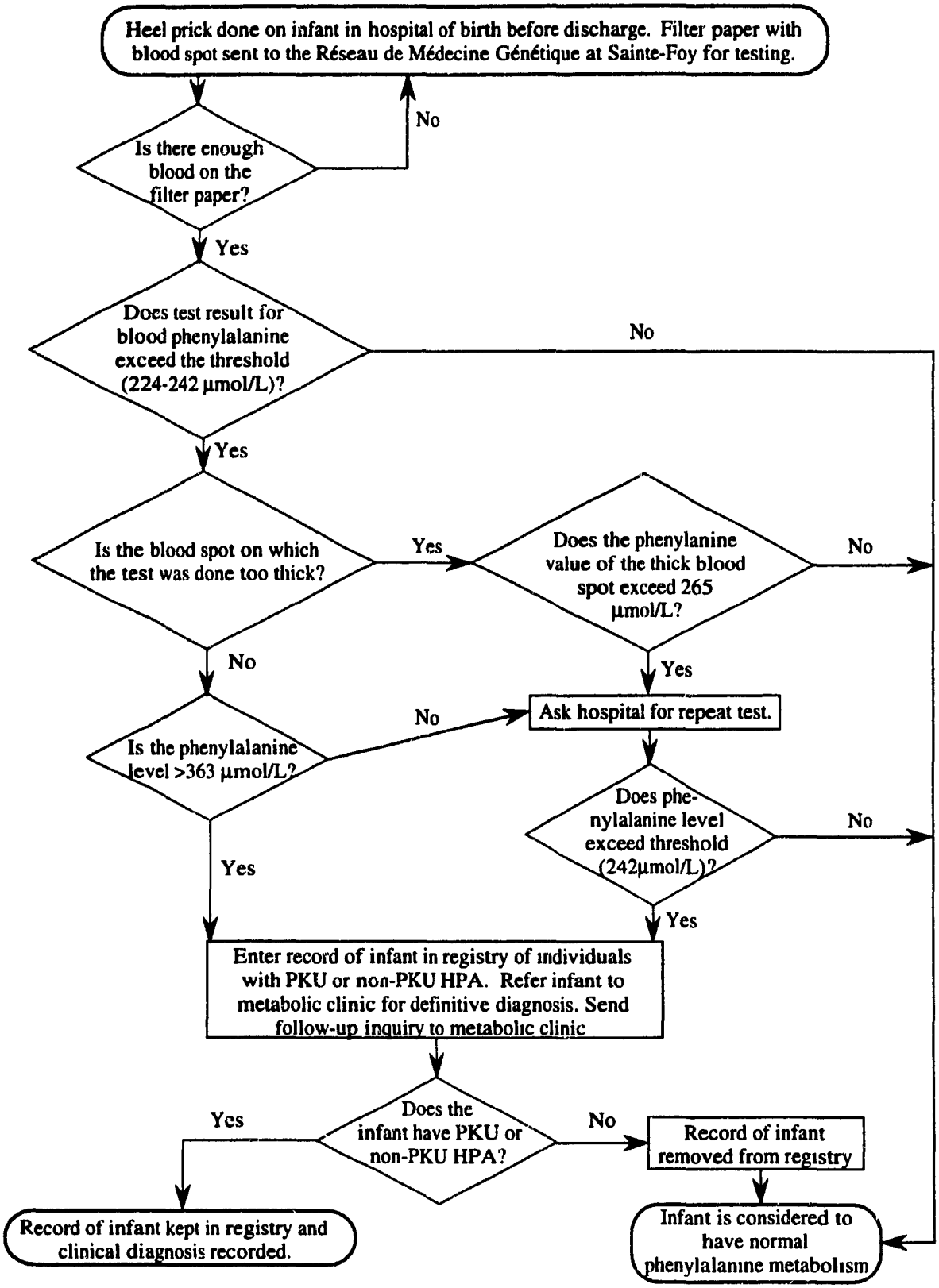

Figure 2. Flow chart of the Quebec Newborn Screening Program. Thresholds specific for age and year that were used by the screening program are described in the text.

registry of individuals with PKU or non-PKU HPA kept by the Newborn Screening Program.

At the time an infant is referred to a metabolic clinic for follow-up, a form is sent to the clinical centre to confirm that the referral was acted upon, and a confirmed diagnosis of transient HPA, PKU, or non-PKU HPA is requested. The clinic confirms or rules out the diagnosis of PKU or non-PKU HPA, informs the Newborn Screening Program of the disease status of the infant, and initiates treatment of the individual if necessary. A diagnosis of PKU or non-PKU HPA is entered into the registry or, if the infant is unaffected, the record is deleted from the registry.

All further contact for follow-up of the patients is through the clinics and not by the Newborn Screening Program. The record of a diagnosis of PKU or non-PKU HPA in the Newborn Screening Program registry is based only on the form returned by the clinic and is never systematically updated, since there is no formal arrangement for the metabolic clinics to notify the Newborn Screening Program about subsequent patient information. The clinics try to maintain contact with their patients for follow-up. If they are not successful, the clinic renews its attempts for clinic visits as the patients approach adolescence in order to provide reproductive and genetic counselling, especially for females. In spite of this effort, patients are still lost to follow-up, even through their reproductive years.

5.1: Population

5.1.1: Participation in screening

A review of the Newborn Screening Program annual reports showed that between January 1, 1973 and March 31, 1990, a total of 1,556,708 blood spotted filter papers were sent to the Québec Newborn Screening Program for analysis. Although the number of infants with analyzed samples cannot be calculated exactly, an estimated 1,513,217 infants had one or more filter papers analyzed. During the same time period 1,575,310 infants were born in the province (Bureau de la Statistique du Québec).

To examine the effectiveness of the Newborn Screening Program, the number of infants who had screening tests analyzed was compared by reporting period to the number of live born infants in Québec (Bureau de la Statistique du Québec). The percentage of live born infants with analyzed samples increased from 86.9% in 1973 to 98.3% in 1987-88 (Figure 3). The difference between the number of live born infants and the number of infants with analyzed samples implies that 64,516 infants born between January 1, 1973 and March 31, 1988 had no samples analyzed.

Explanations for live born infants not having analyzed samples include: infants for whom only insufficient samples were sent, infants born in hospital but not screened, infants not born in hospital, and infants who died before an adequate sample was obtained.

The rapid increase in the percentage of live born infants with analyzed screening test results reflects two trends: the increasing percentage of infants screened in each hospital and the decreasing proportion of insufficient samples received over the past two decades. In the years 1970 to 1972, not all hospitals in the province participated in the

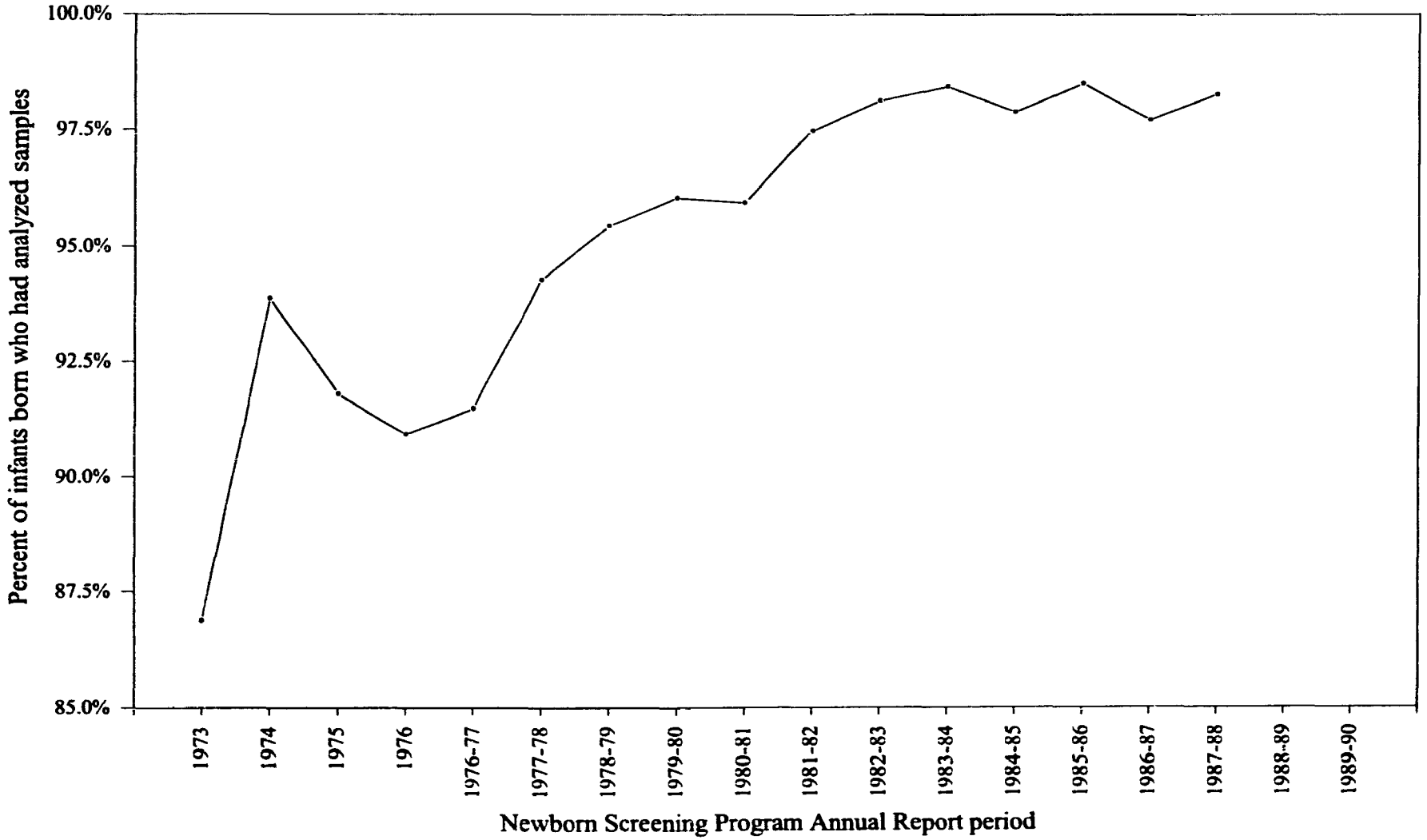

Figure 3. Percent participation (tests completed per live born infant), by year. The source of the number of births is the Bureau de Statistique de Quebec reports.

screening program. However, nearly all hospitals participated for 1973 and subsequent years, as indicated in the annual reports for those years, and there was a sharp increase in the number of infants screened per hospital. The failure of the Newborn Screening Program to reach 100% screening of all infants can be primarily attributed to infants not born in hospitals and infant deaths before screening, as well as to insufficient samples for which a repeat sample is never obtained. Infant deaths before screening should account for a maximum of 0.2% of all live born infants (Costa et al., 1985), based on estimates of infant deaths in the first week of life. There is no consistent mechanism in the province for screening infants born outside of hospital, who depend on the home doctor or local community heath centre to provide blood spotted filter papers. The lack of coordinated effort to screen infants not born in hospital is most likely the largest contributor to the lack of 100% screening effectiveness in the province.

5.2: Samples

The screening program as a diagnostic test cannot be evaluated simply on the basis of a 2-by-2 table to demonstrate sensitivity and specificity because of the multiple stages at which infants are screened. Measures of reliability are presented, instead, as measures at various stages of the screening process. Without any screening later in life to verify the phenylalanine status of all individuals, there is no standard against which to compare the predictions of the Newborn Screening Program. The only indication of incomplete sensitivity would be the detection of people ascertained through their mental retardation or by birth of offspring with characteristic fetal phenotypes of maternal HPA.

5.2.1: Insufficient samples received

The percentage of insufficient samples among the total number of samples received was calculated for each of the Newborn Screening Program annual reports as a percentage of insufficient samples out of total samples received. The percentage of samples received that were insufficient has shown a marked decline, from 5.95% in 1973 to 0.17% in 1987-88, but has since risen to 0.37% (Figure 4). These percentages varied greatly from hospital to hospital, in a seemingly non-random fashion. Certain hospitals tended to consistently have a higher percentage of insufficient samples.

5.2.2: Samples analyzed

The efficiency of the Newborn Screening Program was evaluated by the comparison of the number of filter papers received, by reporting period, to the number of unique infants with at least one sample analyzed. The ratio of samples received to infants analyzed decreased from 1.075 in 1973 to 1.006 in 1987-88 (Figure 5). Thus, averaged over all reporting periods, approximately 1,033 filter papers were received for every 1,000 infants analyzed.

This measure of efficiency reflects two factors. The first is the proportion of insufficient samples, which has declined between 1973 and 1988 (Figure 4). The second is the number of replicate samples sent to the Newborn Screening Program. The number of replicate samples is not described in recent annual reports and thus is difficult to evaluate, but their number is increasing (P. Fiset, personal communication). The frequency of repeat tests when the first screening test is done at a very early age (before two days of age) is increasing, as is the frequency of replicate tests done at separate

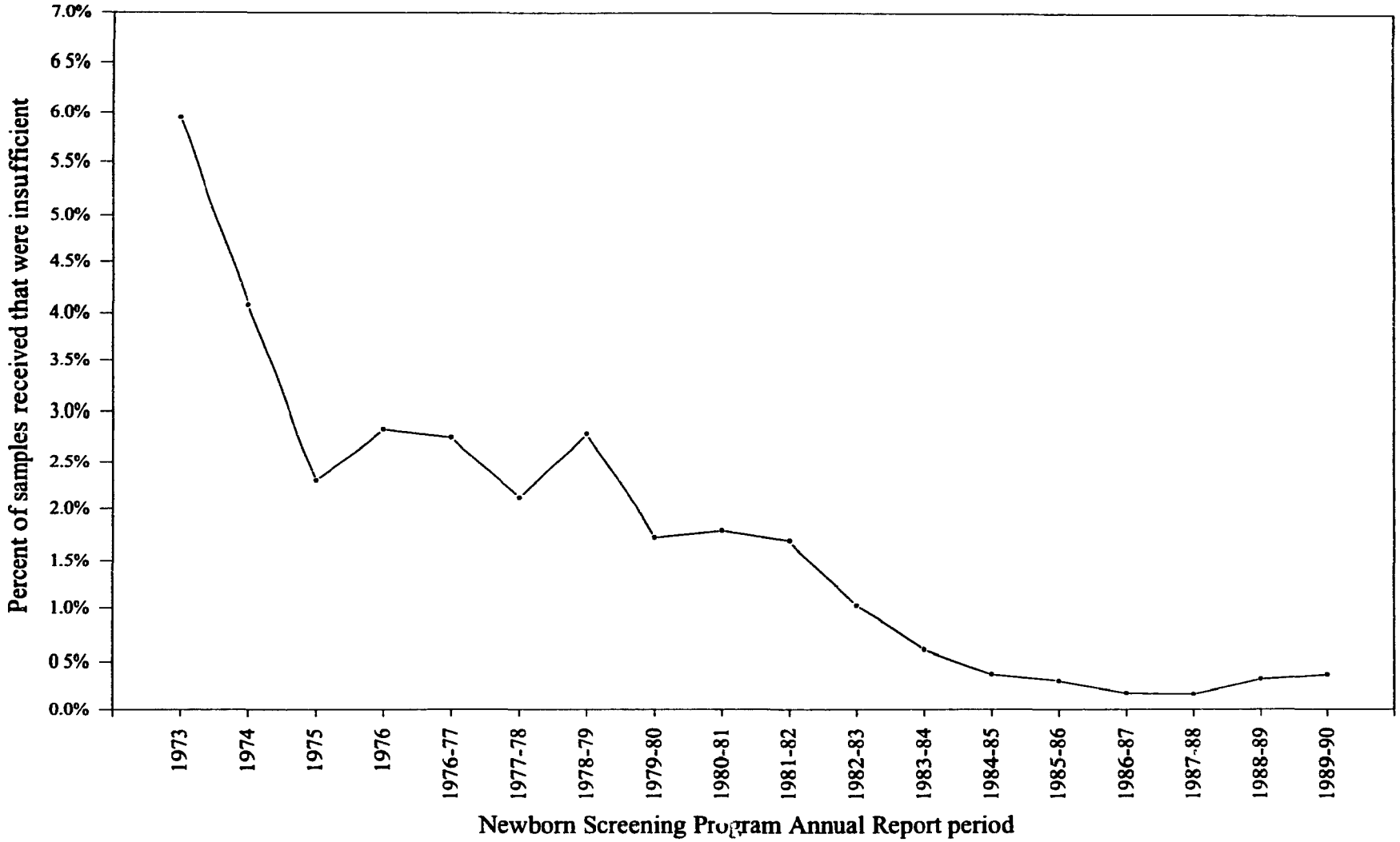

Figure 4. The percentage of unsatisfactory samples received by the Quebec Newborn Screening Program

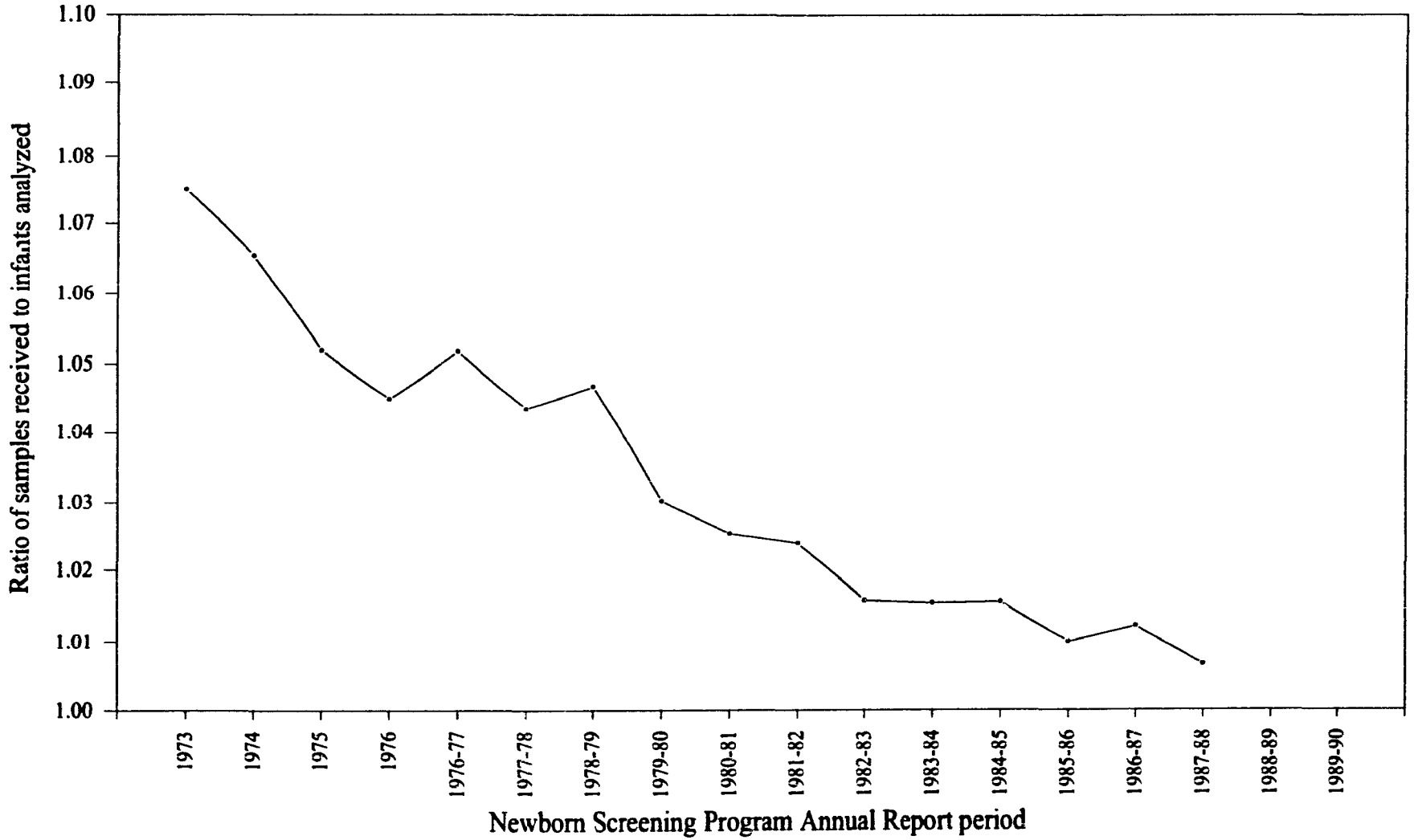

Figure 5. Samples recieved by the Newborn Screening Program for analysis related to the number of infants with at least one sample analyzed.

hospitals when infants are transferred. Although the number of insufficient samples has not increased greatly since 1987-88, the increase in the number of duplicate samples received would cause an increase in the ratio of samples received to infants analyzed.

5.3: Test results: population

5.3.1: Proportion of samples above the screening threshold

Annual reports of the Newborn Screening Program did not provide information about the number of infants with blood phenylalanine values above the threshold, but rather the number of samples (filter papers) with phenylalanine above the threshold. As the incidence of PKU or non-PKU HPA does not vary significantly from year to year (section 5.5.4), variation in the number of samples with phenylalanine above the screening threshold is due to either variation in the number of infants with spuriously elevated phenylalanine, or variation in the number of samples received per infant. The number of samples received per infant will not contribute much to the variation in the number of samples above the threshold, as this accounts for an average of 33 extra samples per 1,000 samples analyzed.

Variation was seen in the number of samples analyzed which had phenylalanine values above the screening threshold. The annual number of samples with phenylalanine above the screening threshold ranged from 27 to 303 samples per twelve month period. Over the years, the change in the proportion of samples analyzed with phenylalanine values above the screening threshold was consistent across all geographic regions, allowing for a province-wide pattern of change to be considered. This corresponded to an average proportion of analyzed samples above the threshold of 1 / 873, and ranged from a

low of 1 / 3,257 for 1981-82 to a high of 1 / 286 samples analyzed for 1983-84 (Figure 6). The annual variation in the number of samples analyzed exceeding the threshold can be partially accounted for by the change in screening threshold value. This is most clearly seen for the period 1976-77, where a decrease in the screening threshold for phenylalanine from 243 μmol/L to 213 μmol/L resulted in a doubling of the number of samples exceeding the threshold over that seen in the previous three years. When this threshold was increased in May 1977, the number of infants with observed elevated phenylalanine declined (Figure 6).

5.3.2: Regional distribution of samples above the screening threshold

The proportion of samples with phenylalanine values above the threshold was taken from annual report data. The proportion of samples above the threshold appeared to vary by geographic region (Figure 7), with highest proportions in the North of Québec and in the Cantons de l'Est; and lowest proportions in the lower St. Lawrence regions.

A χ^2 test was used to test the homogeneity of the proportion of infants above the screening threshold among regions. The proportion of infants screened in each region (1973 to 1989-90) was used to calculate the expected values for that region. The regional distribution of the proportion of samples with phenylalanine values above the threshold differed significantly from expected ($\chi^2_{10} = 109.4$, $p < 0.001$).

5.3.3: Phenylalanine values by age at screening

Phenylalanine value data collected for this study for normal infants with respect to age at screening is discussed in Appendix C.2.4. For the purposes of this thesis,

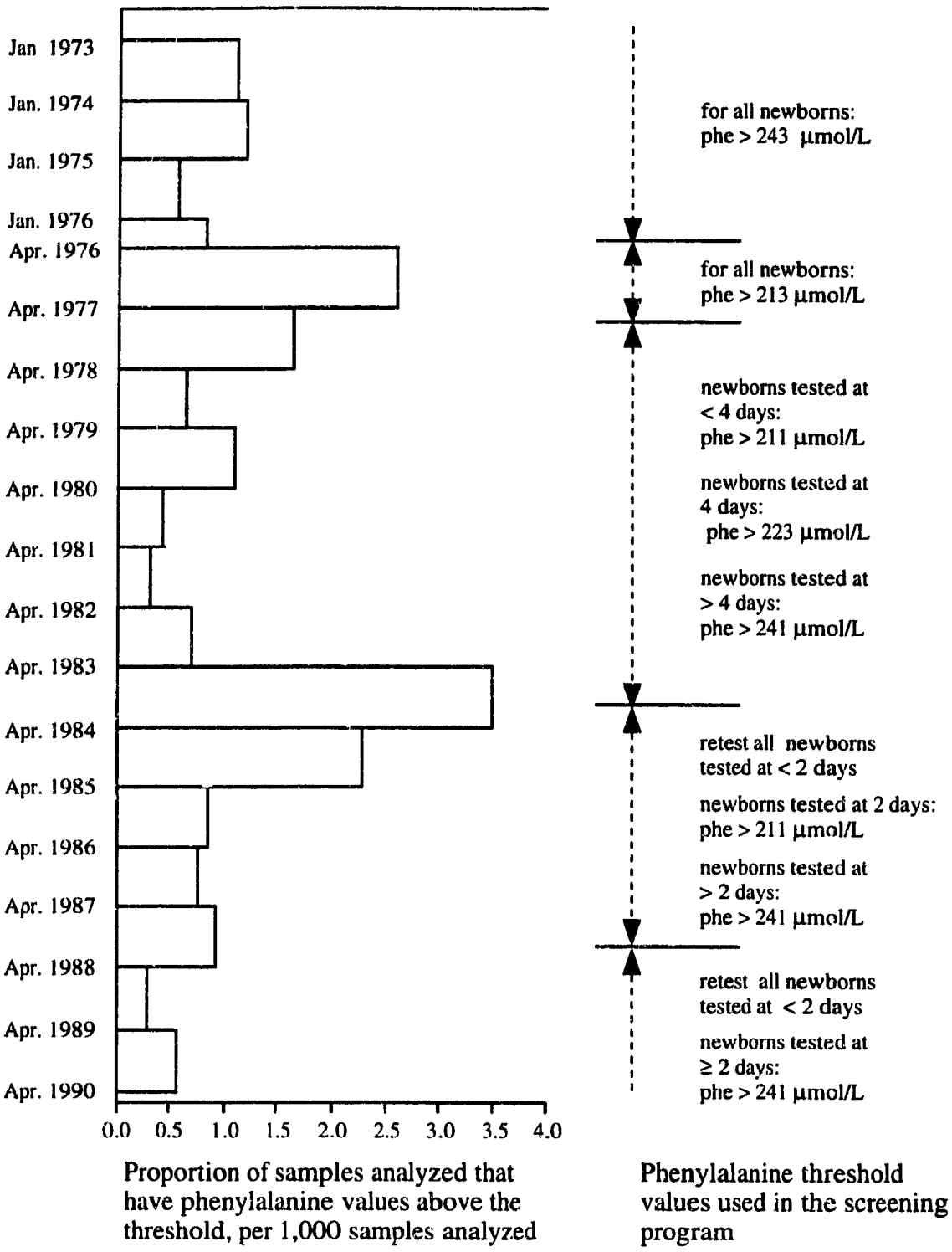

Figure 6. Proportion of samples analyzed with phenylalanine values above the (period specific) screening threshold (per 1000 samples analyzed).

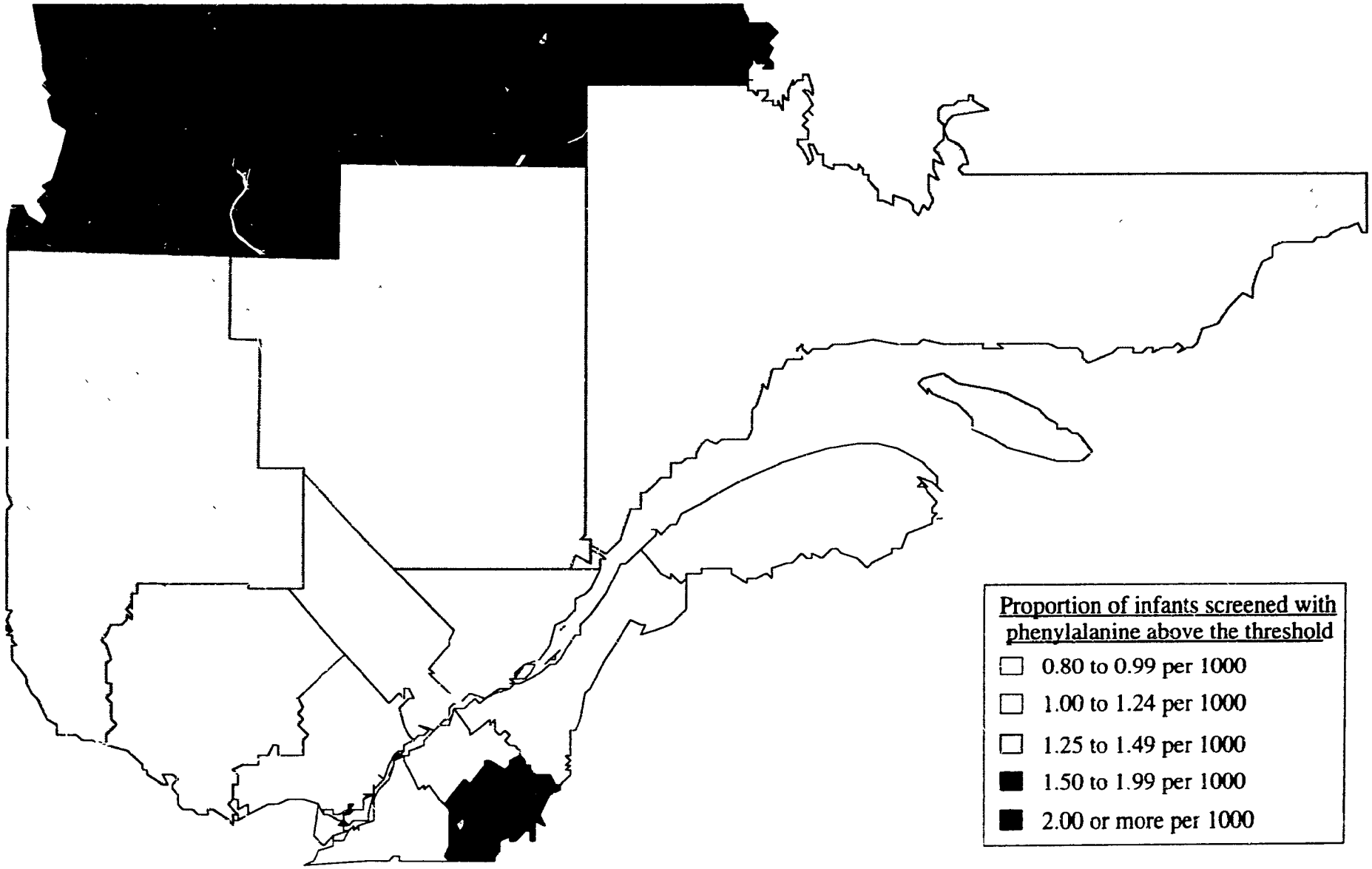

Figure 7. Proportion of infants with phenylalanine above the threshold by the administrative regions in the province of Québec.

reanalysis of the data of McCabe et al. (1983) for serial blood phenylalanine measurements of normal infants used as controls was done. No correlation was seen between age at screening and mean blood phenylalanine for measurements taken between: cord blood and 14 days of life ($p > 0.51$); 12 hours of life and 14 days of life ($p > 0.70$); or 12 hours of life and 7 days of life ($p > 0.51$), as tested by Pearson correlation coefficients. These data suggest that normal infants do not show any rise in phenylalanine values in the first week or first two weeks of life.

Analyses of phenylalanine values of normal infants by age at screening, for data from the Québec Newborn Screening Program, are shown in Appendix C.2.4.

5.4: Test results: affected individuals

5.4.1: Distribution of blood phenylalanine values

All but one of the individuals with PKU or non-PKU HPA who were screened at birth had phenylalanine values above the screening threshold on initial testing. The one individual whose initial phenylalanine value was below the threshold was tested at less than one day of age, so was automatically recalled for a second screening test, at which time the phenylalanine value was above the threshold.

The mean initial phenylalanine value among the 152 individuals with PKU or non-PKU HPA was 733.0 µmol/L, with a standard deviation of 724.6 µmol/L and a median value of 529.5 µmol/L (4 missing values). When divided into PKU and non-PKU HPA subgroups, descriptions of phenylalanine values were as follows: PKU: mean 1,000.6 µmol/L, standard deviation 903.8 µmol/L; non-PKU HPA: mean 403.2 µmol/L, standard deviation 173.8 µmol/L (Figure 8). Neither group had a

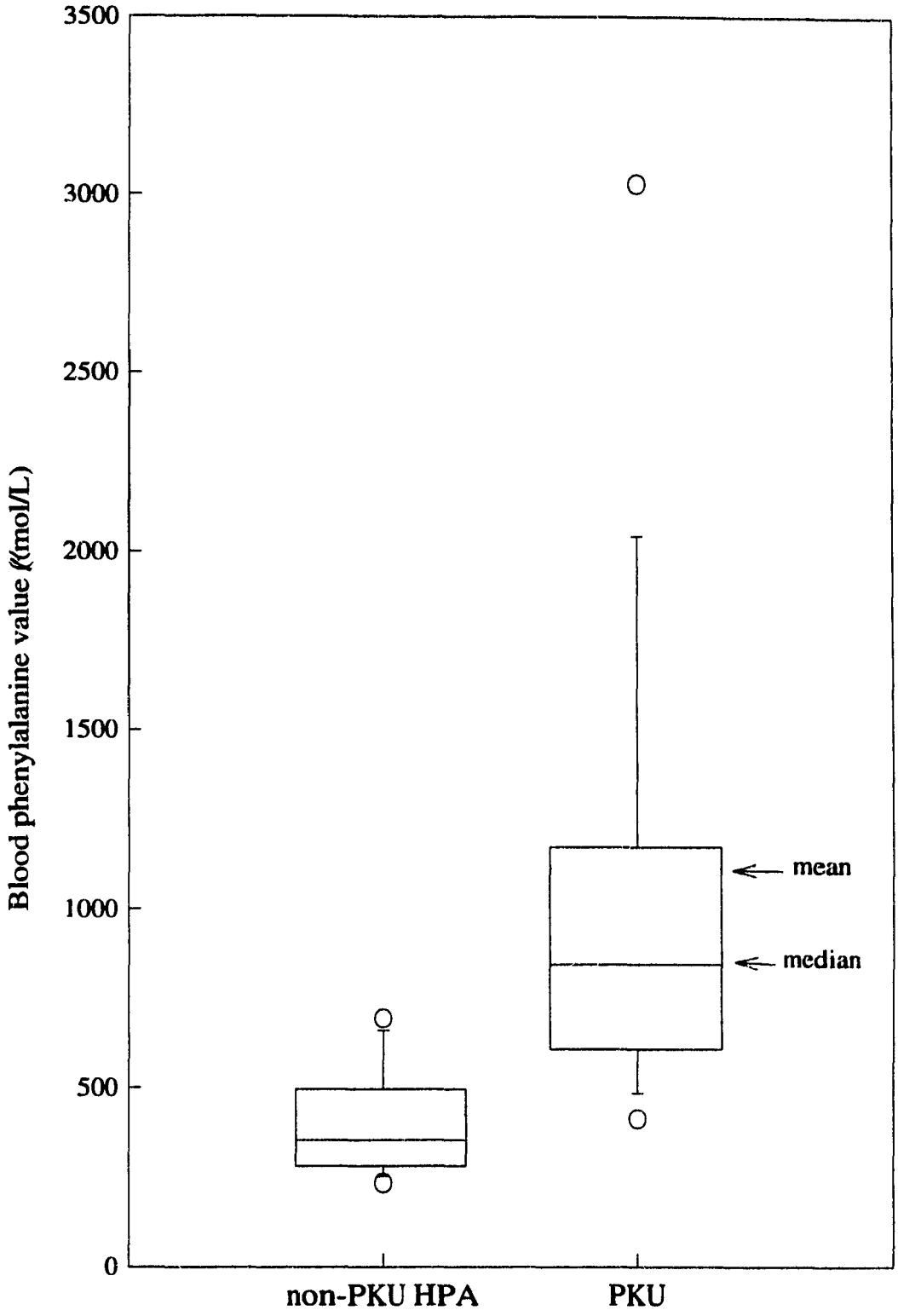

Figure 8. Box plots of distribution of initial phenylalanine values for hyperphenylalaninemia.

distribution of phenylalanine values that was likely to have come from a normal distribution, as shown by the Shapiro-Wilk test ($p < 0.0001$ for both groups).

Analyses of phenylalanine values of normal infants are shown in Appendix C.2.1.

5.4.2: Phenylalanine values by age at screening

The relationship between initial phenylalanine values and age at screening was examined for individuals with PKU or non-PKU HPA. Regression of phenylalanine values for these individuals on age at screening explained more variance in phenylalanine values when phenylalanine values were expressed logarithmically ($R^2 = 0.08$), than as a simple linear regression ($R^2 = 0.05$). Increase in the phenylalanine values in the first week of life for individuals with PKU or non-PKU HPA is best expressed as an exponential function (phenylalanine value = $10^{(2.55 + 0.05\ age)}$), where age is expressed in days. The predicted phenylalanine value, by this model, for infants screened at less than one day of age is 354 μmol/L and at seven days is 794 μmol/L.

When the data were divided into PKU and non-PKU HPA contributions, the regression lines became: (phenylalanine value = $10^{(2.59 + 0.09\ age)}$) ($R^2 = 0.25$) for PKU; and (phenylalanine value = $10^{(2.50 + 0.02\ age)}$) ($R^2 = 0.03$) for non-PKU HPA. The predicted phenylalanine values for infants with PKU tested at less than one day of age is 389 μmol/L and at seven days is 1,660 μmol/L. For infants with non-PKU HPA, the predicted phenylalanine values are 316 μmol/L at less than one day of age and 437 μmol/L at seven days of age (Figure 9).

The increase in phenylalanine values of infants over time is an important factor in determining the age at which infants should be screened. An important note for mean

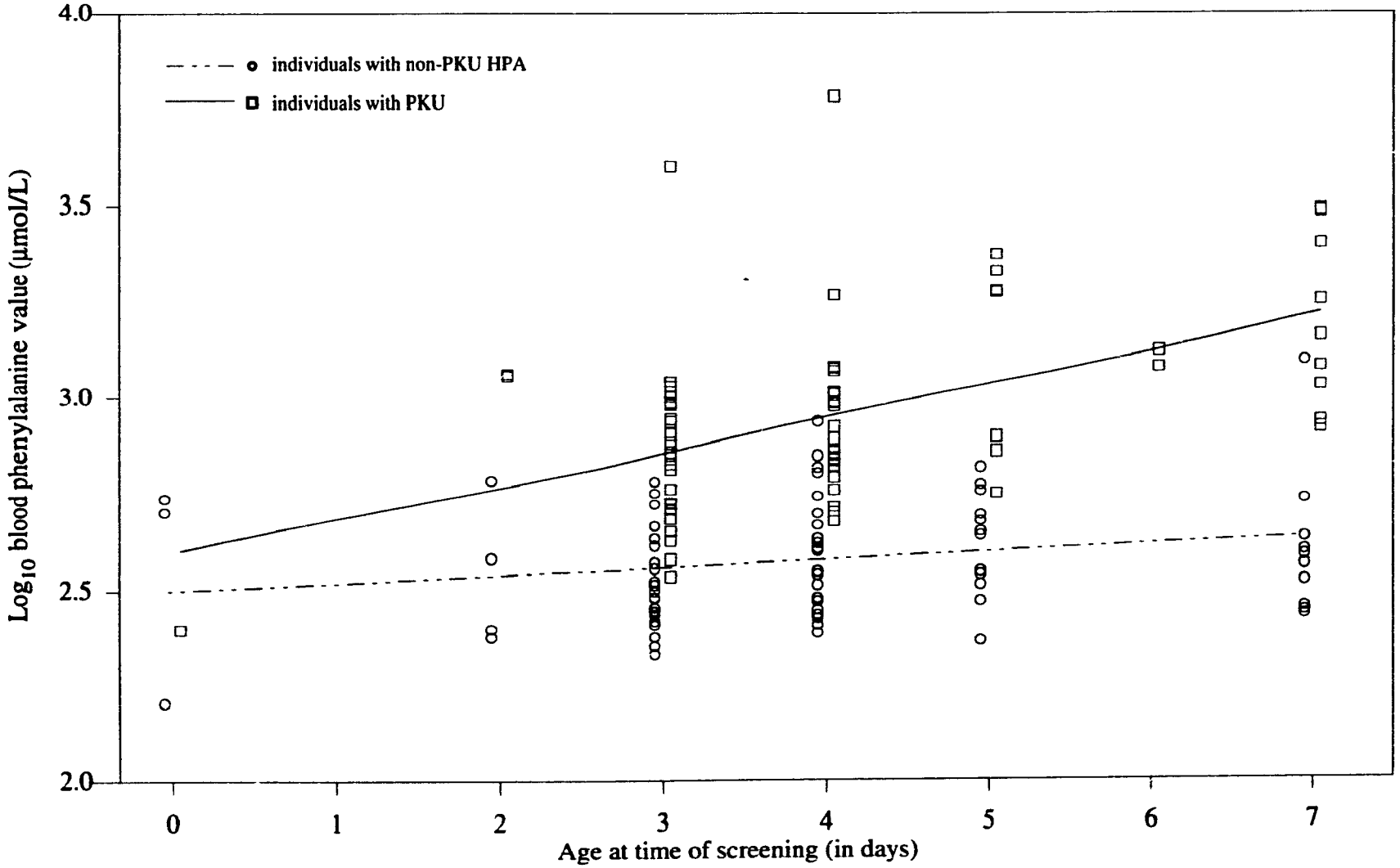

Figure 9. Change in mean blood phenylalanine value by age after birth (day) for individuals with hyperphenylalaninemia.

phenylalanine values calculated here is that they represent the mean phenylalanine values of individuals with PKU or non-PKU HPA *detected by screening*. If any other infants with PKU or non-PKU HPA were missed by the Newborn Screening Program, they would have been missed because their phenylalanine values were below the threshold. If any infants with PKU or non-PKU HPA could be ascertained by other means and their screening phenylalanine values added to the data, the regression lines for non-PKU HPA would be lowered. The regression lines for PKU and their proximity to the screening threshold before two days of age confirms the need to screen newborn infants at no earlier than two days of age.

Analyses of phenylalanine values for normal infants by age at screening is shown in Appendix C.2.4.

5.4.3: Phenylalanine values by gender

Analysis of the initial phenylalanine values by gender for individuals with PKU or non-PKU HPA (Figure 10) showed no significant difference between male and female mean initial phenylalanine values using the Wilcoxon rank-sum test (non-PKU HPA: $p > 0.42$; PKU: $p > 0.50$).

Analyses of phenylalanine by gender for normal infants is shown in Appendix C.2.2.

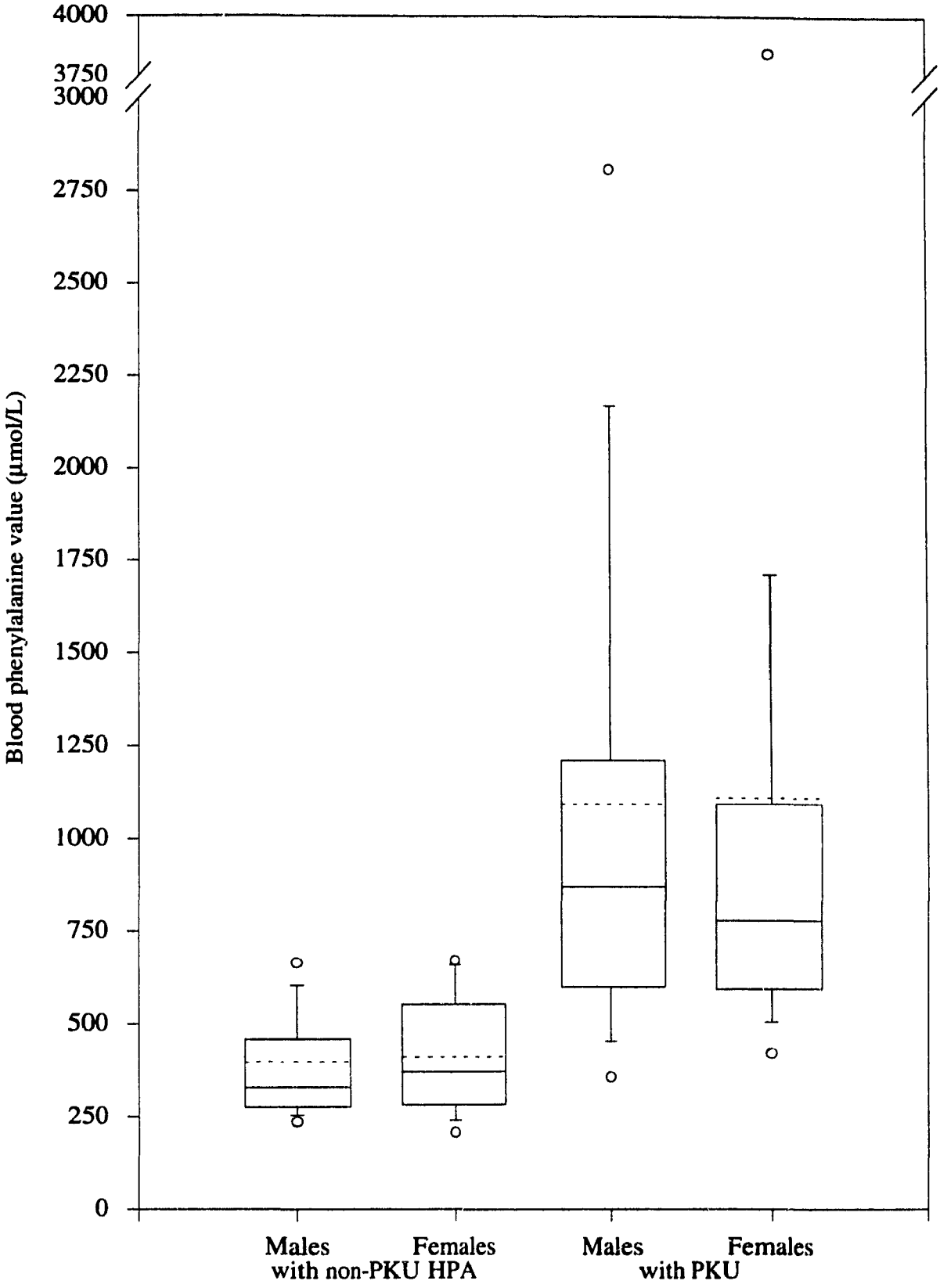

Figure 10. Box plot of initial blood phenylalanine values for individuals with hyperphenylalaninemia, by gender.

5.5: Affected individuals

5.5.1: Classification

Sixty-nine individuals with non-PKU HPA and 73 with PKU were identified by clinical file review at the four hospital centres. Several other groups of individuals were also identified by the clinical file review. These included:

1) an individual with PKU caused by a cofactor defect (who had a registry record indicating PKU, but who was not identified distinctly from an individual who had PKU due to a PAH defect);
2) other individuals with PKU or non-PKU HPA who were born before the start of the Newborn Screening Program, some of whom were screened later in life and thus had registry records;
3) newborns followed for possible HPA who had false positive screening test results or transient HPA;
4) individuals with PKU or non-PKU HPA born and screened outside the province who are now followed by the Newborn Screening Program or a clinic for blood phenylalanine monitoring;
5) family members of individuals with PKU or non-PKU HPA who underwent heterozygote testing.

Information about all of these people, except unaffected family members (who were usually born before the start of the Newborn Screening Program), was collected to verify that these individuals had been included in the Newborn Screening Program registry and to rule out inclusion in the study group. The one individual who had been

detected at the age of two years, because the hospital of birth had not participated in the Newborn Screening Program, was also included in the study group.

One individual with non-PKU HPA was not found in the registry, as were two other individuals who had been identified by the Newborn Screening Program but had since moved out of the province (these two individuals had probably been removed from the registry after they had moved). Several individuals had changed diagnosis (as per their clinic file) from the last time of clinic reporting to the registry, and their disease status was misclassified in the registry. Table 2 gives the classifications of individuals with PKU or non-PKU HPA by their initial registry diagnosis made soon after birth, and by their most recent clinical diagnosis. The misclassifications were due to the nature of the Newboorn Screening Program organization. The first diagnosis of PKU or non-PKU HPA was sent from the clinics to the registry and no notification of the reclassification of the patients based on subsequent clinical findings was ever systematically sent to the Newborn Screening Program. The most up-to-date diagnosis was used in all further analyses of the data.

Comparison of the Newborn Screening Program registry records of individuals with PKU or non-PKU HPA and results of the clinical file review showed discrepancies in diagnosis between the two systems and emphasized a lack of coordination between the Newborn Screening Program and the centres following patients. No common system of identification is used. ID numbers which the registry uses are rarely seen in any clinic file and sometimes change over the lifetime of a patient as the registry is restructured. Current addresses or diagnostic information is not routinely kept by the registry, except for patients seen by the clinic at CHUL which also keeps the registry. Up-to-date

Table 2. Current clinical diagnosis for individuals compared with initial classification by the Newborn Screening Program laboratory. Shaded cells represent individuals misclassified by the registry.

	Clinical diagnosis at last clinic visit			
Classification in the registry	No persistent HPA	Non-PKU HPA	PKU	Total
No registry record	7	2	1	10
Non-PKU HPA	5	*71	2	78
PKU	1	6	70	77
Total	13	79	73	165

* Ten of the individuals classified as non-PKU HPA in the registry had no clinical file, so registry information alone was used as their clinical diagnosis.

information about phenylalanine values is added to the registry only if the centres send blood spots on filter paper to the registry for evaluation, instead of analyzing the blood sample themselves. All centres do, in fact, try diligently to maintain contact with their PKU and non-PKU HPA patients. A lack of coordination with the Newborn Screening Program is evident, however. This presents a problem especially when patients move within the province. One individual had a file in three clinics, all of which assumed that he was currently being followed at another clinic.

File review information was combined with registry information to produce a database of information about these individuals. Where there were discrepancies in information (such as current diagnosis), the information from the file review was used in the analysis. Information missing from the database after combining data from the file review and registry is shown in Table 3.

5.5.2: Incidence of hyperphenylalaninemia

The number of individuals with HPA due to PAH defects identified by file review or the Newborn Screening Program and born in the province between January 1, 1970 and December 31, 1992 was 152 (73 PKU and 79 non-PKU HPA). Of those, 123 were born between January 1, 1973 and March 31, 1990 (the time for which annual reports were produced)(56 PKU and 67 non-PKU HPA). One individual with PKU caused by a cofactor defect (non-PAH HPA) was also identified. One infant born in 1972, who had a clinic file, was not screened and was found to have PKU in 1974. No screened infant has ever been found to have HPA by any other means of ascertainment.

Table 3. Number of files with missing observations. The following variables had no missing values: follow-up, date of birth and gender

Variable	non-PKU HPA	PKU
Ethnicity	13	0
Place of birth	3	0
Low phenylalanine diet	9	0
Date of initial test	0	2
Phenylalanine value on initial test	1	3
Clinic	1	0
Total number of individuals	79	73

An estimate of incidence of PKU and non-PKU HPA can also be derived from the number of infants screened from January 1, 1973 to March 31, 1990. Based on the number of screened infants, the estimated incidence of PKU is 1 / 27,021 and non-PKU HPA is 1 / 22,585, giving a combined incidence of 1 / 12,302. This combined incidence predicts a carrier rate of the PAH gene among screened infants of 1 / 56. Using estimates of the incidence of PKU and non-PKU HPA derived from the number of infants screened, it is possible to calculate the expected number of individuals with PKU or non-PKU HPA that were not screened who may remain undetected in the population. Since an estimated 64,516 infants were not screened between January 1, 1973 and March 31, 1988, the expected number of undetected individuals for this period with PKU is approximately two, and the expected number with non-PKU HPA is approximately three.

Since the population screening from 1973 to 1988-89 was not 100%, estimates of incidence based on population live births would differ from the estimates given above. An estimate of incidence with "live births" as the denominator would give a slightly lower incidence of PKU and non-PKU HPA. These estimates, however, would not take into account that infants who are not screened had no other means of ascertainment if they were not mentally retarded.

Since the start of the Newborn Screening Program, only one individual has been identified with a cofactor defect in phenylalanine metabolism. The incidence of non-PAH HPA is very low, and estimation procedures are again subject to the population denominators chosen. Presumably, any other individual with a cofactor defect would have been ascertained, if not by the screening test then by other means, because of the lethal phenotype involved. No other such individual has been detected in the province.

The denominator used for the incidence calculation is then number of live born infants, not number of infants screened, because of the multiple sources of ascertainment. For the period 1970 to 1992 (inclusive), the estimated incidence of non-PAH HPA is 1 / 2,037,231.

5.5.3: Gender ratios

The overall gender ratio of infants screened between 1973 to 1990 (see Appendix C.3.1) was 1.07 males to 1 female. This ratio was used as the "expected" ratio when comparing gender ratios of other groups of infants. No gender bias in screening was suspected in the normal population of newborn infants.

The null hypothesis that there was no gender ratio distortion in individuals with PKU or non-PKU HPA was tested. The overall ratio of 90 males to 61 females seen among individuals with PKU or non-PKU HPA together (now considered to be affected, at their current age) was significantly different from the expected gender ratio of 1.07 ($\chi^2_1 = 4.3$, $0.05 > p > 0.025$). When the group was subdivided into PKU and non-PKU HPA, there was no significant difference in gender ratio from expected for the PKU infants (40♂:33♀, $\chi^2_1 = 0.4$, $p > 0.25$), but there was a significant difference in gender ratio for the non-PKU HPA infants (50♂:28♀, $\chi^2_1 = 5.2$, $0.025 > p > 0.01$). Although there was no significant difference in gender ratio for individuals with PKU, the distortion of gender ratio was in the same direction for both groups.

Analysis of age at screening by gender for individuals with PKU or non-PKU HPA (combined) showed no significant difference between the mean age at initial screening for males and females, using the Wilcoxon rank-sum test ($p > 0.16$).

5.5.4: Year of birth

The mean number of individuals with PKU identified per year from 1970 to 1992 was 3.17, with 3.44 individuals with non-PKU HPA identified per year in the same time period. The number of individuals that were born in the province each year with PKU or non-PKU HPA is shown (Figure 11).

The distributions of the annual number of individuals with PKU was compared to the annual number of individuals with non-PKU HPA, to test if there was a difference in the number of cases identified annually. There was no significant difference in the number of individuals with PKU or non-PKU HPA detected annually from 1970 to 1992 ($\chi^2_{22} = 26.8, 0.25 > p > 0.10$) Based on the similarity of their annual distributions, the annual number of individuals with PKU or non-PKU HPA were combined for further testing of year of birth.

Homogeneity in the incidence of PKU and non-PKU HPA detected annually was shown by testing the number of individuals with PKU or non-PKU HPA per reporting period against the number of individuals screened per reporting period (Figure 3). There was significant variation in the incidence of PKU and non-PKU HPA per reporting period ($\chi^2_{16} = 29.2, 0.05 > p > 0.01$).

5.5.5: Month of birth

The distribution of individuals with PKU or non-PKU HPA by month of birth is shown in Figure 12. A small sample χ^2 test (Smith, 1986) was done to compare the distribution of the month of birth of individuals with non-PKU HPA to the month of birth

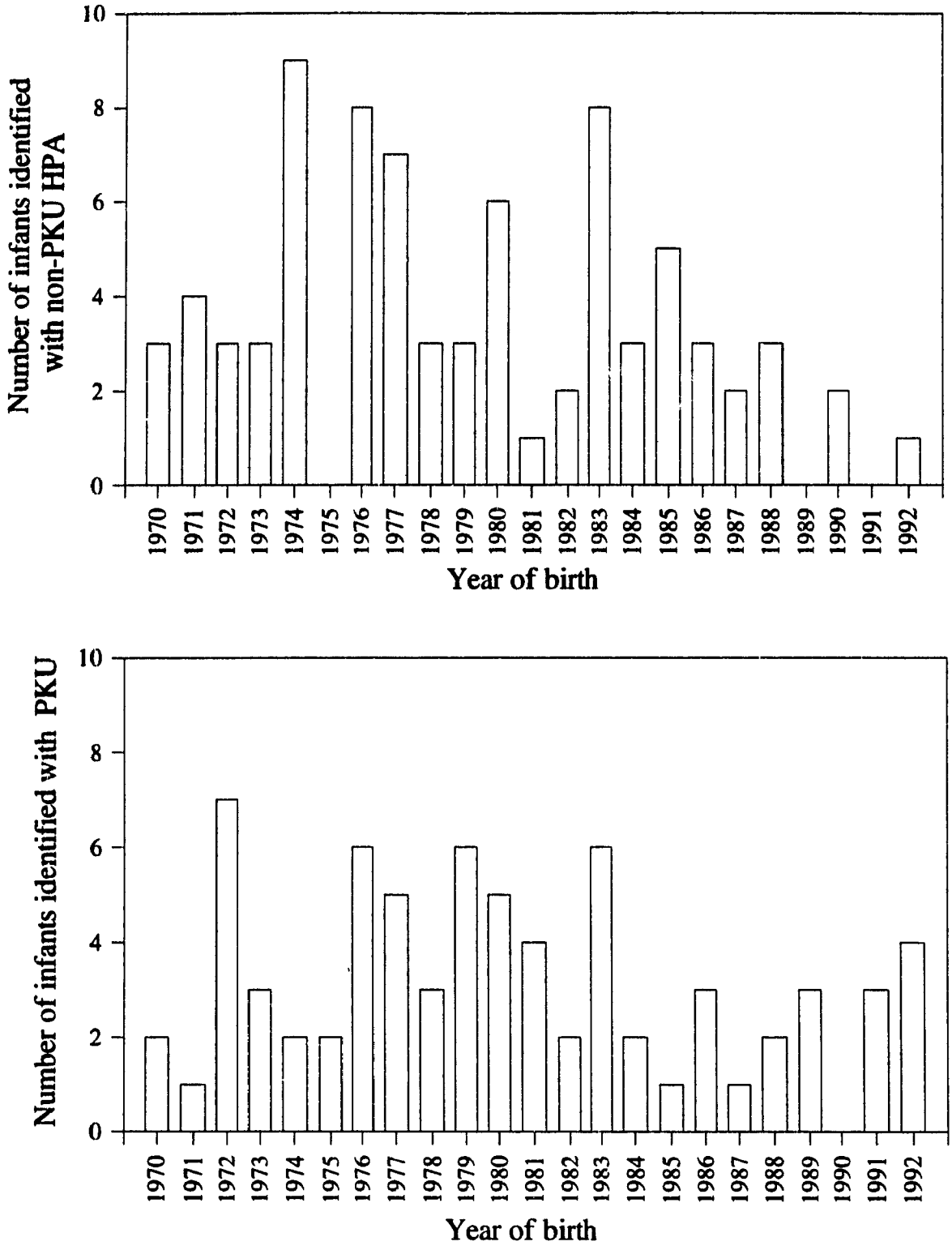

Figure 11. The annual number of individuals with non-PKU HPA or PKU identified by newborn screening in Quebec. Not all individuals born in 1992 have had their disease status confirmed.

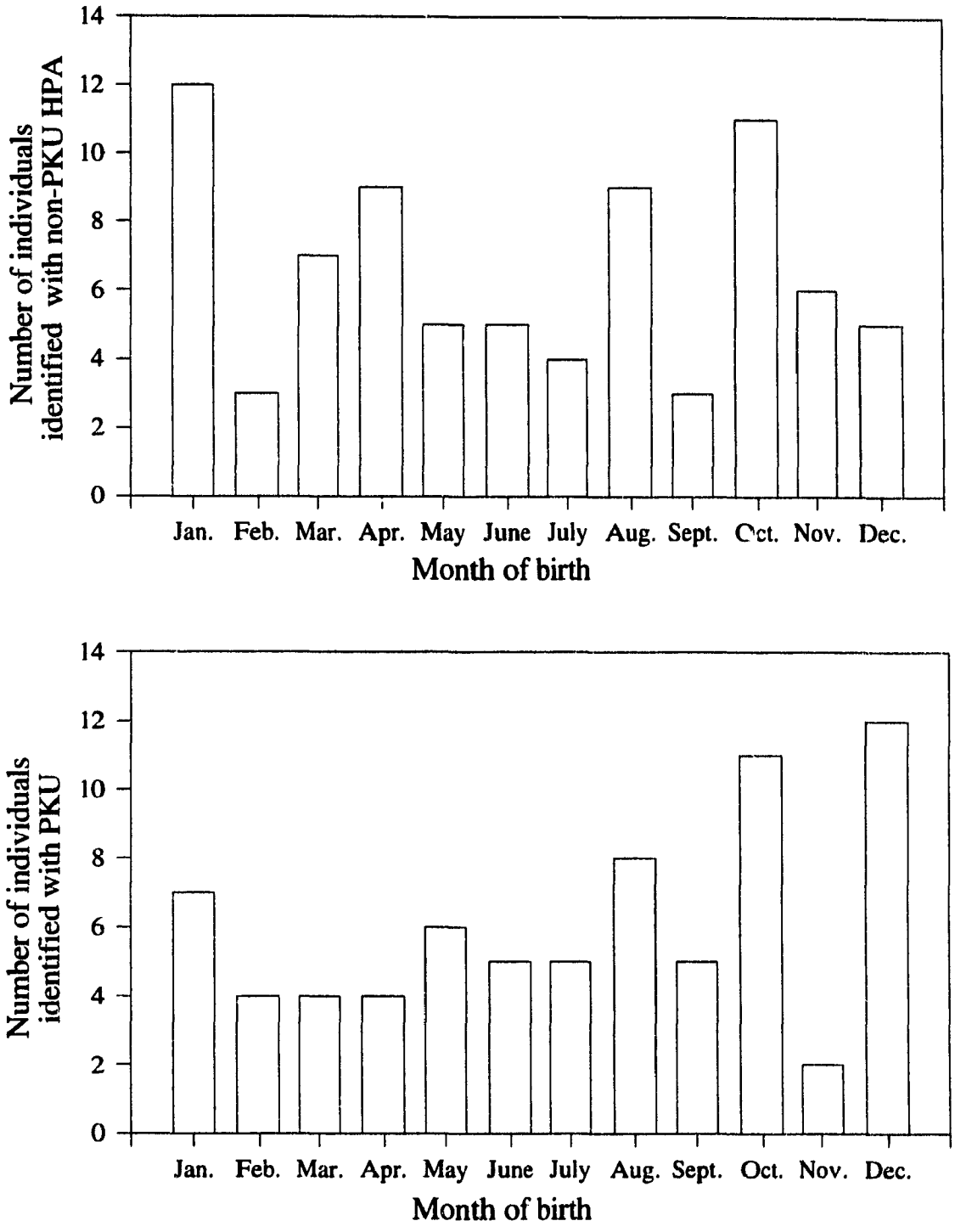

Figure 12. The number of individuals with non-PKU HPA or PKU by month of birth, 1970 to 1992.

of those with PKU. There was no significant difference in the distribution of month of birth between individuals with PKU or non-PKU HPA ($Z = -0.23$, $p > 0.5$). Homogeneity of these two distributions allows them to be combined for further analysis.

The distribution of month of birth of individuals with PKU or non-PKU HPA was compared to the number of infants born in each month (1970 - 1992). This was done to test the hypothesis that the number of individuals with PKU or non-PKU HPA born each month does not differ significantly from the number expected, based on the number of births per month. There was a significant difference in the number of individuals identified with PKU or non-PKU HPA each month ($\chi^2_{11} = 22.7$, $0.025 > p > 0.01$). This test is not an efficient test of the seasonality of the distribution, but only states that the distribution differs from month to month.

Analyses of phenylalanine values by month of birth for normal infants are shown in Appendix C.2.3.

5.5.6: Age at screening

The distributions of age for individuals with PKU or non-PKU HPA at the time of screening were compared (Figure 13). Both distributions were significantly different from a normal distribution as indicated by the Shapiro-Wilk test (PKU: $p < 0.0001$; non-PKU HPA: $p < 0.0001$). There was no significant difference in mean age at screening between individuals with PKU or non-PKU HPA as determined by Wilcoxon rank-sum test ($Z = 0.16$, $p > 0.87$).

Similar age at screening in individuals with PKU or non-PKU HPA would suggest that PKU and non-PKU HPA are equally identifiable at a given age. A comparison of the

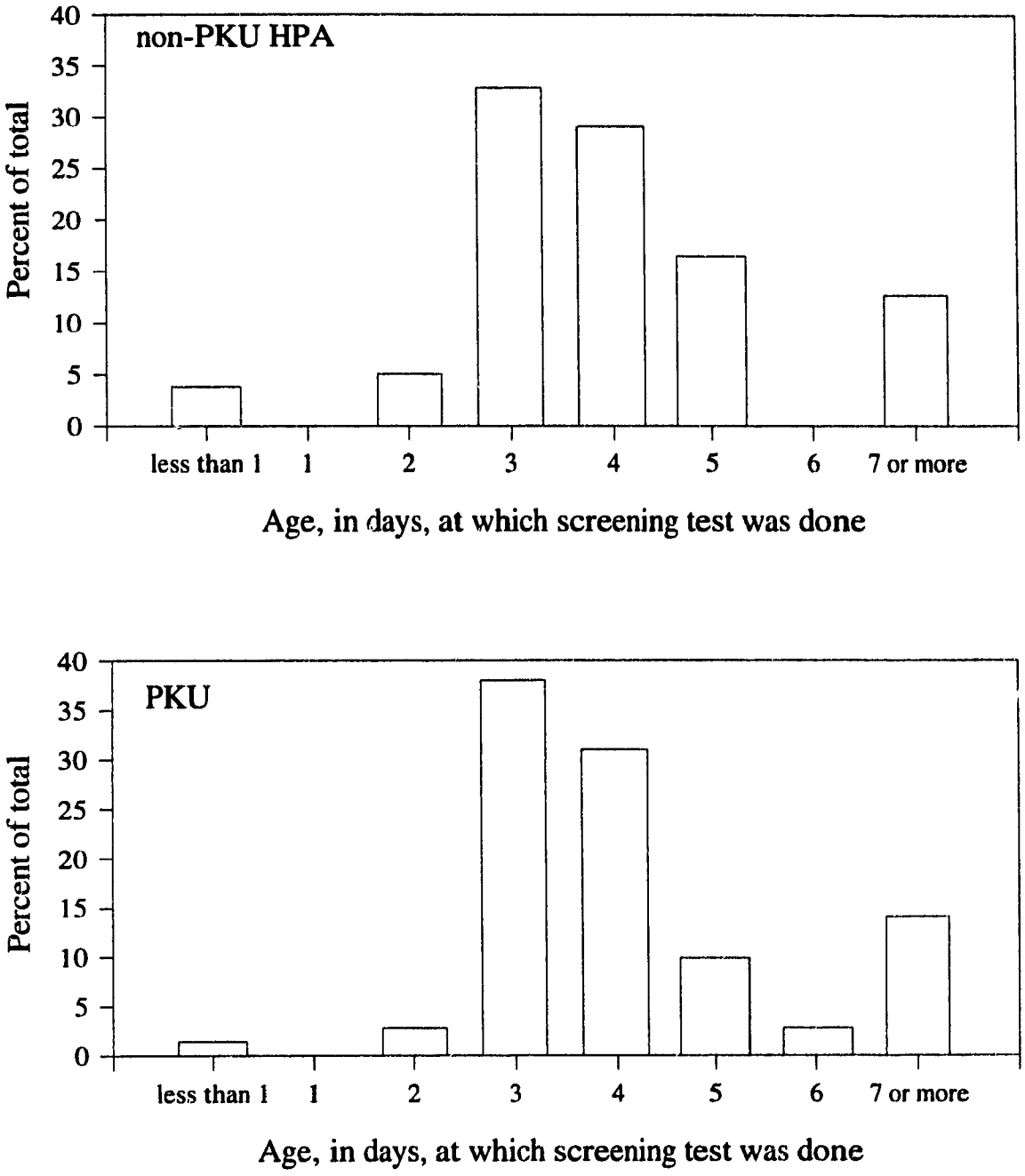

Figure 13. The distribution of the age (days) at which the screening test was done for individuals with hyperphenylalaninemia.

distribution of the age at screening of PKU and non-PKU HPA (combined) to the distribution of age at screening for all screened infants would give an indication of whether infants with PKU or non-PKU HPA are screened at the same age as normal infants. If infants with PKU or non-PKU HPA were screened later in life than other infants, it would be suspected that cases of PKU or non-PKU HPA had been missed because of early screening.

Analyses of age at screening of normal infants are shown in Appendix C.3.2.

5.5.7: Ethnicity

The ethnic distributions of the individuals with PKU or non-PKU HPA were compared (Table 4). There was no significant difference in the distribution of ethnicity between individuals with PKU or non-PKU HPA as tested by a small sample χ^2 test (Smith, 1986) ($Z = 1.03$, $p > 0.15$). The similarity of the distributions for PKU and non-PKU HPA allowed the two distributions to be combined for further analysis. The combined distribution of ethnicities for individuals with PKU or non-PKU HPA was compared to the ethnic population distribution (1991) of individuals under the age of 25. This was done to test the hypothesis that these individuals were identified randomly from the general population of the province. There was a significant difference in the ethnic composition of the group of individuals with PKU or non-PKU HPA ($Z = 2.23$, $p < 0.0001$) as tested by a small sample χ^2 test (Smith, 1986).

Among the cases of non-PKU HPA, the number of individuals of Mediterranean origin was more than four times the expected number. Also, in the category "French and

Table 4. The ethnicity of individuals with non-PKU HPA and PKU, and the ethnic composition of the Québec population (1991) under age 25.

Ethnicity	HPA		PKU		Québec Population
French Canadian	52%	(41)	74%	(54)	73.1%
British	4%	(3)	4%	(3)	3.0%
Mediterranean	11%	(9)	4%	(3)	3.8%
Other European	0%	(0)	1%	(1)	1.0%
Jewish	1%	(1)	0%	(0)	1.0%
Black	1%	(1)	0%	(0)	0.8%
Asian	0%	(0)	0%	(0)	1.4%
French and other	8%	(5)	4%	(3)	3.4%
English and other	3%	(2)	4%	(3)	4.4%
Other	0%	(0)	4%	(3)	8.1%
Missing	21%	(17)	4%	(3)	--
Total	100%	(79)	100%	(73)	100.0%

other" for individuals with non-PKU HPA, four of the five individuals have Mediterranean origins for "other".

The large number of individuals with PKU or non-PKU HPA missing ethnicity information compromises the conclusions that can be drawn from this analysis. It is unclear whether there was any bias in the recording of ethnicity since it is possible that ethnicity was not recorded when the parents were English or French Canadian like the majority of the population seen at that centre. Ethnicity was missing for cases where no clinic file could be found, thus the larger number of individuals with non-PKU HPA that were missing this information.

The missing information about ethnicity of non-PKU HPA individuals leaves the comparison of ethnic distributions of non-PKU HPA with the general population and PKU inconclusive. If all "missing" ethnicities are actually French Canadian, the ethnic distribution of individuals with non-PKU HPA would be as expected from the population. If a large proportion of "missing" are not French Canadian, this would indicate a large distortion of the ethnic distribution for non-PKU HPA individuals from that of the general population. Only one association could not be weakened by the assignment of any ethnicities to "missing" values: the excess of individuals of Mediterranean origin. The combined contribution of at least 14% (counting a contribution of 50% of four individuals from the "French and other" category) shows a significant increase over the expected population prevalence of people of Mediterranean origin.

5.5.8: Regional distribution

The distributions of regions of birth for the number of individuals with PKU and the number of individuals with non-PKU HPA were compared (Figure 14, Figure 15). The number of registered live births in each region (from Bureau de Statistique de Québec statistics) was used as a population comparison for individuals with PKU or non-PKU HPA. For these comparisons, Region 00 was excluded since it is not a Québec territory. Region 00 comprises the Queen Elizabeth Island nursing stations north of Québec, and thus not included in the registry of live births for the province of Québec (Bureau de la Statistique du Québec).

For PKU and non-PKU HPA, heterogeneity of the distribution of regions of birth compared to provincial births might indicate clustering of different mutations in different regions. Results of comparisons showed that the distribution of the incidence of PKU is significantly different from the distribution of the incidence of non-PKU HPA ($\chi^2_8 = 30.0$, $p < 0.001$). The distribution of the incidence of non-PKU HPA differs significantly from the expected distribution (that of live born infants in each region) ($\chi^2_9 = 25.1$, $p < 0.01$), but the distribution of the incidence of PKU did not differ significantly from the expected distribution ($\chi^2_9 = 12.6$, $p > 0.1$). The distributions of region of birth for PKU and non-PKU HPA were also compared to the distribution of the proportion of samples with phenylalanine values above the threshold (Section 5.3.2). The distribution of the proportion of samples with phenylalanine values above the threshold differed significantly from the distribution of the incidence of PKU ($\chi^2_{10} = 24.2$, $p < 0.01$), and was nominally significantly different from the distribution of the incidence of non-PKU HPA ($\chi^2_{10} = 20.5$, $p < 0.05$).

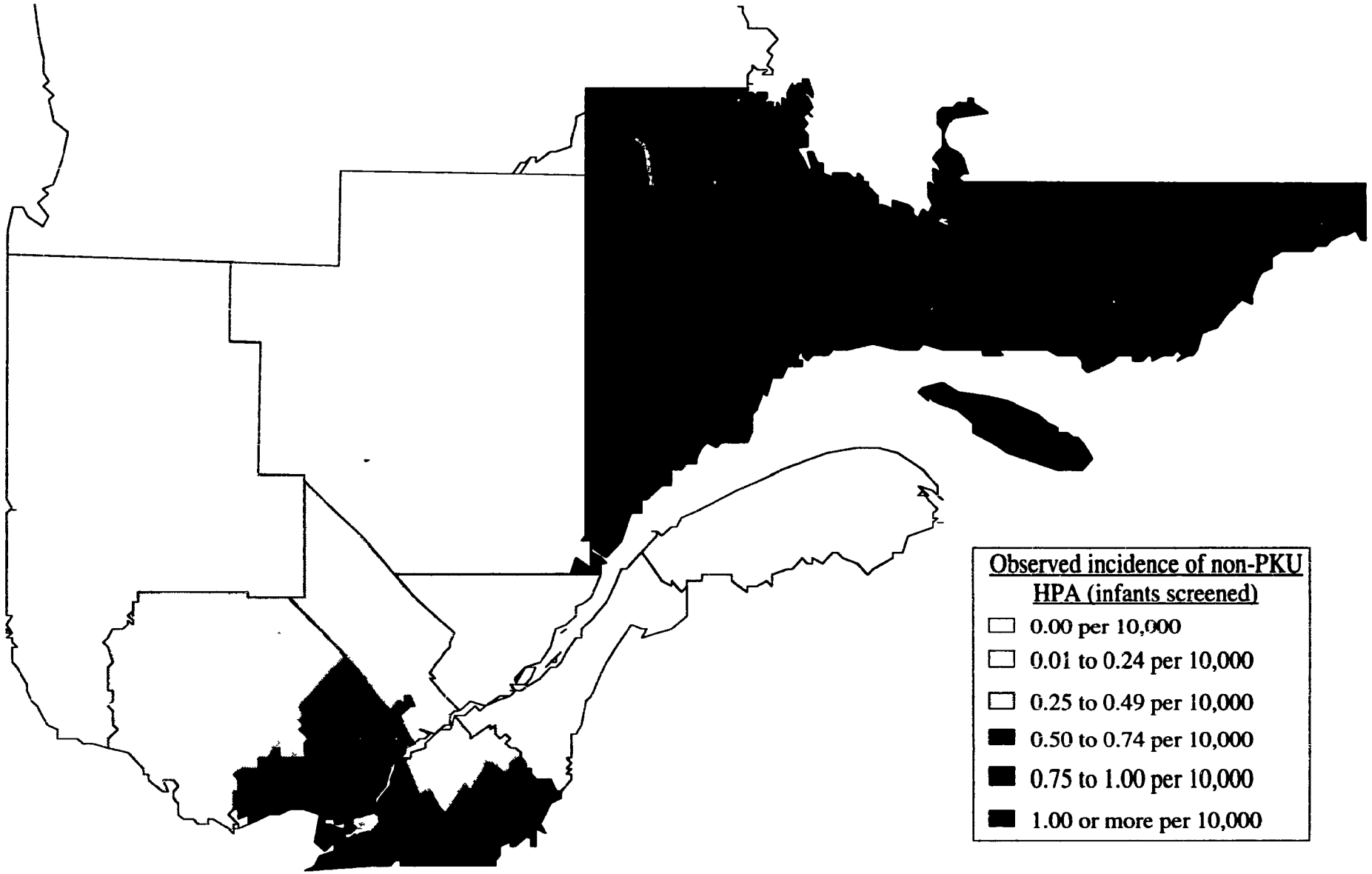

Figure 14. Observed incidence of non-PKU HPA by administrative region in the province of Québec.

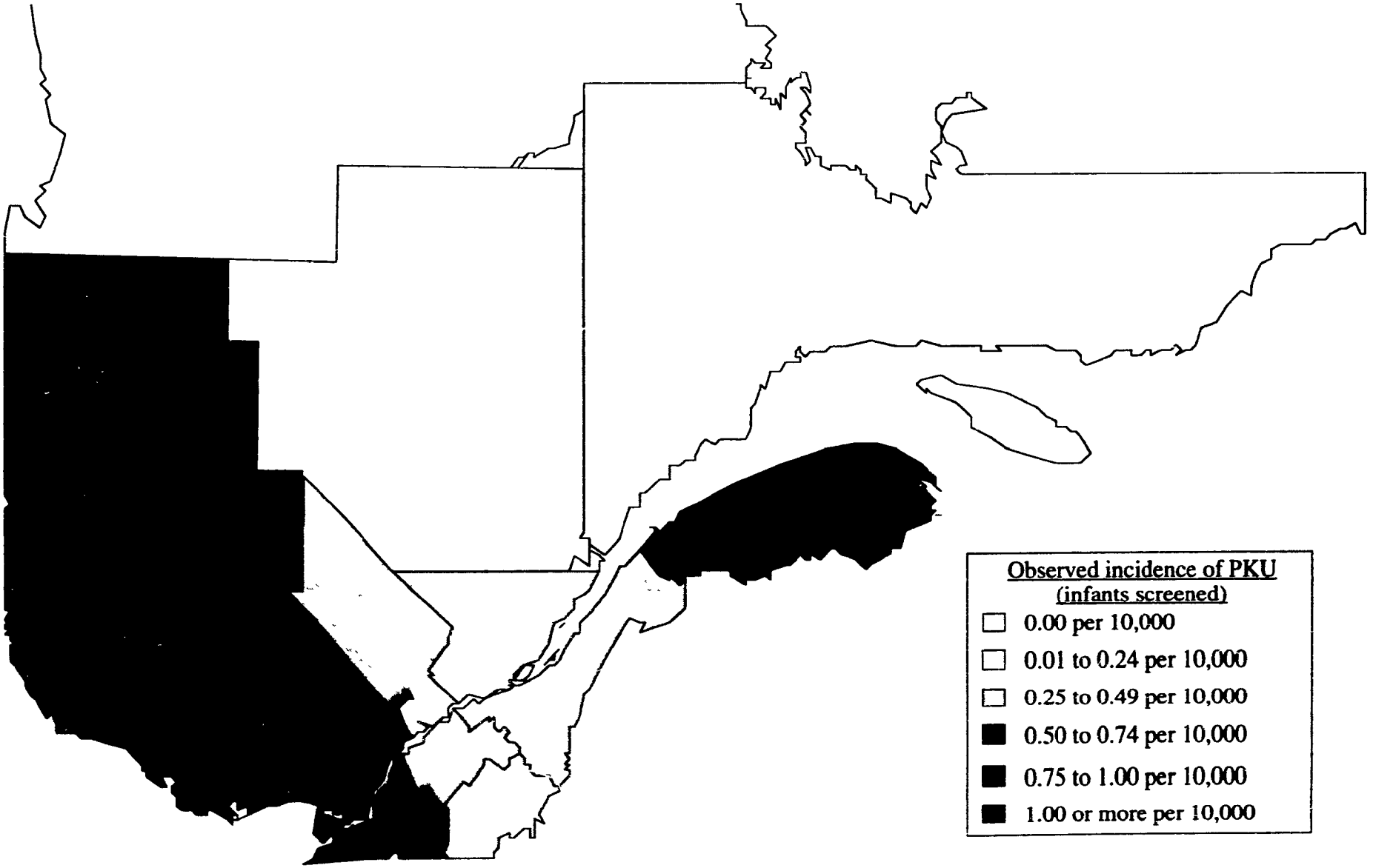

Figure 15. Observed incidence of PKU by administrative region in the province of Québec.

5.6: Follow-up

5.6.1: Follow-up of affected individuals

Ten of the individuals with PKU or non-PKU HPA listed in July 1992 in the Newborn Screening Program registry had no clinic files at any of the four centres. These individuals were lost to follow-up in the strictest sense of the term, as none of their clinic files could be found. A list of the names and birth dates of individuals with no files was sent to the Montreal Children's Hospital and Hôpital Sainte-Justine, but this did not result in retrieval of any of the ten files. At Hôpital Sainte-Justine, the nine missing individuals not only had no file in the clinic, but also had no hospital file. All ten were born after the start of the Newborn Screening Program and had non-PKU HPA (Table 5). They had been followed for an average of 1.6 years based on information in the registry. Two had sufficiently elevated phenylalanine values at their final visit to be considered as truly having non-PKU HPA; four had phenylalanine values within the normal range at their final clinic visit; and four had abnormally elevated tyrosine as well as phenylalanine values, possibly indicating tyrosinemia. These ten people were included in all analyses because they were initially classified by the registry as hyperphenylalaninemic, and had been followed (as indicated by several phenylalanine test results in the registry). The eight individuals who did not have clearly elevated phenylalanine values were not excluded from the analyses due to their similarity to those individuals with borderline phenylalanine who had a clinical file confirming the diagnosis of non-PKU HPA. For example, one individual with confirmed non-PKU HPA did not have a record in the Newborn Screening Program registry because his phenylalanine values had been sufficiently low at one point that he was reported to the registry as having "transient

Table 5. Follow-up of individuals identified (non-PKU HPA or PKU) 1970 to 1992, by clinic.

Diagnosis	No clinic file	Lost to follow up	Being followed	Uncoop-erative	Moved or died	Total
Clinic: The Montreal Children's Hospital						
non-PKU HPA	1	13	7	1	1	23
PKU	0	2	9	0	2	13
Clinic: Le Centre Hospitalier de l'Université de Sherbrooke						
non-PKU HPA	0	0	3	0	2	5
PKU	0	0	4	1	0	5
Clinic: L'Hôpital Sainte-Justine						
non-PKU HPA	9	4	11	2	1	27
PKU	0	7	19	1	3	30
Clinic: Le Centre Hospitalier de l'Université de Laval						
non-PKU HPA	0	10	12	1	0	23
PKU	0	0	20	4	1	25

HPA" at an early age, although further follow-up by the clinic proved that he had non-PKU HPA.

Individuals with PKU were significantly more likely to be followed (71%) than those with non-PKU HPA (42%) ($\chi^2_1 = 5.9$, $p < 0.025$). Analysis of follow-up by age and gender is calculated as Kaplan-Meier survival curves where the survival probability is calculated as the probability of still being followed at any given age (Figure 16). Data for individuals whose files could not be found, who were lost to follow up, or who were uncooperative, had uncensored data values in the analysis. Data for individuals who were followed, had moved, or had died were right censored. Survival age was calculated as the age at last clinic visit.

The survival curves (Figure 16) illustrate that females with non-PKU HPA are more likely to be followed than males with non-PKU HPA from two years of age, and are at least 50% more likely to be followed after nine years of age. The median age of follow-up for individuals with non-PKU HPA is 8 years for males and 13 years for females. Success of follow-up is more evident for individuals with PKU, for whom follow-up is 100% until age 12 years for females and until age 14 years for males. Males with PKU have a greater chance of being followed than females with PKU while they are between the ages of 12 and 18 years. The median age of follow-up for both males and females with PKU is about 20 years. Over the age of 20, follow-up of individuals with PKU or non-PKU HPA is similar, but the numbers of individuals being followed are very small.

The Kaplan-Meier survival curves for Québec patients are consistent with other studies which suggest that loss to follow-up occurs at the time at which dietary

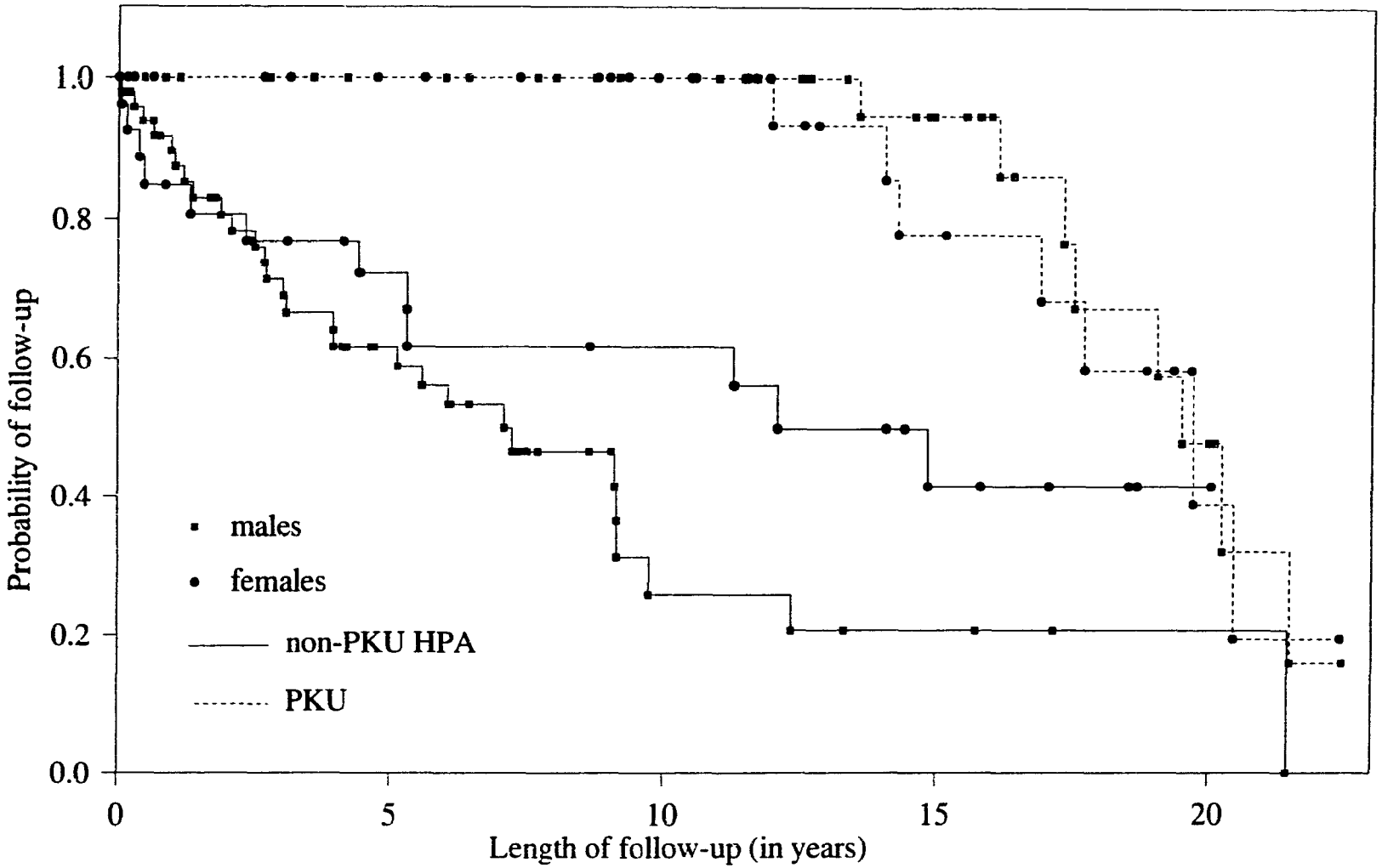

Figure 16. Kaplan Meier survival curves for the follow-up of individuals with hyperphenylalaninemia by gender. Data for individuals who were still being followed at the time of clinical file review are right censored values. The age at last clinic visit is the age used in calculations and diagnosis is based on the most recent information available (clinic or registry).

intervention is stopped or relaxed. This occurs soon after birth for individuals with non-PKU HPA and after approximately 12 years of age for individuals with PKU. Recent follow-up and dietary recommendations will hopefully encourage individuals with PKU or non-PKU HPA to maintain contact with the clinics for lifelong monitoring and dietary management, resulting in increased surveillance of individuals with non-PKU HPA as well as those with PKU.

A frequent reason for loss to follow-up was a change in address and telephone number without notifying the clinic. Some individuals were not pursued for follow-up by centre personnel because of the perceived lack of necessity for follow-up of individuals with non-PKU HPA who did not require dietary therapy. Most of all, busy centre schedules do not allow for active pursuit of every patient who did not volunteer to be followed. Some individuals with PKU or non-PKU HPA who had not been recently seen at a centre had their clinical files set aside after the file review conducted as part of this research, and an effort was made to contact them. The individuals who were to be contacted, both males and females, were mostly over the age of 12 years and would be offered reproductive counselling and biochemical evaluation.

5.6.2: Prediction of disease classification based on initial values

The range of phenotypic variation in both initial and subsequent phenylalanine values for individuals with PKU or non-PKU HPA is show in Figure 17. Although both PKU and non-PKU HPA are caused by defects in the same gene, there was large variation in the blood phenylalanine levels within and between these two groups. Phenylalanine levels during an individual's life were calculated from the average of all the blood

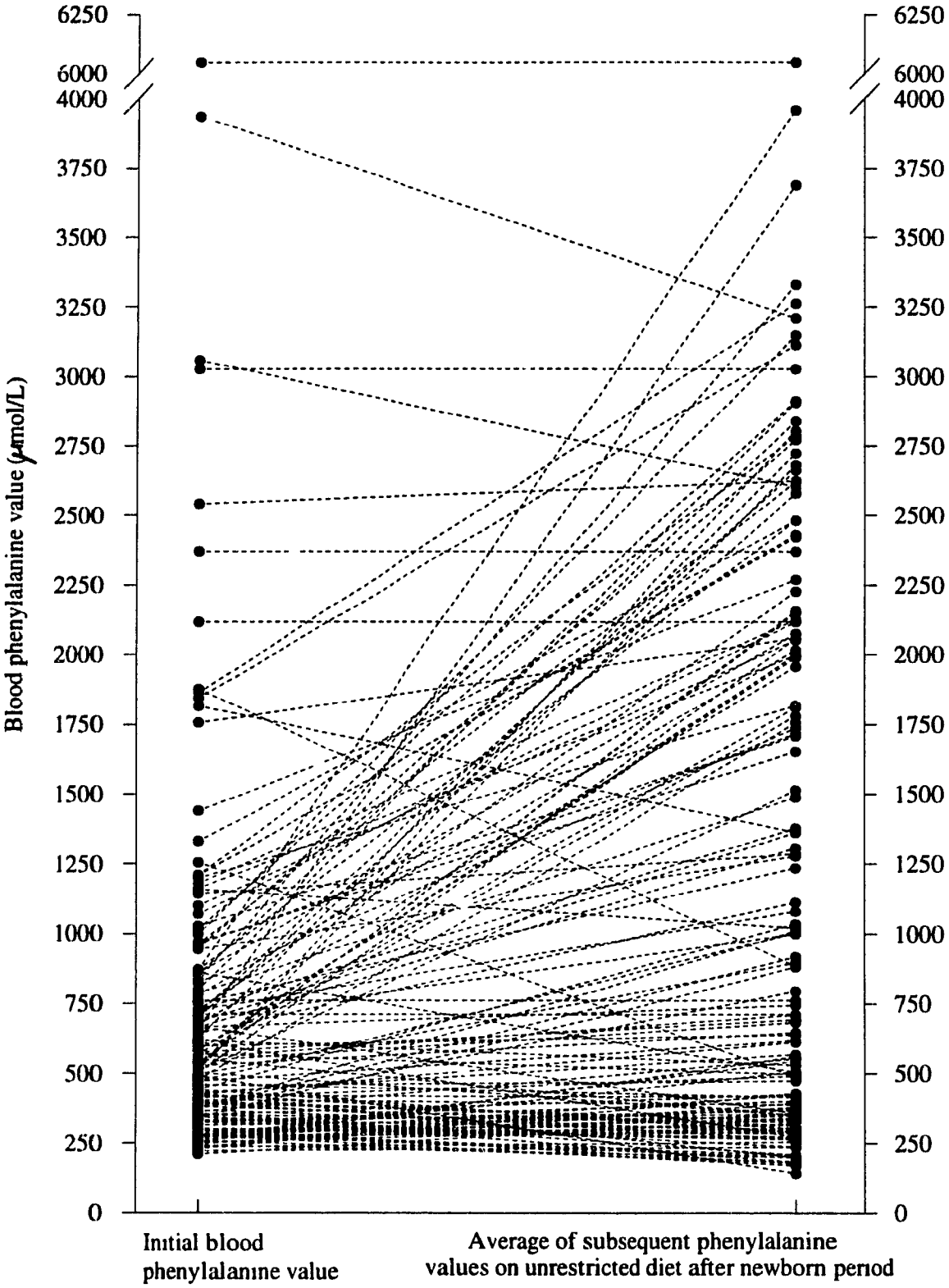

Figure 17. Comparison of initial blood phenylalanine values (screening test) and the average of phenylalanine value later in life for untreated individuals with PKU or non-PKU HPA.

phenylalanine values given (except for the initial screening value) for all tests that were performed while the individual was not on a low phenylalanine diet. It can be seen (Figure 17) that the initial blood phenylalanine did not uniformly reflect the eventual blood phenylalanine concentrations for the individual.

Discriminant analysis quantitatively demonstrated the role of initial phenylalanine values in predicting eventual disease classification. Discrimination between individuals with PKU or non-PKU HPA by phenylalanine values at first screening resulted in an overall 22% misclassification as to eventual clinical classification as PKU or non-PKU HPA. Only 2% of individuals with actual non-PKU HPA were misclassified as PKU, but over 41% of individuals with PKU were misclassified as non-PKU HPA. The R^2 value for this analysis indicated that initial phenylalanine values alone accounted for 23.3 % of the variance between the PKU and non-PKU HPA categories.

Including the age at screening did not decrease the misclassification rate of individuals with PKU or non-PKU HPA by the initial phenylalanine value since 23% were misclassified, primarily due to the error rate of misclassification of individuals with PKU (Table 6). Age at screening did little to explain the variance in the difference between the two categories ($R^2 = 0.0005$), whereas initial phenylalanine value retained the high R^2 of 0.23.

The estimation procedure by discriminant analysis gave an underestimate of the misclassification rates for the models, since they were estimated from the same data that was used to establish the estimation coefficients for the models.

Table 6. Classification of individuals with hyperphenylalaninemia by discriminant analysis based upon initial phenylalanine value and age at first screening test, versus final diagnosis. Four individuals remain unclassified because of missing initial phenylalanine value or age at first screening test.

	Classification by initial phenylalanine value and age at first screening test		
Final diagnosis	non-PKU HPA	PKU	Total
non-PKU HPA	73 94%	5 6%	78 100%
PKU	28 40%	42 60%	70 100%
Total	101 68%	47 32%	148 100%

5.6.3: Genotype-phenotype correlations

Nineteen individuals were identified who had been identified by the Newborn Screening Program since 1970 and had both their PAH mutations genotyped. Their mutations had known PAH enzyme activity (Section 4.5). One additional individual, not used in this analysis, had both mutations genotyped, but with unknown enzyme activity. The PAH enzyme activity of an individual was taken as the mean of the predicted enzyme activity for each of the two mutations belonging to that individual. All but one individual had PKU. The one remaining individual had severe non-PKU HPA and had previously been classified as having PKU. Fifteen of the nineteen individuals (79%) had a PAH genotype that conferred 0% PAH enzyme activity. This selection bias in the sample made statistical analysis unfeasible. Examination of the data and the relationship between PAH enzyme activity and blood phenylalanine values suggests that linear analysis of their correlation is inadequate to describe the distribution of values (Figure 18).

Individuals with a predicted enzyme activity of 0% show large variation of initial phenylalanine values ranging from 500 to 4,000 µmol/L. No relationship was noted between age at screening of these individuals and the magnitude of their initial phenylalanine values. All individuals with predicted PAH activity greater than 0% have initial phenylalanine values between 500 and 900 µmol/L. Genotyping of more such individuals is necessary to demonstrate any correlation between their PAH enzyme activity and their initial phenylalanine values.

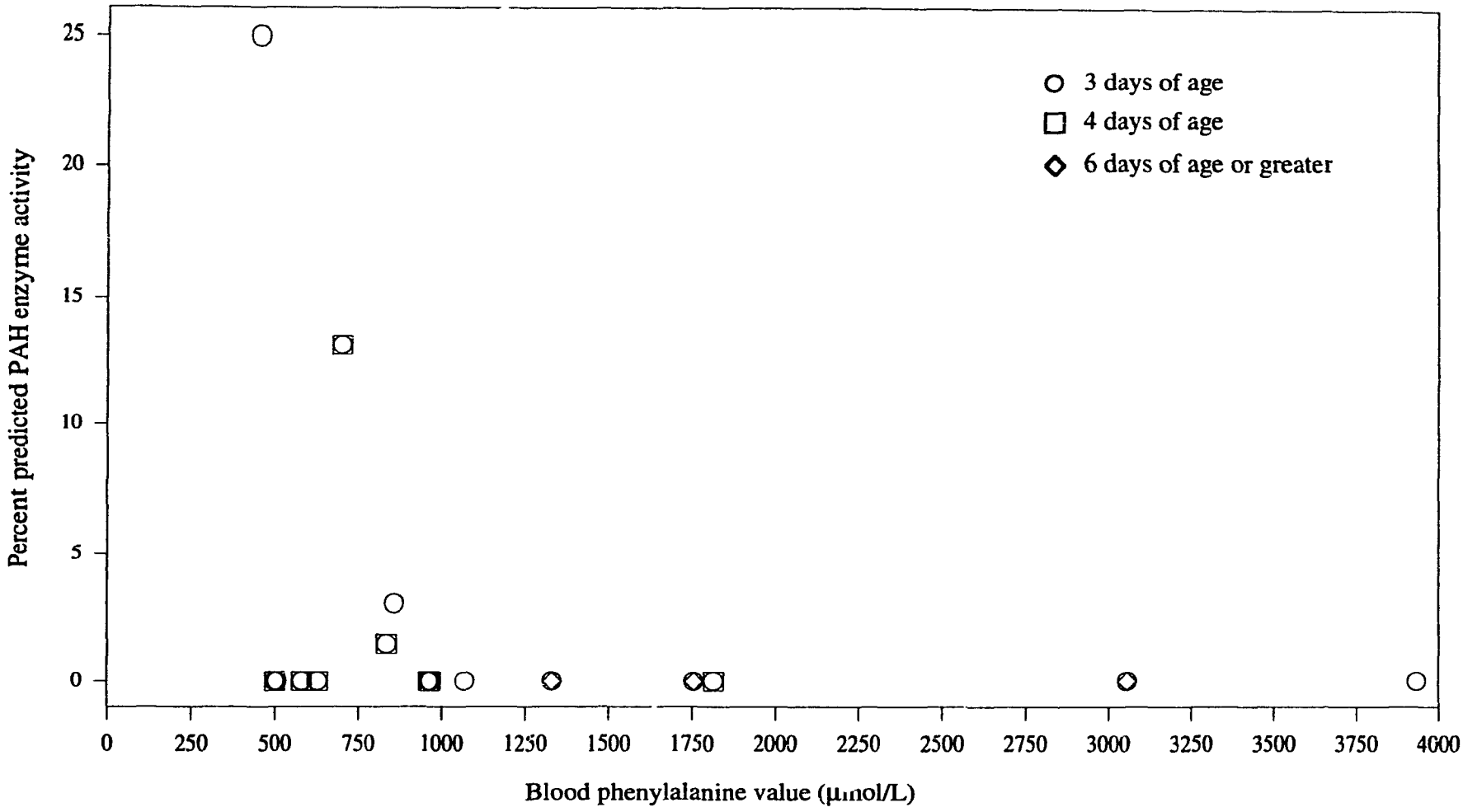

Figure 18. Relationship between blood phenylalanine values (initial screening) and predicted phenylalanine activity (% normal by expression analysis) in PKU cases. Results are grouped by the age at which the infants were screened. The infants six days of age or greater were six, seven and thirty days old, in order of increasing phenylalanine value.

Chapter 6: Discussion

Many issues concerning the implications of the results of the study have already been presented. Limitations and biases in the data have also been described. This discussion will address overall issues raised by this thesis, and will present some ideas for future research.

6.1: Population

It is necessary to consider, as context, the population of Québec as a whole in which the Newborn Screening Program operates. Even if the Newborn Screening Program were to screen 100% of the hospital born population, there are other potential sources of maternal HPA in the province. Québec has a growing immigrant population and women from other countries with elevated phenylalanine levels may not have been screened. Even if they were screened, they may not realize that they are at risk of giving birth to children with maternal HPA effects. The bulk of the child-bearing population of Québec is still too old to have been screened, although this number decreases every year. With midwifery becoming an accepted practice, more children will be born at home without being screened. If screening for hyperphenylalaninemias is to be truly effective, it must be effective in the population of the province at large, as well as among infants born in Québec hospitals.

6.2: Samples

The efficiency and effectiveness of the Newborn Screening Program can be put into perspective by examining such measures as population participation, insufficient

samples and samples analyzed over time. Although 64,516 infants born between 1973 and 1990 were not screened, over 1 / 6 of these infants were born in 1973. At the current rate of screening in live born infants, less than 1,500 infants per year remain unscreened (based on estimates for years when the number of unique infants was recorded). Similar improvement over time is seen in the percentage of sufficient samples and the proportion of infants with test results per samples received. Knowledge and acceptance of screening as a valuable routine test for newborns has grown since 1970, and with it has grown proficiency at completing the screening test in hospitals (J. Morrisette, A. Grenier, personal communication). An example of the screening program's adaptability is demonstrated by a video (in both French and English) that will soon be circulated to hospitals in the province demonstrating how to properly spot blood onto filter paper and avoid unsatisfactory samples (A. Grenier, personal communication).

6.3: Test results: population

The proportion of infants identified with phenylalanine values above the threshold is also a measure of how well a screening program is functioning. In general, if only 1 in 873 babies is recalled for a second blood test in the population, the emotional strain of repeat testing (Sorenson et al., 1984) has been imposed upon very few parents of potentially affected children. Although the high proportion of infants identified with phenylalanine values above the threshold is unsatisfactory in some years (1 in 286 infants in 1983-84), responsive changes in the screening threshold have corrected for the high rate of detection of elevated phenylalanine values.

The proportion of samples exceeding the threshold varies greatly from screening program to screening program. Walker et al. (1981) reported a fluctuation of annual recall rate from 1 / 300 to 1 / 8,000 infants screened over 9 years, also using a threshold of 240 μmol/L. Morris et al. (1983) stressed that with variation in mean phenylalanine values and in the number of individuals above the screening threshold observed in some screening programs, the MRC (Great Britain) recommended threshold of 240 μmol/L may be sufficient to detect all cases of PKU, but may not detect all cases of non-PKU HPA and other PKU variants.

6.4: Test results: affected individuals

Together, the similarity of both age at screening and initial phenylalanine values between genders can be interpreted as a similarity in the detection of males and females with PKU or non-PKU HPA. If females had either lower phenylalanine values or later ages at screening, it might indicate that females with PKU or non-PKU HPA had lower blood phenylalanine values for a given age at screening. On the other hand, the similarity of age at screening and phenylalanine values does not exclude the possibility that individuals of either gender have been missed in screening. If there is a real deficit of females with PKU or non-PKU HPA identified by screening, ascertainment of affected females by other means might reveal them to differ in initial phenylalanine value (the reason they would be missed by screening). This would lower the mean initial phenylalanine value for females with PKU or non-PKU HPA.

6.5: Affected individuals

Classification

Misclassification of diagnosis of PKU or non-PKU HPA in the registry, while not a danger for the affected individuals, demonstrates the lack of coordination between the registry and clinics who treat affected individuals. The discrepancy in data from these two sources, however, does show the need for follow-up of affected individuals because of changing diagnosis at a later date. The dynamic nature of the registry (loss of records due to moving, changing diagnosis, and addition of records of affected immigrants diagnosed elsewhere) indicates its function as a record of prevalent cases and not as a record of incident cases born in the province.

Incidence

Québec is one of the few populations in the world where non-PKU HPA is as frequent as PKU. Certain PAH mutations in combination with other mutations can lead to different severity of phenotypes and, thus, there could be some correlation in incidences of PKU and non-PKU HPA. Comparisons of the rates of PKU in Canadian provinces illustrate this point. Québec has a low incidence of PKU (1:27,021) equal to that of Manitoba and Ontario (1:32,812 and 1:27,038, respectively). Other provinces have higher incidences of PKU ranging from 1:9,229 (Nova Scotia) to 1:16,564 (Alberta), giving an overall incidence of 1 newborn with PKU for every 20,943 births in Canada (Ferreira, 1987). The national incidence of non-PKU HPA is 1:28,433: not much lower than seen in Québec. For most provinces there is a higher incidence of PKU than non-PKU HPA, resulting solely from variation in PKU incidence.

Another perspective by which to examine the provincial rates of PKU and non-PKU HPA is a comparison with the rates in some of the populations from which Québecers originate. Northern France, from 1972 to 1981, reported a PKU incidence of 1 per 13,535 births and a non-PKU HPA incidence of 1 per 70,000 (Dhondt and Farriaux, 1983). No records of non-PKU HPA incidence were available from southern France, although the overall incidence for hyperphenylalaninemias is 1 per 16,000 (both "classical" and "atypical" forms) (Farriaux, 1987). England, from 1984-1988, had a PKU incidence of 1 per 15,000 and a non-PKU HPA incidence of 1 per 24,000 (Smith et al., 1991). These estimates are also affected by what is defined as "non-PKU HPA" in each population.

The estimates of incidence of PKU and non-PKU HPA derived from the number of infants screened is an approximation of the true incidence of these diseases. The presence of other sources of ascertainment for PKU and non-PKU HPA would have allowed live births to be used as the denominator to calculate the estimate. Knowledge of the number of screened infants from 1970 to 1973, or the use of live births for the denominator, would have permitted an estimation of incidence over a longer time period (1970 - 1990) and thus give a more stable estimate. The estimate of incidence per infants screened (as calculated) is the most reasonable, as it allows for the possibility of undetected cases in the population.

Two undetected cases of PKU is less likely than three undetected cases of HPA, as cases of PKU may be ascertained by mental retardation. The exact probability of ascertainment of PKU by mental retardation is not known. The extent of the foreseen effects of maternal HPA caused by these five undetected cases, while real, is much

smaller than the foreseen effects of maternal HPA that may be caused by women identified with PKU or non-PKU HPA who have been lost to follow-up before reproductive age.

Gender

The difference in the number of males and females identified with PKU or non-PKU HPA may be a true phenomenon, or may result from bias in follow-up of females with HPA. It is possible that the 10 non-PKU HPA males whose clinical files were never found, or the non-PKU HPA males that were lost to follow-up, have since become cases of transient HPA. This would reduce the gender ratio distortion. If six or more of the 50 presumed non-PKU HPA males actually do not have non-PKU HPA, there would be no significant difference in gender ratio in individuals with non-PKU HPA. It is possible that due to the very short follow-up time for the individuals with no clinic file (mean age is 1.6 years), these males with suspiciously low phenylalanine values may have had transient HPA. Previous reports (Knox and Kang, 1970) suggest that an excess of males with non-PKU HPA may be caused by transient HPA, with the individual not becoming normal until after the age of two. A similar situation may have happened with individuals identified in Québec, with inadequate follow-up of males to determine their final disease status.

Year and month of birth

PKU and non-PKU HPA were distributed similarly for year and month of birth but, together, differed from expected population distributions based on infants screened per year and infants born per month. The small sample of individuals with HPA makes their occurrence a rare event in the population. Temporal variation in the incidence of HPA may simply be a result of the random occurrence of rare events. Unfortunately, the test of monthly variation in affected individuals did not describe seasonality among months of birth, but showed only a significant departure from expected number by month. Analysis of seasonal variation could reveal if the trend in month of birth of affected individuals matched the seasonal trend of phenylalanine values of normal infants (section C.2.3).

Region and ethnicity

Although a large proportion of ethnicity data was missing for individuals with non-PKU HPA, the proportion of individuals of Southern European origin was striking. This association could not be weakened by the assignment of missing ethnicities to any ethnic group. This large contribution from Southern Europe is seen in other multi-ethnic populations such as Israel, where there is 14% of Italian ("Roman") ancestry among individuals with PKU or non-PKU HPA (Kleiman et al., 1993). A similar deviation from the expected distribution of ethnic origins in children with non-PKU HPA was reported in Massachusetts (Levy et al., 1971). In that study, 53% of children with non-PKU HPA were Italian where 15% were expected, with other ethnicities represented being Jewish and English/Scottish, but not the expected Irish or Polish. The ethnic distribution seen in

Québec shows what is known about PKU and non-PKU HPA: different mutations are present in different populations, at different frequencies.

Such ethnic variation in PKU and non-PKU HPA frequency may be in part responsible for the variation seen in regional incidence of PKU and non-PKU HPA. (The number of cases was too small to examine region - ethnicity correlations). Founder effect may also contribute to regional incidence of PKU and non-PKU HPA.

6.6: Follow-up

Both detection of all individuals with PKU or non-PKU HPA, as well as follow-up of these individuals to prevent disability in current and future generations, is necessary for effective screening. Over the first 20 years of its operation, the Newborn Screening Program has developed an increasingly efficient system for screening nearly all live born infants in the province. Unfortunately, the information recorded for the screening test varies from individual to individual, and it is impossible to determine all the screening decisions made, especially those made towards the start of the Newborn Screening Program. Follow-up of individuals with PKU or non-PKU HPA has improved in the past few years in light of recent findings about the need for continued blood phenylalanine monitoring over a lifetime. Unfortunately, this study has indicated that some individuals with PKU or non-PKU HPA identified by screening in the early years of the Newborn Screening Program are not being followed, and probably cannot be found.

Kaplan-Meier survival curves are useful for demonstrating follow-up of individuals with PKU or non-PKU HPA, but provide only a cross-sectional view of the clinic follow-up system. Infants identified with PKU or non-PKU HPA in the past ten

years are more likely to be followed (for longer and more rigorously) than infants identified previously because of new knowledge about the long-term effects of elevated blood phenylalanine (Anon., 1993b; Smith et al., 1990a; Smith et al., 1990b). A Kaplan-Meier survival curve for infants born in the past ten years would show a higher probability of follow-up across all ages.

Individuals that are lost to follow-up can be divided into two categories: those who refuse to come to clinic (but whose whereabouts are known); and those for whom the clinic has no address. Individuals who refuse to come to the clinic can at least be approached at a later date to be reminded of the importance of reproductive counselling. Individuals for whom the clinics had no forwarding address, however, cannot be contacted by any means, and the clinics must rely on the individuals to initiate contact, which in many cases is unlikely.

The New England Maternal PKU Project reported similar difficulties in re-establishing contact with women with PKU or non-PKU HPA for reproductive counselling, within a screening program that identifies newborn individuals with elevated blood phenylalanine values but whose current record is known only in clinics (Waisbren et al., 1988). The New England study stressed the need for coordination of newborn screening program and clinics, in order to keep information up-to-date about PKU and non-PKU HPA patients. Re-establishing contact with women who were lost to follow-up was especially difficult for those with non-PKU HPA.

Both examination of actual phenylalanine values and discriminant analysis have demonstrated that initial phenylalanine values often do not reflect phenylalanine values later in life for individuals with PKU or non-PKU HPA. These findings reinforce the

need for close follow-up of individuals identified by screening for diagnostic changes later in life. Discriminant analysis indicates that if initial phenylalanine values (with or without age at screening) are used to make decisions about whether or not dietary therapy is necessary, a serious lack of treatment would occur among infants who will later be determined to have PKU. Current practices ensure that the Newborn Screening Program receives a confirmatory diagnosis made within a year for all individuals who are seen by a clinical centre because of elevated phenylalanine values, but there is no guarantee that infants with PKU or non-PKU HPA will be followed past the time when the diagnosis is made. The lack of correlation between initial phenylalanine values and eventual diagnosis for infants with PKU should caution clinics not to hastily dismiss an initial phenylalanine value in the range of 500 - 1,000 μmol/L as a benign condition not requiring parental and clinical attention, until a diagnosis has been well established. The need to retest infants with PKU or non-PKU HPA later in life in order to reassess phenylalanine dietary requirements and to check for normalization of phenylalanine values has been stressed by others (Howell, 1970).

Genotype - phenotype correlations can be helpful in prediction of eventual disease severity in PKU and non-PKU HPA (Svennson, 1993). The large range of initial blood phenylalanine values that correspond to a predicted PAH enzyme activity level is not surprising considering the lack of a clear cut boundary between initial phenylalanine values and eventual diagnosis of PKU or non-PKU HPA. Phenylalanine values in the newborn period are dependent not only on PAH enzyme activity (as determined by genotype), but also by the phenylalanine load ingested since birth. If phenylalanine values of infants with PKU or non-PKU HPA in the newborn period were tested under

identical dietary conditions, there would be a higher correlation of blood phenylalanine and genotype. Genotype determination can be an important aid in determining the eventual severity of disease, and could be a valuable prognostic indicator in the clinics for diagnosis of HPA in the newborn period when mutation-enzyme correlations are known.

6.7: Implications of this study

This thesis provides an encouraging note for the Newborn Screening Program on the improvement of screening effectiveness over the years, and the ability of threshold adjustment to determine the proportion of infants with phenylalanine values above the screening threshold. Of note is the decreasing age at which screening is done, which may require adaptation on the part of the Newborn Screening Program. Discrepancies between the information held by the clinics and that held by the Newborn Screening Program show the weakness of a screening program that is not centralized for both screening and follow-up. In addition, the number of births occurring out of hospital is an important consideration towards reaching 100% newborn screening. A means of sensitizing home visitors to the necessity of screening and the creation of an organized reporting system should be considered.

Clinics can also use information presented in this thesis. It is important for clinics and the Newborn Screening Program to recognize the 70 μmol/L difference shown by different methods of measuring blood phenylalanine in their interpretations of phenylalanine values obtained from different sources. The need to re-evaluate blood phenylalanine elevation later in life to monitor changes in diagnosis and appropriate treatment is also evident. The importance of the clinics' role in maintaining contact with

affected individuals in the absence of a centralized registry for purposes of reproductive counselling is emphasized here.

6.8: Suggestions for further research

Transient hyperphenylalaninemia

The designation of "transient HPA" remains a catch-all phrase for any apparent phenylalanine elevation at birth which at any time becomes normal, whether on the next test or several years later. Careful follow-up of infants with transient HPA until phenylalanine value normalization could provide information on the different causes of transient HPA. Follow-up of these infants born in the province over several years would be necessary as approximately 100 infants per year with phenylalanine values above the threshold would be identified. Such a study could provide estimates of transient HPA due to causes such as: prematurity, high protein diet, laboratory error and inadequate blood spots, neonatal tyrosinemia or other aminoaciduria, other illnesses, as well as transient HPA possibly caused by 4-A-carbinolamine dehydratase deficiency. By adding a phenylalanine blood test for the mother of the affected child to the investigation it would be possible to determine if any transient HPA is caused by unrecognized hyperphenylalaninemia in the mother. Examination of the contributing factors by region would demonstrate regional variation in the causes of elevated phenylalanine screening values.

Maternal hyperphenylalaninemia

This study was not able to elucidate the possible incidence or effects of maternal HPA in the province. The data used were limited by their cross-sectional view of the population. However, rescreening for hyperphenylalaninemias later in life is very costly for the number of cases detected. It would not be feasible to ascertain incidence of PKU or non-PKU HPA in the adult population of Québec, although this study has estimated that approximately five undetected individuals with PKU or non-PKU HPA have been born since 1973.

A means of estimating the incidence of non-PKU HPA that is severe enough to cause maternal HPA associated birth defects would be to ascertain mothers whose offspring had microcephaly, mental retardation and developmental delay. Testing for elevated blood phenylalanine values in these women would identify those whose offspring had congenital anomalies as a result of the mothers' maternal HPA phenotype. Such a study could be done retrospectively to increase sample size and reduce study cost. Identifying mothers of affected children for the study would not allow complete ascertainment of all non-PKU HPA women, or even all women with severe non-PKU HPA. Women that would not come to the attention of the study would be those whose children, by chance, had no birth defects or minor defects that would go unrecognized; women who never became pregnant; and women whose blood phenylalanine was elevated enough so that they would have had spontaneous abortions instead of giving birth to malformed babies. A study limiting the population of interest to mothers with more than one child with associated birth defects would further restrict the study scope but would increase the likelihood of finding mothers with elevated phenylalanine. A

study such as this could estimate the magnitude of the effect of these conditions on the child-bearing population and thus on the next generation.

6.9: Concluding remarks

This study has evaluated the Québec Newborn Screening Program on several levels: population, samples, test results, individuals and follow-up. The development of the Newborn Screening Program since its beginning over two decades ago has been monitored. The test results, demographic characteristics and follow-up of individuals identified with PKU or non-PKU HPA have been examined. Both an efficient screening program and adequate follow-up of identified individuals are necessary for screening to be successful, not only for the benefit of the individual but for prevention of maternal HPA as well. Screening for HPA in Québec has become an efficient process for infants born in hospital, though not for those born at home. Follow-up of individuals with PKU or non-PKU HPA is now more rigorous, and all individuals being followed are encouraged to maintain close contact with the clinics for metabolic monitoring and reproductive counselling.

Introduction to the Appendices

The original goal of this research was to provide a comprehensive description of individuals with PKU or non-PKU HPA and the test results by which they were identified. The intention was to use the file review and the registry of individuals with PKU or non-PKU HPA to provide information about affected individuals and to use annual reports, as well as demographic and test result data from the Newborn Screening Program, to provide a comparison group and population baseline. Data collection and analysis were performed based on this research plan.

Extensive examination of the data for screened newborn infants and results of the analyses revealed certain serious biases. It was presumed that data about normal newborn infants screened by the Newborn Screening Program would be easily retrievable and in a format which would readily provide responses to research questions. However, data kept by the Newborn Screening Program are for auditing and monitoring the performance of the screening program, and not for research purposes. The data for normal newborn infants, including their phenylalanine values, proved too unrepresentative to be considered accurate without extensive adjustment and reanalysis, an undertaking beyond the scope of this thesis.

For this reason, the data for normal newborn infants screened and the original, biased analyses appear in these Appendices instead of in the main text of this thesis. The results illustrate the analysis that was attempted and the general trends that can be obtained from the data. They also describe the screening test data kept by the Newborn Screening Program; this has not been completely documented before. Appendix A

describes the information kept by the Newborn Screening Program in the form of annual reports. Appendix B describes preliminary adjustments made because of biases in the data. Appendix C describes analyses of data from the population and from other sources.

Appendix A: Québec Newborn Screening Program annual reports

Documents produced by the Québec Newborn Screening Program as annual reports are included and described in this Appendix. As discussed in the results section, annual reports are produced for each hospital, which is identified by a unique code in annual reports. The coding system from 1973 to 1987-88 was a five character identifier, with the first two characters (from 00 to 10) representing the administrative region in which the hospital is located. Some codes do not represent actual hospitals, but represent categories of infants not ascertained by hospital of birth. For example, the hospital codes that end in the digits "99" are screening tests for which only the region and no other location information is known. These are usually screening tests performed at community health centres or by doctors or nurses during visits to infants born in the home.

Hospital identification codes are consistent across all years, with one minor modification. Starting in 1988-89, a four digit code was translated from the old five character code by removing the third character; for example, hospital code 06523 became hospital code 0623.

Document A: Hôpital 5 - Sang

This document was produced for the years 1973 to 1987-88. It documents the number and results (insufficient, abnormal) of **first samples** of blood sent to the Newborn Screening Program for analysis. Data are given by the individual hospitals in the province and is organized in the following way:

Variable	Name	Description
1	numero plaque	Hospital code
2	nombre d'echantillions sanguins recus	Number of first blood samples received. This is the total number of filter papers received by the screening program for which it is the infant's first contact with the screening program.
3	nombre d'echantillions sanguins insuffisants	Number of first blood samples that were received that were judged to have insufficient blood on the filter paper for analysis.
4	nombre d'echantillions sanguins: % recus	The percentage of insufficient samples among first blood samples received. ((variable 3 ÷ variable 2) * 100)
5	nombre d'echantillions sanguins analyses	Number of first blood samples that were actually analyzed. This is the number of samples remaining after subtracting those that were insufficient. (variable 2 - variable 3)
6	prelevements analyses normaux (normaux)	Number of samples, among those analyzed, which were normal with respect to blood phenylalanine, tyrosine and thyroxine values.
7	prelevements analyses normaux: % analyses	Percentage of samples analyzed that were normal with respect to blood phenylalanine, tyrosine and thyroxine values. ((variable 6 ÷ variable 5) * 100)
8	prelevements analyses anormaux: phenyl	Number of samples that were analyzed and had abnormal blood phenylalanine values.
9	prelevements analyses anormaux: % analyses	Percentage of samples analyzed that had abnormal blood phenylalanine values. ((variable 8 ÷ variable 5) * 100)

Document A

HOPITAL 5 — BRNO — DU 01 JANVIER 75 AU 31 DECEMBRE 75

NUMERO PLAQUE	NOMBRE D'ÉCHANTILLONS SANGUINS RECUS	INSUFFISANTS	% / RECUS	PRELEVEMENTS ANALYSES NORMAUX	% / ANALYSES	PRELEVEMENTS ANORMAUX PHENYL	% / ANALYSES	TYROS	% / ANALYSES	THYR	% / ANALYSES
10907	0	0		0	0	0		0		0	
10908	1	1	100%	0	0	0		0		0	
10909	0	0		0	0	0		0		0	
10910	0	0		0	0	0		0		0	
10999	0	0		0	0	0		0		0	
OUFREC	89457	1878		87579	86070	51		749		749	

① ② ③ ④ ⑤ ⑥ ⑦ ⑧ ⑨

Document B: Reprises - Sang

This document was produced for annual reports from 1973 to 1987-88. It documents the number of **second and subsequent samples** sent to the Newborn Screening Program for analysis. Hospitals sent subsequent samples at the request of the Newborn Screening Program for several reasons:

1. There was insufficient blood on the first sample.
2. The infant was too young at the time of the first test.
3. There was an abnormal first test result.

Variable	Name	Description
1	numero plaque	Hospital code
2	nombre d'echantillions sanguins recus	Number of subsequent samples received.
3	nombre d'echantillions sanguins insuffisants	Number of subsequent samples received that were judged to have insufficient blood on the filter paper for analysis.
4	nombre d'echantillions sanguins: % recus	The percentage of insufficient samples among the subsequent samples received. ((variable 3 ÷ variable 2) * 100)
5	nombre d'echantillions sanguins analyses	Number of subsequent samples that were actually analyzed. This is the number of samples remaining after subtracting those that were insufficient. (variable 2 - variable 3)
6	prelevements analyses normaux (normaux)	Number of subsequent samples, among those analyzed, which had normal blood phenylalanine, tyrosine and thyroxine values.
7	prelevements analyses normaux: % analyses	Percentage of subsequent samples analyzed which had normal blood phenylalanine, tyrosine and thyroxine values. ((variable 6 ÷ variable 5) * 100)
8	prelevements analyses anormaux: phenyl	Number of subsequent samples that were analyzed and had abnormal blood phenylalanine values.
9	prelevements analyses anormaux: % analyses	Percentage of subsequent samples analyzed that had abnormal blood phenylalanine values ((variable 8 ÷ variable 5) * 100).

Document B

REPRISES - SANG — PERIODE = DU 01 JANVIER 75 AU 31 DECEMBRE 75

NUMERO PLAQUE	NOMBRE D'ECHANTILLONS SANGUINS			PRELEVEMENTS ANALYSES NORMAUX		PRELEVEMENTS ANALYSES ANORMAUX					
	RECUS	INSUFFISANTS	% / RECUS	ANALYSES	NORMAUX	% / ANALYSES	PHENYL	% / ANALYSES	TYROS	% / ANALYSES	THYR	% / ANALYSES
10907	0	0		0	0		0		0		0	
10908	1	0	0%	1	1	100%	0	0%	0	0%	0	0%
10909	0	0		0	0		0		0		0	
10910	0	0		0	0		0		0		0	
10999	0	0		0	0		0		0		0	
QUEBEC	3641	260		3381	3081		12		56		238	

① ② ③ ④ ⑤ ⑥ ⑦ ⑧ ⑨

Document C: Tableau Global des Activités du Reseau Provincial de Médécine Génétique

This document was produced for each of the annual reports for 1973 to 1987-88; one page per hospital, each page containing both hospital and summary information. This was distributed to each hospital to inform them of their screening success relative to the province as a whole.

Information provided on this summary sheet is for the **samples** that were received *and analyzed* by the screening program. Information is given in three columns: Québec, Hôpital 5 and Reprise. The numbers in the Hôpital 5 and Reprise columns correspond to the information given for each specific hospital for variables shown in **Document A** and **Document B** respectively. The Québec column corresponds to the sum of the Hôpital 5 and Reprise documents for the province of Québec. For example, the number for "Nombre d'echantillions sanguins recus - Québec" is obtained by adding the "nombre d'echantillions recus - total" for the two documents "Hôpital 5" and " Reprise"; that is, adding the values in column 2 for the row titled "Québec" on both **Document A** and **Document B.**

Document C

CONCERNANT LES NAISSANCES VIVANTES DU 01 JANVIER 75 AU 31 DECEMBRE 75

POUR L'ENSEMBLE DU QUEBEC

AINSI QUE POUR HOTEL DIEU DE LEVIS

DEPISTAGE DE MALADIES METABOLIQUES HEREDITAIRES - SANG

		QUEBEC	HOPITAL 5	REPRISES
NOMBRE D'ECHANTILLONS SANGUINS	RECUS :	93098	1574	44
	INSUFFISANTS :	2138	12	4
	ANALYSES :	90960	1562	40
NOMBRE DE PRELEVEMENTS SANGUINS ANALYSES	RESULTATS NORMAUX :	89151	1539	36
	* ANORMAUX EN PHENYLALANINE :	63	0	0
	** ANORMAUX EN TYROSINE :	805	11	0
	*** ANORMAUX EN THYROXINE :	987	12	4

5 OCTOBRE 1976

RESEAU PROVINCIAL DE MEDECINE GENETIQUE DU QUEBEC

CENTRE HOSPITALIER DE L'UNIVERSITE LAVAL

03215

* SUPERIEURS A 4.0 MG EN PHENYLALANINE
** SUPERIEURS A 7.5 MG EN TYROSINE
*** INFERIEURS A -2.4 ECART-TYPE

Document D: Tableau de la Participation au Reseau Provincial de Médécine Génétique

This document was produced for each of the annual reports from 1973 to 1987-88. The document is a companion to **Document C** and was distributed to hospitals to provide hospital specific and summary statistics of the Newborn Screening Program.

Information supplied on this summary sheet is for **distinct newborns with one or more blood samples.** Multiple samples may be sent for one infant for several reasons:

1) if an infant is transferred from one hospital to another, (because of prematurity or illness) both hospitals may send blood samples;

2) if an infant was tested for the first time very early in life, another sample may be sent when the child is several days older (unsolicited by the screening program); or

3) if a metabolic problem is suspected, (for example if the infant's sibling has PKU) more than one sample may be sent.

The information provided is a summary of both "Hôpital 5" and "Reprise" reports (Document A and B). Information for "naissances vivantes" (live births) is supplied by each hospital, where available. The number of live births per hospital is often missing. The variables are defined below:

1) nombre de nouveau-nes distincts avec un ou plusieurs echantillions sanguins recus: the number of distinct newborns for whom the Newborn Screening Program received at least one sample. This number includes all distinct infants represented on the "Hôpital 5" or on the "Reprise" list, or on both (an infant with an insufficient sample on the "Hopital 5" form may also appear on the "Reprise" form.)

2) nombre de nouveau-nes distincts avec un ou plusieurs echantillions sanguins insuffisants: the number of distinct newborns who after their first and possibly subsequent samples, still had insufficient blood spots for analysis. These infants would never have had an analysis done.

3) nombre de nouveau-nes distincts avec un ou plusieurs echantillions sanguins analyses: the number of distinct newborns with one or more samples analyzed. This is a measure of the number of infants in the province that the screening program actually tested for hyperphenylalaninemia.

4) nombre de nouveau-nes distincts avec un ou plusieurs resultats d'analyses sanguines normaux: the number of distinct newborns with one or more normal blood tests. This includes individuals with one abnormal test, followed by a normal test.

5) nombre de nouveau-nes distincts avec un ou plusieurs resultats d'analyses sanguines anormaux en phenylalanine: the number of distinct infants who have persistently abnormal phenylalanine values and have never had a normal phenylalanine value. These infants have been referred to a metabolic clinical centre for investigation of hyperphenylalaninemia.

Document D

TABLEAU DE LA PARTICIPATION AU RESEAU PROVINCIAL DE MEDECINE GENETIQUE

CONCERNANT LES NAISSANCES VIVANTES DU 01 JANVIER 75 AU 31 DECEMBRE 75

POUR L'ENSEMBLE DU QUEBEC

AINSI QUE POUR HOTEL DIEU DE LEVIS

DEPISTAGE DE MALADIES METABOLIQUES HEREDITAIRES - SANG

		QUEBEC	HOTEL DIEU DE LEVIS LEVIS #SAHQ=0730001	/NAISSANCES VIVANTES +	
NOMBRE DE NOUVEAU-NES DISTINCTS AVEC UN OU PLUSIEURS ECHANTILLONS SANGUINS	RECUS :	89237	1574	/ 1652	(95.3%)
	INSUFFISANTS :	720	3	/ 1652	(0.2%)
	ANALYSES :	88517	1571	/ 1652	(95.1%)
NOMBRE DE NOUVEAU-NES DISTINCTS AVEC UN OU PLUSIEURS RESULTATS D'ANALYSES SANGUINES	NORMAUX :	88483	1570		
	ANORMAUX :	34	1		
	* ANORMAUX EN PHENYLALANINE :	2	0		
	** ANORMAUX EN TYROSINE :	4	0		
	*** ANORMAUX EN THYROXINE :	27	1		

5 OCTOBRE 1976

RESEAU PROVINCIAL DE MEDECINE GENETIQUE DU QUEBEC

[redacted] HOSPITALIER DE L'UNIVERSITE LAVAL

03215

+ ESTIMATION DU NOMBRE DE NAISSANCES D'APRES LE RESEAU PROVINCIAL DE MEDECINE GENETIQUE SECTEUR INFORMATIQUE

* SUPERIEURS A 4.0 MG EN PHENYLALANINE

** SUPERIEURS A 7.5 MG EN TYROSINE

*** INFERIEURS A -2.4 ECART-TYPE

Document E: Cumulatif du rapport annuel

This document has been produced for each annual report from 1988-89 to 1992-93. It is not a final report, but is a draft version because the information has yet to be verified against the screening database for the years in question. Only the number of samples, and not the number of distinct infants screened, are reported in this document. The variables listed on the document, in order, are as follows:

No. hopital: the hospital code in the new form.

Nom: the name of the hospital or area corresponding to the hospital code.

Nais. Hop.: the number of births in the hospital, as declared by the hospital. Missing values are reported as -1.

1er prel: the number of first samples received. When there are samples received from a hospital with no births, it is a hospital which receives infants for intensive care.

Inadequats: the number of samples with insufficient amount of blood among those received.

Rap. recus: the number of subsequent tests received (Reprises).

TSH an: number of samples with abnormal thyroid stimulating hormone (TSH).

Rvn: the number of samples with abnormal TSH that yielded a normal result on subsequent tests.

Dep: the number of samples with abnormal TSH that remained abnormal upon subsequent samples. The infants contributing these samples were referred to a metabolic clinical centre for investigation.

Phe an: number of samples with abnormal phenylalanine.

Rvn: the number of samples with abnormal phenylalanine that yielded a normal result on subsequent tests.

Dep: the number of samples with an abnormal phenylalanine that remained abnormal upon subsequent samples. The infants contributing these samples were referred to a metabolic clinical centre for investigation.

Tyr an: number of samples with abnormal tyrosine.

Ryn: the number of samples with abnormal tyrosine that came back normal on subsequent tests.

Dep: the number of samples with an abnormal tyrosine that remained abnormal upon subsequent samples. The infants contributing these samples were referred to a metabolic clinical centre for investigation.

Document E

CUMULATIF DU RAPPORT ANNUEL 88-89

NO. HOPITAL	NOM	NAIS. HOP.	1ER PREL.	INADE-QUATS	RAP. RECUS	TSH AN.	RVH.	DEP.	FHE. AN.	RVH.	DEP.	TYR. AN.	RVH.	DEP.
0643	C. H PIERRE BOUCHER	2388	2355	2	1	0	0	0	0	0	0	1	1	0
0644	CENTRE HOSPITALIER ANNA LABERGE	0	0	0	0	0	0	0	0	0	0	0	0	0
0699	REGION 06	-1	143	4	1	0	0	0	0	0	0	0	0	0
0700	C. H DE BUCKINGHAM	279	269	1	0	0	0	0	0	0	0	0	0	0
0701	C. H REGIONAL DE L OUTAOUAIS	1240	1242	3	2	3	2	1	0	0	0	0	0	0
0702	PONTIAC COMMUNITY HOSPITAL	103	103	0	0	0	0	0	0	0	0	0	0	0
0703	CENTRE HOSPITALIER GATINEAU MEMORIAL	43	36	1	1	0	0	0	0	0	0	0	0	0
0705	C H DE MANIWAKI	127	124	0	0	0	0	0	0	0	0	0	0	0
0706	HOPITAL NOTRE-DAME DE STE-CROIX	302	302	0	0	0	0	0	0	0	0	0	0	0
0707	C H DES LAURENTIDES	43	42	0	0	0	0	0	0	0	0	0	0	0
0708	C H DE GATINEAU	1240	1224	13	10	0	0	0	0	0	0	0	0	0
0799	REGION 07	-1	30	7	3	0	0	0	0	0	0	0	0	0
0800	CENTRE HOSPITALIER HOTEL-DIEU D'AMOS	528	521	0	0	0	0	0	0	0	0	0	0	0
0802	C. H ST-FRANCOIS D'ASSISE	313	311	0	0	1	1	0	0	0	0	0	0	0
0803	C. H. ROUYN-NORANDA	713	716	1	1	1	1	0	1	1	0	0	0	0
0804	C. H ST-SAUVEUR	818	810	3	3	1	1	0	0	0	0	0	0	0
0805	CENTRE DE SANTE STE-FAMILLE	142	154	0	0	0	0	0	0	0	0	0	0	0
0806	CENTRE DE SANTE LEBEL	6	7	0	0	0	0	0	1	0	0	0	0	0
0807	CENTRE DE SANTE ISLE-DIEU	4	3	0	0	0	0	0	0	0	0	0	0	0
0899	REGION 08	-1	0	0	0	0	0	0	0	0	0	0	0	0
0900	CENTRE DE SANTE DE LA BASSE-COTE NORD	6	16	6	1	0	0	0	0	0	0	0	0	0
0902	CENTRE DE SANTE ST-JEAN EUDES	39	39	1	0	0	0	0	0	0	0	0	0	0
0903	CENTRE DE SANTE DE LA HAUTE-COTE NORD	21	21	0	0	0	0	0	0	0	0	0	0	0
0904	C H REGIONAL BAIE COMEAU	564	564	1	1	1	1	0	0	0	0	0	0	0
0905	HOPITAL SEPT-ILES	547	544	1	0	0	0	0	0	0	0	0	0	0
0999	REGION 09	-1	17	0	0	0	0	0	0	0	0	0	0	0
1000	HOPITAL DE L'UNGAVA	77	71	4	4	0	0	0	0	0	0	0	0	0
1001	HOPITAL CHISASIBI	55	65	2	1	0	0	0	0	0	0	0	0	0
1004	C.H BAIE D'HUDSON DISP POVUNG.	120	119	0	0	0	0	0	0	0	0	0	0	0
1099	REGION 10	-1	2	0	0	0	0	0	0	0	0	0	0	0

EN DATE DU 94/01/17

Document F: Depistage néonatal sanguin des maladies génétiques, rapport annuel

This document was produced for each of the annual reports for 1988-89 and 1989-90, and may yet be produced for subsequent years. A page is produced for each hospital in the province and is sent to the hospitals to provide information about their participation in the screening program relative to the province as a whole. The provincial summary is provided for each variable as well as information for the hospital in question. Variables provided are as follows:

Variable	Name	Description
1	Nombre de naissances	Number of births. If this value is not available for the hospital, the column contains a "-".
2	Premiers prelevements	Number of first samples received from the hospital.
3	% de participation	Percent of births for which samples were received. This number may exceed 100% if duplicate samples are sent. ((variable 2 ÷ variable 1) * 100)
4	Prelevements inadequats	Number of insufficient samples received on the first samples.
5	Rappels recus	Number of subsequent samples received requested because of insufficient samples, screenings done at less than 2 days of age and abnormal test results on the first test.
6	% de qualite de prelevement	The percent of first samples received that were not insufficient. (((variable 2 - variable 4) ÷ variable 2) * 100)
7	Test de depistage en phenylcetonurie	Number of samples identified with abnormal blood phenylalanine values. - anormaux: initially testing abnormal - presentment normalises: normal after subsequent tests - presentment depistes: remains abnormal after subsequent tests, referred to metabolic clinic

Document F

DÉPISTAGE NÉONATAL SANGUIN DES MALADIES GÉNÉTIQUES
RAPPORT ANNUEL 1988-1989
DU 1 AVRIL 88 AU 31 MARS 89

HOPITAL ST-FRANCOIS D'ASSISE
10 RUE DE L'ESPINAY
QUEBEC, (QC)

	Province de Québec	Votre Hôpital	Remarques
(1) Nombre de naissances:	87478	1952	
(2) Premiers prélèvements:	86655	1997	
(3) % de participation:	99,1%	102,3%	
(4) Prélèvements inadéquats:	287	7	
(5) Rappels reçus:	199	4	
(6) % de qualité du prélèvement:	99,7%	99,6%	

Evaluation de la bonne qualité de vos prélèvements

Tests de dépistage en:		Anormaux		Présentement Normalisés	Dépistés
(7) *	Hypothyroïdie:	81	2	2	0
**	Phénylcétonurie:	27	0	0	0
***	Tyrosinémie:	5	1	0	1

Nombre d'enfants dépistés positivement, d'avril 88 à mars 89 inclusivement, par le programme sanguin du Réseau de Médecine Génétique du Québec en :

Hypothyroïdie:	16
Phénylcétonurie:	2
Hyperphénylalaninémie:	2
Tyrosinémie:	4

Seuil de rappel:

* TSH supérieure à 14 mU/L
** Phénylalanine supérieure à 241 µmol/L
*** Tyrosine supérieure à 247 µmol/L et succinylacétone supérieur à 4,9 µmol/L

Remarque:

S.V.P. Voir à faire indiquer le code postal sur vos cartes patients, si ce n'est déjà fait.
Merci!

le 90/11/16

Appendix B: Datasets of infants screened, 1973 to 1990

B.1: Preface

In addition to data collected from the annual reports of the Newborn Screening Program and a clinical file review, phenylalanine values in samples for all newborn infants were also obtained for the years 1973 to 1990 from the Newborn Screening Program. The intention was to use these data to describe and to compare two groups of infants: infants screened who were classified as not having PKU or non-PKU HPA (normal infants) because their results were below the threshold, and those with screening test results above the threshold but who did not have PKU or non-PKU HPA by subsequent tests or investigation. These two groups would serve as comparisons for individuals with PKU or non-PKU HPA, and present an overall perspective on the Newborn Screening Program.

These appendices follow the structure of results outlined in the main body of the thesis: population, screening, test results, individuals and follow-up. Appendix B discusses the population from which the data were drawn, and how the screening program has kept data for screened infants. Appendix C presents results about phenylalanine values (test results) and individuals. No follow-up information was available for infants whose test results were examined in these appendices. The relevance of results from these appendices are discussed in context of the main body of this thesis in the Discussion (Chapter 6).

B.2: Datasets of the Newborn Screening Program

The Newborn Screening Program keeps demographic data and test analysis information about infants screened. These data have been used in past years by the Newborn Screening Program to evaluate trends in screening. From 1970 to 1982-83 (using the same reporting periods as the annual reports), laboratory personnel recorded phenylalanine values for one or two samples (along with corresponding demographic information) for every batch of 40 samples processed. Unfortunately, if there were too many samples to process, the recording of information was done less systematically (sometimes stopping completely) because of time constraints. This frequently occurred in the period of April to June when there were higher birth rates and consequently larger numbers of samples were received by the Newborn Screening Program to be analyzed. In addition, information was also preferentially recorded for samples with phenylalanine values above the threshold. This was done so that if the infant whose phenylalanine was above the threshold was later shown to have PKU or non-PKU HPA, the information about the initial screening test would be available. The recording of information for samples with phenylalanine values above the threshold continued in time periods where fewer or no other phenylalanine values were recorded. For the years 1983 to present, demographic information was kept in computer files for all filter papers received. For the years 1983-1984 to 1987-88, laboratory test results were also kept, but in separate files. Since then, test results are kept in the same files as demographic information for all infants.

Different data were available for different reporting periods. The Service Informatique of the Newborn Screening Program retrieved information about as many infants as possible for the time period January 1, 1973 to March 31, 1990, merging demographic information with test results for the reporting periods 1983-84 to 1987-88. Names were removed from the records of all datasets to protect confidentiality, and all blood phenylalanine values were converted to μmol/L. Merged information could not be obtained for varying numbers of infants, and these unmerged records were not available for analysis. The data received for different reporting periods ranged from 0.6% to 100% of infants screened per reporting period.

Each file provided by the Newborn Screening Program contained records corresponding to one reporting period of the Newborn Screening Program annual reports. The number of records contained in each dataset are shown in Table 7. Datasets contained the following variables:

ID number
father's initial
date of birth
gender
date of screening test
hospital of birth
weight at time of screening
initial phenylalanine value

Table 7. Number of records contained in datasets received from the Newborn Screening Program and made available for analysis.

Time period	Number of records
January 1973 to December 1973	1,947
January 1974 to December 1974	6,198
January 1975 to December 1975	8,277
January 1976 to March 1976	1,355
April 1976 to March 1977	3,838
April 1977 to March 1978	4,727
April 1978 to March 1979	5,909
April 1979 to March 1980	6,151
April 1980 to March 1981	6,302
April 1981 to March 1982	6,570
April 1982 to March 1983	6,395
April 1983 to March 1984	86,552
April 1984 to March 1985	1,081
April 1985 to March 1986	562
April 1986 to March 1987	725
April 1987 to March 1988	17,678
April 1988 to March 1989	86,520
April 1989 to March 1990	92,449

The code written to import the data from its original form as a text file and convert it to a dataset is shown as a pseudocode algorithm below:

read I.D. number
read father's initial
read date of birth
    if not a valid date, then classify as 'missing'
read sex
    if not a valid sex, then classify as 'missing'
read date of screening test
    if not a valid date, then classify as 'missing'
    if earlier than birth date, then classify as 'missing'
read region of birth
    if not a valid region, then classify as' missing'
read hospital of birth
    if not a valid hospital, then classify as 'missing'
read phenylalanine value
    if not a valid phenylalanine value, then classify as 'missing'
read year of birth
    if not a valid year, then classify as 'missing'
read month of birth
    if not a valid month, then classify as 'missing'
calculate: age at first screening test = (date of test - date of birth)
calculate truncated age categories for infants tested after 7 days of life:
    if age at first test is greater than 7, then age category = 7, else
      age category = age at first test

decide if phenylalanine value is above threshold:

get screening threshold for this test date and age at first test

if phenylalanine value is greater than the threshold for this test date and age at first test, then:

person exceeds the threshold

calculate: amount in excess of threshold

end.

Three additional variables were calculated from the data as stated above. The age at screening test was calculated as the difference between the date of birth and date of screening test where both these dates were present. Two variables were constructed based on the phenylalanine values and the (period-specific) threshold value (Table 1). The phenylalanine value was compared to the threshold phenylalanine value and judged to be above or below the threshold. For phenylalanine values that were above the threshold, the difference between the threshold and the phenylalanine value was calculated.

To maximize the information made available in records which were partially missing date information, year, month and date were read as separate variables. In this way, dates with a missing day value still may have given information about month and year of birth.

The datasets were screened for multiple records with the same ID number, which would indicate several tests, or duplicate entries for one infant. The record that was retained for an infant had the earliest dated test result.

The distribution of tested infants by month of birth and mean monthly phenylalanine values are given in Table 8. Distributions are shown for gender, region of

Table 8. Relationship between season and blood phenylalanine values (April 1985 to March 1986).

Month of birth	Number	Percent of non-missing data	Mean blood phenylalanine value (μmol/L)
January	68	12.1	203.4
February	69	12.3	243.8
March	125	22.3	220.5
April	47	8.4	193.8
May	26	4.6	208.0
June	50	8.9	144.8
July	6	1.1	175.2
August	56	10.0	165.9
September	18	3.2	184.5
October	31	5.5	207.8
November	28	5.0	207.8
December	37	6.6	175.6
Missing	1	NA	175.0

birth, age at test and phenylalanine above and below the threshold in Figures 19, 20, 21, and 22 . These univariate distributions illustrate some of the exploratory analyses done to look for biases in the data.

B.3: Biases in the data

The univariate distributions of the data make evident the biases created by non-random recording of test results in the data. Mean phenylalanine values, month of birth, and number of individuals above and below the threshold show large variation from dataset to dataset. These are not independent biases, but arise from the same source. Individuals with phenylalanine in excess of the threshold are more likely to be recorded in the Newborn Screening Program's database than "normal" individuals. This bias is particulary apparent in months with an usually low number of records (Table 8). The over-representation of phenylalanine values above the threshold in datasets with small numbers of records increases the mean phenylalanine value for that reporting period.

Another bias in the data becomes apparent when comparing the number of infants with phenylalanine values above the threshold for 1988-89 and 1989-90 to the number stated in the annual reports for those reporting periods (Table 9). This difference is wholly attributable to thick blood spots on the filter papers. A thick blood spot can cause an otherwise normal phenylalanine level to exceed the screening threshold, as discussed in section 3.3.4. The Newborn Screening Program database contains information for recent years as to whether a blood spot contains an acceptable volume of blood so it can be determined if an individual is exceeding the screening threshold for this reason. This

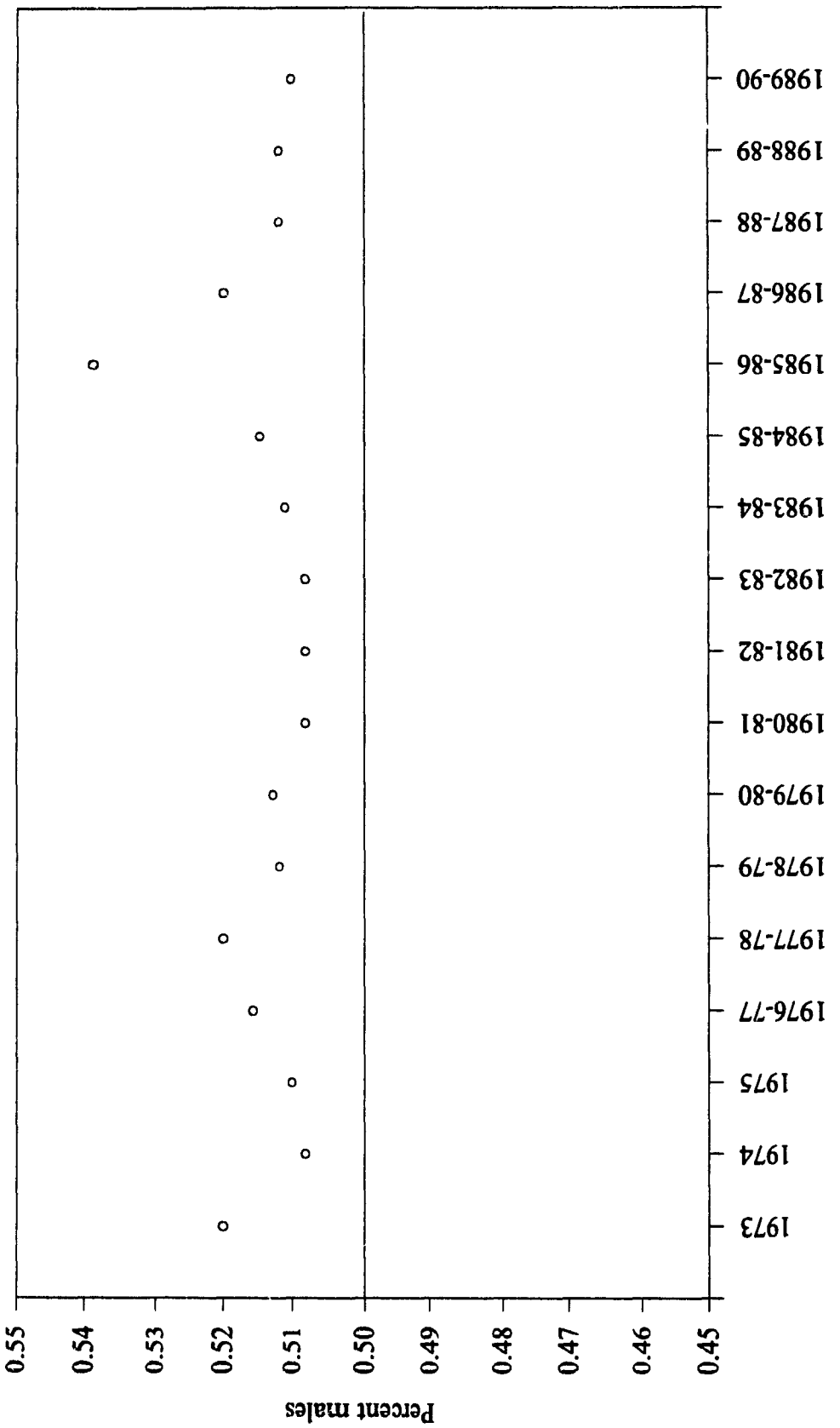

Figure 19. Sex ratio (in each dataset), by reporting period.

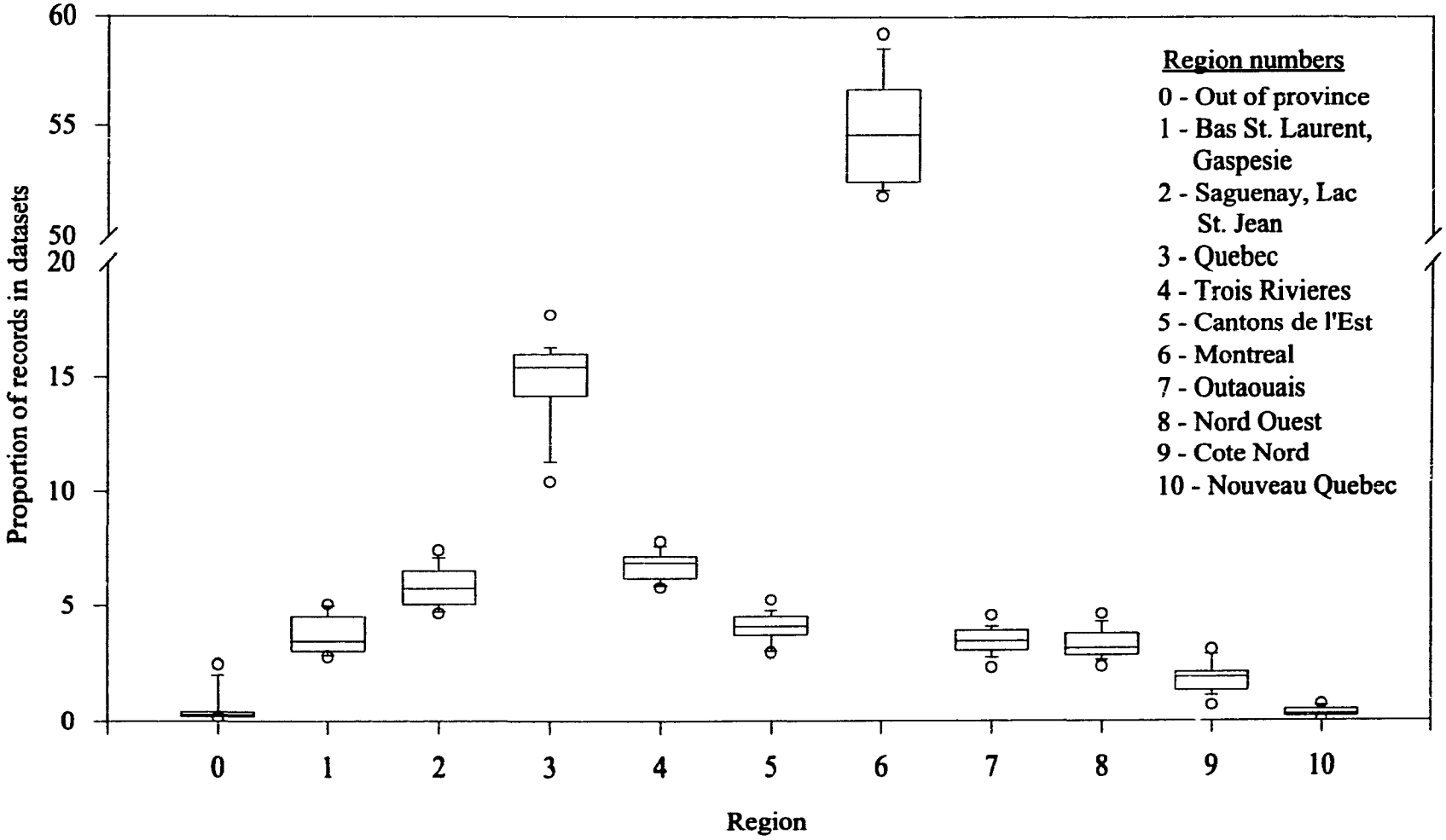

Figure 20. Proportion of records from each region in each dataset

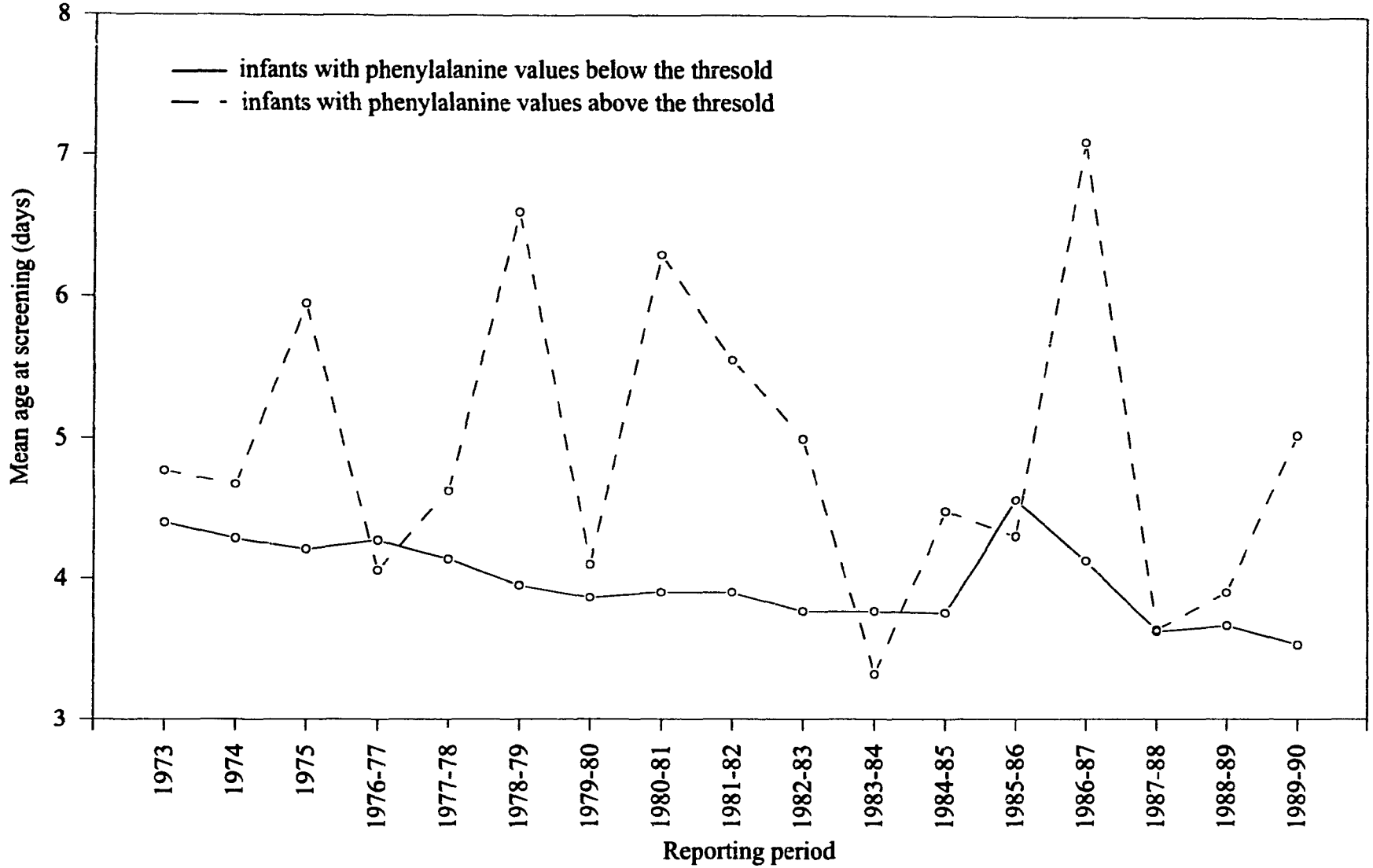

Figure 21. Mean age at screening test for infants with phenylalanine values above and below the screening theshold, by reporting period.

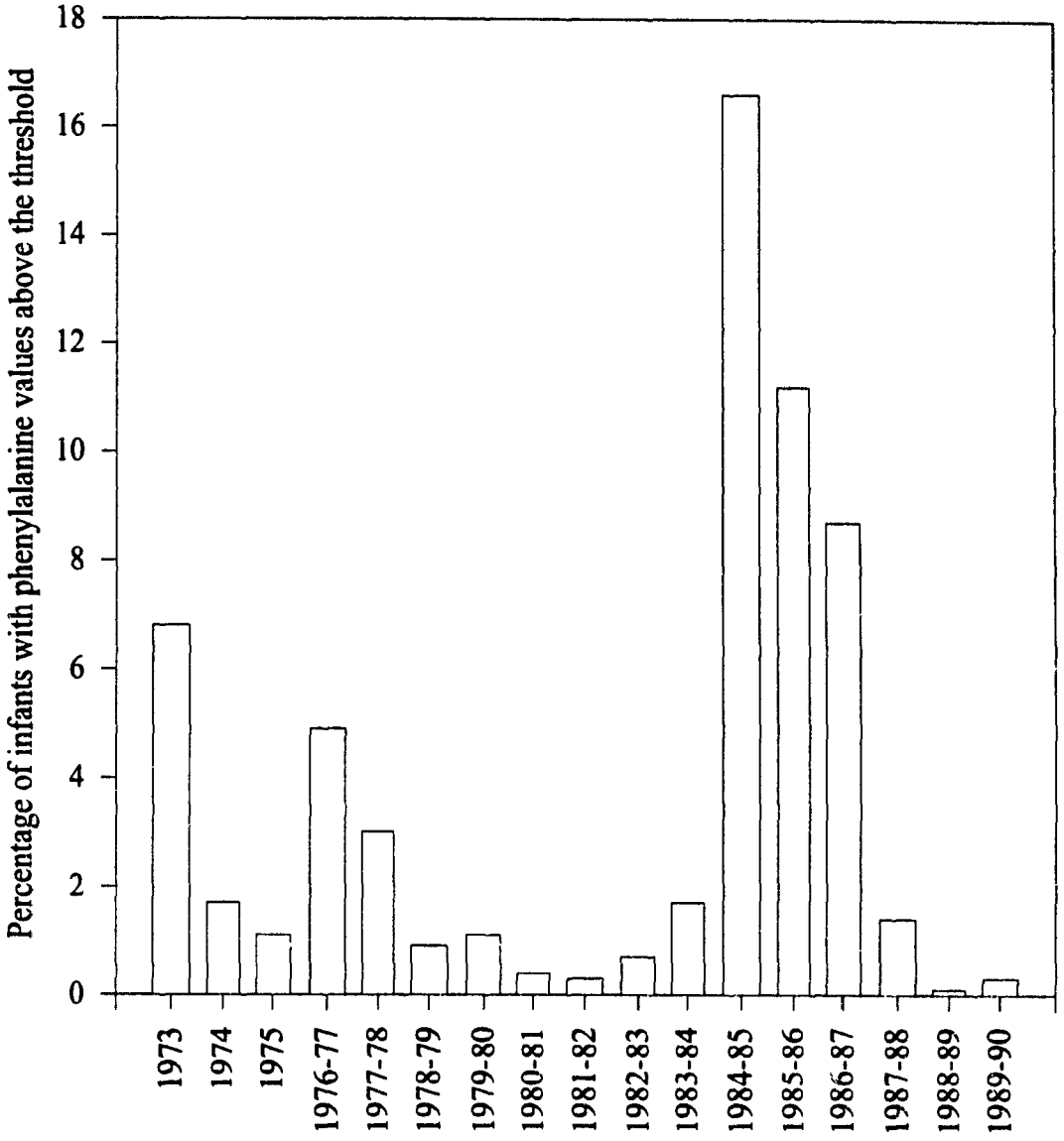

Figure 22. Percentage of infants with phenylalanine values above the period specific threshold in datasets, by reporting period.

Table 9. The number of screened infants above and below the threshold in the datasets, and expected values based on the number of infants above and below the threshold in annual reports

	Number of records in datasets		Number of samples from annual reports	
Year	Below threshold	Above threshold	Normal phenylalanine	Abnormal phenylalanine
1973	1,811	135	73,518	81
1974	6,083	114	80,144	96
1975	8,182	95	87,528	51
1976-77	4,923	270	109,752	252
1977-78	4,575	152	90,527	150
1978-79	5,847	62	90,822	59
1979-80	6,047	77	94,992	102
1980-81	6,264	38	92,658	43
1981-82	6,546	24	91,172	28
1982-83	6,350	45	86,933	60
1983-84	67,494	1,186	86,244	303
1984-85	892	189	85,358	195
1985-86	496	66	84,639	72
1986-87	658	67	83,111	66
1987-88	16,809	235	82,676	77
1988-89	86,434	83	86,341	27
1989-90	92,163	282	92,264	57

information was not available for this project, so that both individuals who were truly and those falsely above the threshold contribute to the excessive number of individuals above the screening threshold. The missing variable to determine if an elevated phenylalanine was due to a thick blood spot was available for the datasets for the reporting periods of 1988-89 and 1989-90. However, this would not have completely resolved the problem of elevated phenylalanine values associated with thick blood spots, as no similar information is available for infants with blood phenylalanine values below the threshold. For this reason, the overall shift upwards of phenylalanine values in these data due to thick blood spots could not be directly estimated.

B.4: Adjustments made to the data

To make sample data as representative as possible of the normal population from which they were selected, two adjustments were made to the data: the removal of records of individuals with PKU or non-PKU HPA; and the removal of the excess number infants with phenylalanine values above the threshold. After the adjustments, these datasets were used to represent the "normal" infants, that is those newborn infants who had phenylalanine values below the threshold and those who had initial phenylalanine values above the threshold, but who did not have HPA.

Initial examination of the basic statistics for phenylalanine values for each year showed large standard deviation, skewness, and kurtosis for some reporting periods. It was also noticed that standard deviation increased with sample size, which was contrary to what was expected. Individuals with PKU or non-PKU HPA appeared to cause this

increase and had a large impact on the descriptive parameters of phenylalanine values despite their small proportion of these records in the datasets. Individuals with PKU or non-PKU HPA do not represent normal variation in phenylalanine metabolism, so their records were removed from the data files. Their removal reduced the standard deviation, skewness and kurtosis without drastically changing the mean of the sample. For example, in one set of 801 newborns, removing one record of an individual with PKU (phenylalanine value of 824 µmol/L) from the set reduced the standard deviation from 65.4 µmol/L to 30.9 µmol/L, skewness from 19.2 to 0.8, and kurtosis from 474.4 to 2.63, while the mean changed only from 150.6 µmol/L to 148.3 µmol/L.

The over-representation of individuals with phenylalanine values above the threshold created an artifactual increase in the phenylalanine mean, as discussed in section B.3. A random sampling of individuals exceeding the threshold in each dataset was done so that the proportion of individuals with phenylalanine values above the threshold corresponded to the proportion of individuals with phenylalanine in the normal range in each dataset. For example, if the number of infants with phenylalanine values represented in the data for that reporting period was 25% (from annual reports), a sample of the infants with phenylalanine values above the threshold was taken so their number was also 25% of that expected. The proportions derived from Table 9 were used to determine the proper adjustment by which to reduce the number of individuals over the threshold. An example of the data selection process is shown in Figure 23.

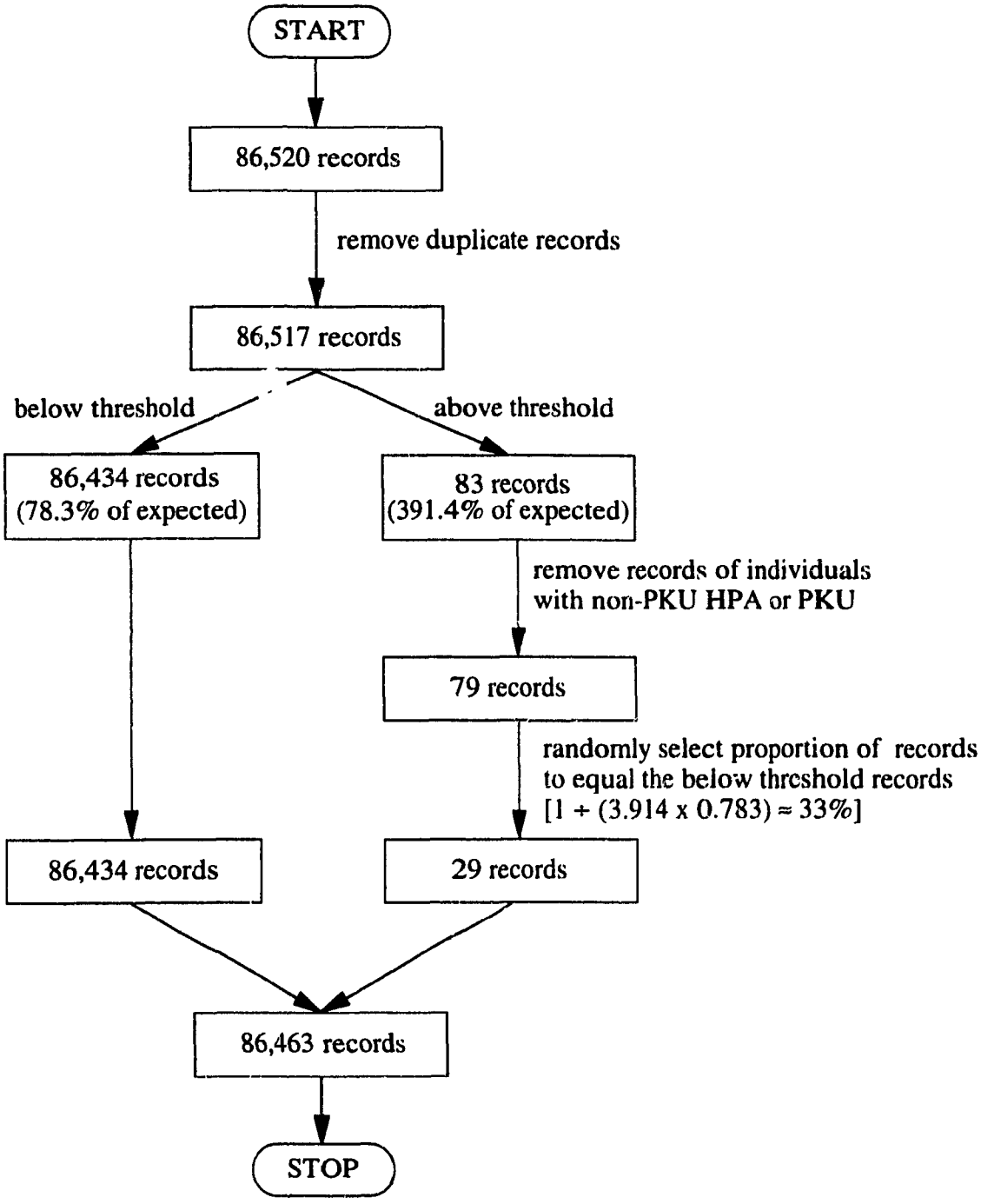

Figure 23. Flowchart of steps undergone in the selection of data. Illustrated is the data selection for the dataset of April 1988 to March 1989.

Because of the artificially inflated mean phenylalanine values in some months, as shown in section B.3, not all data was used in analysis. Data included in the following analyses were for the time periods:

October 1973 to July 1974

December 1974 to February 1975

June 1975 to March 1976

July 1976 to March 1984

April 1988 to March 1990.

Appendix C: Datasets of infants screened: analyses

Datasets containing demographic information and phenylalanine values were a large, valuable source of information about infants screened by the Newborn Screening Program in general. Extensive analyses were undertaken to characterize the completeness of the data and identify biases and to provide preliminary estimates of blood phenylalanine distributions in newborn infants over an eighteen year period. Parametric analyses were undertaken without a thorough verification of the assumptions of normality and homoscedasticity necessary for reliable statistical inference. The preliminary results reinforce the necessity for reanalysis to obtain reliable estimates from the samples of records. Potential problems for the testing of certain hypotheses, which were originally part of this thesis, due to inadequacies of the present datasets are documented. Results of non-parametric analyses given in this Appendix are considered more reliable because the tests are more robust with respect to the distribution of the data.

C.1: Records for newborn infants with phenylalanine values above the threshold

Records of infants with phenylalanine values above the threshold were analyzed to compare this group of infants to normal infants and individuals with PKU or non-PKU HPA. A separate dataset was created containing only infants whose phenylalanine exceeded the threshold value but who were eventually diagnosed as normal (they did not have PKU or non-PKU HPA on follow-up). This file was made as informative as possible by the inclusion of the maximum number of infants with phenylalanine values

above the threshold, without exceeding the number in the annual reports of the Newborn Screening Program. All infants were used in the analyses for years where the datasets contained 100% or less than the number of infants with phenylalanine values above the threshold. In datasets which contained more records of infants with phenylalanine values above the threshold, a simple random sample of these infants was taken so that their number did not exceed that in the annual report. For example, from April 1989 to March 1990, there were 5.3 times the number of infants with phenylalanine values above the threshold, so a 20% random sample of these 267 infants was used in the analyses so that their number would approximate the 57 in the annual report.

C.2: Analyses of population phenylalanine values

C.2.1: Phenylalanine values

The overall mean phenylalanine value of normal infants was 127.7 μmol/L and ranged from 111.1 to 150.3 μmol/L annually (Table 10). The hypothesis of normality of the distributions of phenylalanine values was tested based on the Shapiro-Wilk test for the 1973 dataset and by the Kolmogorov test for all other datasets. The probability of phenylalanine values observed in normal infants being normally distributed given the phenylalanine values in these datasets was less than 1% for every reporting period. The data for normal infants from April 1988 to March 1990 is shown in Figure 24 as an example of a representative distribution of phenylalanine values.

The dataset of infants with phenylalanine values above the threshold included 1,230 individuals (Table 10). The distribution of phenylalanine values in this group was

Table 10. Descriptive statistics of phenylalanine values for normal infants by reporting period and for infants with phenylalanine values above the threshold (1973 to 1990)

Sample, by year or type	Mean phe (µmol/L)	Standard Deviation	Skewness	Kurtosis	Minimum (µmol/L)	1st Quartile (µmol/L)	Median (µmol/L)	3rd Quartile (µmol/L)	Maximum (µmol/L)	Sample Size
1973	139.9	39.5	0.1	-0.1	6	115	139	163	242	1,811
1974	111.1	42.8	0.3	0.3	6	84	108	139	290	6,086
1975	117.4	37.4	0.5	1.3	6	90	115	139	405	8,186
1976	128.6	40.3	0.6	0.7	6	102	121	151	393	4,937
1977	138.7	44.8	3.0	59.5	6	108	133	163	1,162	4,584
1978	128.0	36.2	0.2	0.4	6	102	127	151	302	5,852
1979	126.4	37.9	0.2	0.7	6	102	127	145	363	6,079
1980	114.0	36.1	0.3	0.5	6	90	115	133	290	6,269
1981	112.9	31.4	0.4	1.1	6	90	115	133	302	6,549
1982	120.0	33.9	0.8	6.7	6	96	121	139	569	6,356
1983	138.3	30.0	1.4	19.7	6	121	139	157	848	67,391
1988	134.3	28.5	0.7	4.6	0	114	132	151	833	86,463
1989	150.3	27.7	0.5	1.1	0	131	148	167	596	92,220
elevated phe	273.9	76.7	5.5	64.0	212	230	254	290	1,525	1,230

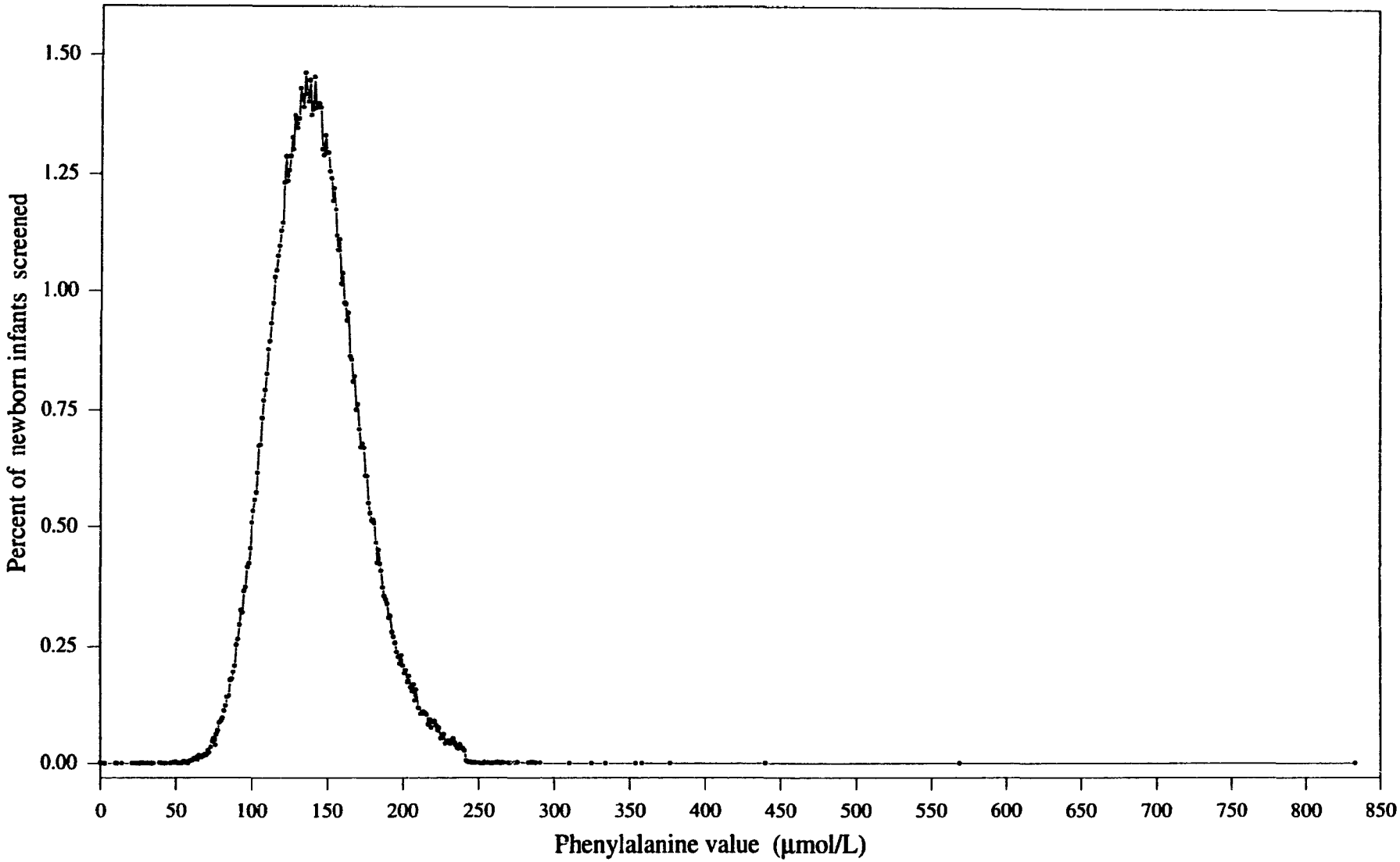

Figure 24. The distribution of phenylalanine values in normal newborn infants for April 1988 to April 1990 (n = 178,683).

highly skewed (Figure 25) and the probability of a normal distribution of phenylalanine values of these individuals was less than 0.01% by the Shapiro-Wilk test.

The amount by which an infant's phenylalanine was elevated above the threshold was also examined for infants with phenylalanine values above the threshold (Figure 25) because of the variable threshold which was used to determine normal phenylalanine. The median phenylalanine value above the threshold was 24 μmol/L.

C.2.2: Phenylalanine values by gender

The Wilcoxon rank-sum test was used to test the hypothesis that phenylalanine mean values, by gender, were not significantly different. No significant difference was seen for tests of the distribution of phenylalanine values in males and females within each dataset. There was no significant difference in mean phenylalanine values of males (272.7 μmol/L) and females (275.0 μmol/L) with phenylalanine values above the threshold, as tested by the Wilcoxon rank-sum test ($p > 0.12$).

The lack of statistical difference between the distributions of phenylalanine values for males and females yields evidence against the hypothesis that more males with PKU or non-PKU HPA are detected because males, on average, have higher phenylalanine values than females. As the distributions of phenylalanine values above the threshold were also found not to differ between males and females, the excess of affected males detected suggests a truly higher incidence of hyperphenylalaninemia (transient or persistent) in newborn males.

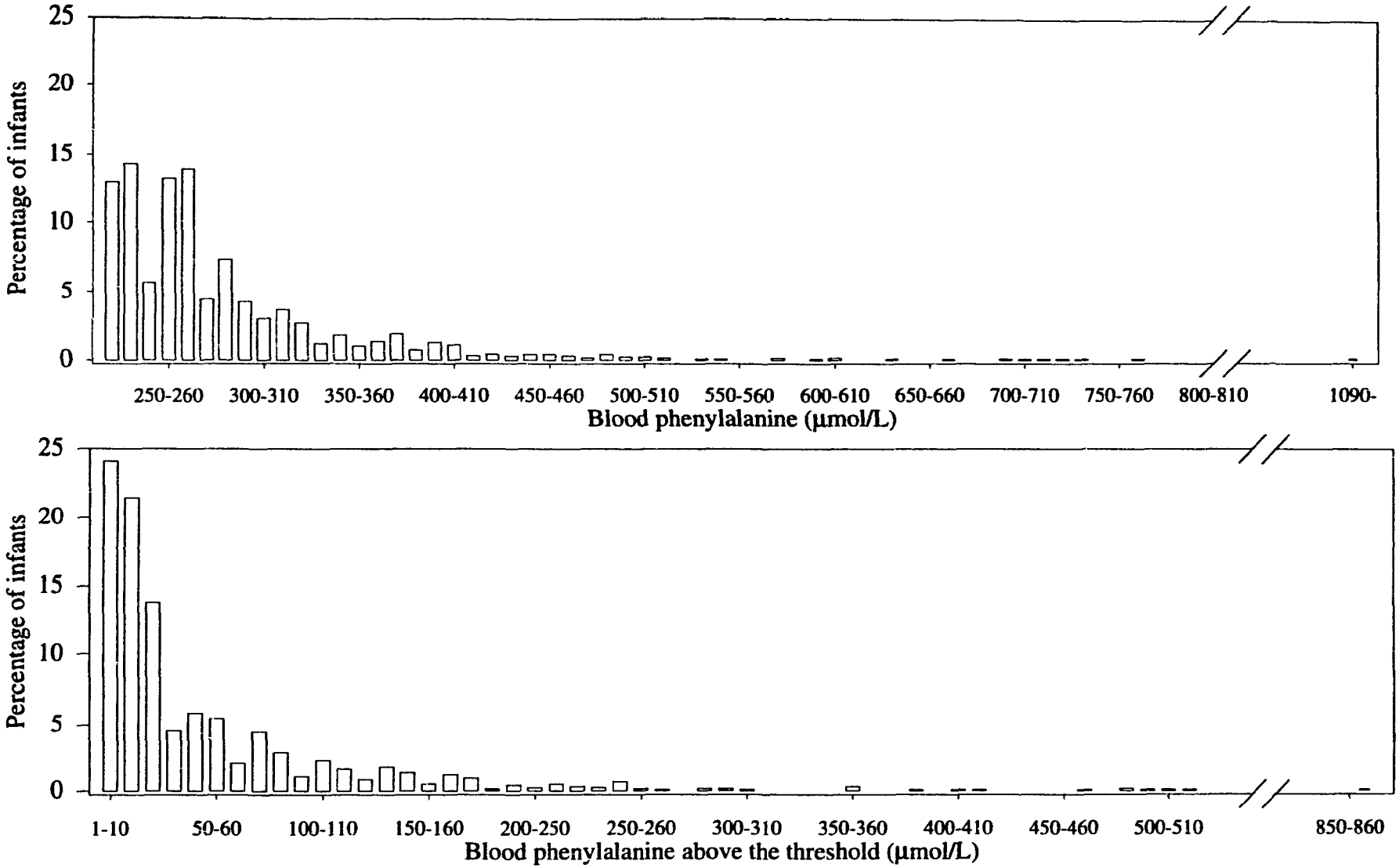

Figure 2[illegible] Distribution of phenylalanine values (top) and the amount exceeding the period specific threshold (bottom) for infants with phenylalanine values above threshold (n = 1230) (January 1973 to April 1990)

C.2.3: Phenylalanine values by month

Friedman's two way analysis of variance by ranks was done by ranking the average monthly phenylalanine value for every year to examine the variation in monthly mean phenylalanine. This was done to take into account the fact that there was more between-year variation in the mean phenylalanine than within-year variation. The resulting χ^2_{11} (= 21.3) differed significantly ($0.05 > p > 0.025$) from the null hypothesis that all months held equal ranks of mean phenylalanine over the years 1973 to 1990. The months May through August consistently ranked lower (July and August being the lowest) than other months. December and January had the highest ranks of mean phenylalanine values. This suggests a seasonal trend in mean phenylalanine values. This seasonal change could be due to humidity (see section C.2.6), temperature or variation in screening methodology, as discussed in Chapter 3.

C.2.4: Phenylalanine values by age at screening

The mean phenylalanine values of normal newborn infants were examined by age at screening. No discernable patterns of change in mean phenylalanine values of normal infants were noted when mean phenylalanine values were compared by age at screening for each year. A paired t-test was done to test if infants screened at less than two days of age had lower phenylalanine values than infants screened at two days or greater by pairing the mean phenylalanine values of these groups of infants for 14 reporting periods. There was no significant difference in mean phenylalanine between infants screened at

less than two days of age and those screened at two days or more ($t_{13} = 1.83$, $0.1 > p > 0.05$).

The variance of the mean phenylalanine values of infants screened at the age of less than two days is very large because of the very few infants tested at one day of age. It is possible that infants screened this early are done so for different reasons: illness, transfer from one hospital to another, or suspicion of a metabolic disease. If this is the case, these infants are not comparable to infants screened at a later age. Serial determination of phenylalanine values for a group of infants during the first week of life better estimates the change in phenylalanine values in the newborn period (see section 5.3.3).

The mean phenylalanine of infants with phenylalanine values above the threshold showed significant change with increasing day of screening test ($F_6^1 = 46.7$, $p < 0.0005$). A simple linear regression of the mean phenylalanine of these infants on age at screening showed that for every additional day of age until screening, there was a 10.3 µmol/L increase in the mean phenylalanine value. The increase in mean phenylalanine could be due to the higher screening threshold for later days of life. The variable threshold was adjusted for, and the trend of mean phenylalanine of infants with phenylalanine values above the threshold over time was again significantly different from the null ($F_6^1 = 21.0$, $p < 0.004$). This indicated that after adjustment for the variable threshold, there was still a 6 µmol/L per day increase in mean phenylalanine of infants with phenylalanine values above the threshold. The mean phenylalanine of infants with phenylalanine values above the threshold screened at less than one day of age was 25.0 µmol/L above the threshold,

while at the seventh day of life the mean phenylalanine was 67.7 μmol/L above the threshold.

C.2.5: Combined effects of variables on normal phenylalanine values: multiple linear regression

Multiple linear regression of age at screening, gender, month of birth and place of birth on phenylalanine values of normal infants was done to estimate the contribution of these four covariates to the overall variance in phenylalanine values. Data for individuals born within the period April 1988 to April 1990 was used. A 60% random sample of the data from this time period was used to establish the model, while the other 40% of the data was used to confirm the parameter estimates of the established model.

Results of a Friedman's two way analysis of variance by ranks for month of birth (see section C.2.3) and regional data for normal infants were used to create dummy variables to reduce the number of variables to be included in the model. For both month of birth and place of birth, the lowest ranked group was used as the reference dummy variable. Groups established for season of birth were: *i)* May, June, July and August (reference); *ii)* September, October, November and December; *iii)* January, February, March and April. Four groups were established for region of birth: *i)* Québec, Saguenay - Lac St. Jean and Trois Rivieres (reference); *ii)* Nord Ouest, Cote Nord and Nouveau Québec; *iii)* Montreal; *iv)* Outaouais, Cantons de l'Est and Gaspésie.

Regression analysis of the data set containing 60% of the data for normal infants for the period April 1988 to April 1990 yielded significant results in favour of the full model ($F^{7}_{103,935} = 612$, $p < 0.0001$), but the parameter for gender was not significant

(t_l = -0.28, p = 0.78). All other parameters achieved a p-value of less than 0.0001. A reduced model without the covariate gender gave a significant result ($F^6_{104,081}$ = 715.4, p < 0.0001) with all coefficients being significantly different from zero (Table 11). The model, however, explained only four percent of the total variation in phenylalanine values in the sample of normal infants.

Tolerance and collinearity diagnostics were estimated for the best model (which included the covariates age, season of birth, and region of birth) to verify the independence of all variables. Collinearity of variables was especially a concern for categorical variables created by groupings. All tolerances were acceptable and in the range of 73.2% to 99.7%. Collinearity diagnostics identified eigenvalues ranging from 1 to 11, indicating no collinearity problems.

Data for normal infants not used in the initial analysis (40% of infants born between April 1988 and April 1990) were used to test the stability of the regression model. Analysis of the regression of age, place of birth and month of birth on phenylalanine value using the 40% data sample gave R^2 = 0.038, very similar to the R^2 of 0.040 obtained from the model using the original data. All parameters were of the same sign and magnitude as the original regression model. Changes in parameter estimates ranged from 0% (for the months September to December) to a decrease of 13.7% (for the regions of Outaouais, Cantons de l'Est and Gaspésie, from 5.1 to 4.4 μmol/L) from the original regression model.

Although the parameter estimates of the covariates age, region of birth and month of birth were significantly different from zero, this can be attributed to the very large

Table 11. Multiple linear regression best model: the effect of age at screening, place of birth and month of birth on normal phenylalanine values

Variable	Parameter Estimate (μmol/L)	Standard Error	*t* for Null Hypothesis
Intercept	138.8	0.4	348.3*
Age at screening	-1.6	0.1	-17.2*
Region 2: Nord Ouest, Cote Nord, Nouveau Quebec	7.0	0.4	16.0*
Region 3: Montreal	3.2	0.2	15.5*
Region 4: Outaouais, Cantons de L'Est, Gaspesie	5.1	0.3	15.8*
Season 2: September to December	12.8	0.2	58.9*
Season 3: January to April	7.4	0.2	34.4*

R^2 for the model = 0.040

Adjusted R^2 for the model = 0.040

$F^{6}_{104,081} = 715.4$, $p < 0.0001$

* $p < 0.0001$

Reference groups were as follows: Region 1: Quebec, Saguenay Lac St. Jean, Trois Rivieres

Season 1: May to August

number of observations used to estimate the parameters. Not all the parameter estimates are clinically significant, but those approaching or exceeding 10 μmol/L are worth consideration as factors that may have a clinically meaningful effect on phenylalanine value determination.

Covariate effects could arise from various sources, as discussed in section 2.3. "Region of birth" effects could be due to regional differences in newborn diet, weather differences, length of time the samples take in mailing, etc. "Month of birth" effects could be due to humidity or screening methodology such as age of the filter papers and delays in the time of analysis of samples (due to holidays, vacations, and periods of elevated birth rate). A more in depth and controlled examination of factors that contribute to "Region" and "Season" would be necessary to disentangle their effects.

The very small proportion (4%) of the variation in phenylalanine values explainable by the covariates in the model indicates that there are other factors which account for the majority of variation in phenylalanine values of newborns. Some of the variation in phenylalanine value is attributable to the variation in blood spot thickness which is not controlled for by the screening procedure. The remainder of the variation in phenylalanine values is attributable to either extrinsic factors which affect phenylalanine interpretation (such as diet), or true inter-individual variation in blood phenylalanine as a result of variation in phenylalanine metabolism.

C.2.6: Association between phenylalanine values and humidity

Monthly means for percent humidity for the years 1973 through 1992 collected at Dorval Airport, Montréal were used to examine the effect of humidity on the results of the screening test. Newborn phenylalanine data from April 1983 to March 1984 and from April 1988 to March 1990 were correlated to the percentage of screened infants born in the Montréal region with phenylalanine values exceeding 211 µmol/L. The threshold of 211 µmol/L was chosen since this was the minimum screening threshold used during these time periods and had been used in a previous study (Hill, 1969). There was no apparent relationship between the mean monthly humidity and the percentage of infants in the datasets with blood phenylalanine exceeding 211 µmol/L.

Monthly mean phenylalanine values of normal infants in the Montréal region from 1973 to 1990 were correlated with their respective mean monthly humidities. No correlation was found between the mean monthly phenylalanine of infants born in the Montréal region and the mean humidity for that month ($F'_{133} = 0.39$, $p > 0.5$).

The lack of correlation between humidity and phenylalanine values in these data, is in sharp contrast to results on the same subject demonstrated by Hill (1969). Hill was able to show correlations of relative atmospheric humidity and phenylalanine values both experimentally and with screening data, making a very strong case for their association. One explanation for the discrepancy of the results may be that atmospheric humidity is not a good measure of the humidity to which screening filter paper is exposed in Québec. The North Carolina screening program which Hill evaluated in 1969 could have had much less indoor climate control than has been present in Québec hospitals for the past

20 years. Today, both air conditioning and heating make atmospheric humidity a poor indicator of indoor building humidity. The types of heating and cooling systems vary widely from hospital to hospital making the true humidity to which filter paper is exposed unpredictable for an entire region.

C.2.7: Phenylalanine values in putative twin pairs

An attempt was made to identify twin pairs in order to estimate the heritability of phenylalanine metabolism. Information was taken from the adjusted data sets (as described in section B.4). Putative twin pairs (or sets of infants) were extracted who had matching data for: date of birth, date of test, hospital of birth and father's initial, as well as having consecutive ID numbers.

No information in the data sets could be used to predict the zygosity of the twin pairs, so twins were categorized according to gender: both female, both male, or one female and one male. The female-female and male-male pairs are referred to as "like-sex pairs", and the male-female pairs as "unlike-sex pairs". It should be noted that these are putative twin pairs, identified as reliably as possible from the data, but without independent confirmation of their twin status.

Analysis of file review data for individuals with PKU or non-PKU HPA showed one pair of twins with non-PKU HPA. Analysis of phenylalanine value data from the pair of twins showed no significant difference in their phenylalanine values taken on the same days over six years (paired $t_{10} = 0.28$, $p > 0.78$).

Extracting sets of individuals from data of normal infants that matched the above criteria yielded 5,442 twin pairs, 222 sets of triplets, 9 sets of quadruplets and 2 sets of quintuplets. There were 1,513 male-male twin pairs, 1,465 female-female twin pairs and 2,464 male-female pairs.

The number of monozygotic (MZ) and dizygotic (DZ) twins in the set was estimated using the formulae (Sofaer, 1990):

$$\textit{proportion of MZ pairs} = \frac{(\textit{number of like pairs} - \textit{number of unlike pairs})}{(\textit{number of like pairs} + \textit{number of unlike pairs})}$$

$$\textit{proportion of DZ pairs} = \frac{2 \times (\textit{number of unlike pairs})}{(\textit{number of like pairs} + \textit{number of unlike pairs})}$$

based on the assumption that non-identical twins will occur with equal probability in the two groups. The estimated proportion of MZ twins was 9.4% of all the twins, or 514 of the 2,978 like-sex pairs.

Heritability of a continuous trait can be measured as a function of variance within (V_W) and variance between (V_B) twin pairs (Sofaer, 1990):

$$V_W = \frac{\sum (X - Y)^2}{2N}$$

$$V_B = \frac{1}{N-1}\left[\frac{\Sigma(X+Y)^2}{2} - \frac{(\Sigma(X+Y))^2}{2N}\right]$$

where X is the phenylalanine value of the first twin, Y is the phenylalanine value of the second twin, N is the number of twin pairs, and the summations are over the N twin pairs.

The proportion of variance (either V_W or V_B) in the like-sex individuals that can be ascribed to dizygotic twins can be predicted from the estimated proportion of dizygotic twin pairs in the like-sex sample because of the additivity of variance. The estimate of the proportion of like-sex variance attributable to like-sex twins was based on the weighted average of the sum of squares of DZ and MZ twins. The estimate of the DZ sum of squares was a weighted average of the estimated average sum of squares of the unlike-sex twins.

The variance within pairs (V_W) for the like-sex pairs was 494.8 $(\mu mol/L)^2$ and for the unlike-sex pairs 646.7 $(\mu mol/L)^2$. This indicated that like-sex pairs were more similar than the unlike-sex pairs. The MZ twin V_W was calculated to be 83.6 $(\mu mol/L)^2$ and the DZ twin V_W was 1,057.9 $(\mu mol/L)^2$.

Variance between pairs (V_B) was almost the same for like-sex twins 2,067 $(\mu mol/L)^2$ and unlike-sex twins 1,958 $(\mu mol/L)^2$. The estimate of MZ twin V_B was 349.3 $(\mu mol/L)^2$ and for DZ twins was 3,675.7 $(\mu mol/L)^2$.

To estimate heritability of phenylalanine values, the intra-class correlations (I.C.C.) for both dizygotic and monozygotic twins were calculated. The intra-class correlation was calculated by:

$$I.C.C. = \frac{(V_B - V_W)}{(V_B + V_W)}$$

The I.C.C. for MZ twins was 0.613, and for DZ twins was 0.553. An upper limit for heritability of phenylalanine values was then calculated as:

$$h^2_{UL} = 2 \times (I.C.C._{MZ} - I.C.C._{DZ})$$

The upper limit of the hereditability of phenylalanine values in twins was calculated to be 12.0%.

The approximate expected incidence of twins is 11 per 1,000 births (Sofaer, 1990), and gives an estimate of 3,600 twins for the 300,000 infants in the datasets from which the twins were extracted. The 5,442 twin pairs and 233 other multiple births exceeded the expected number of twins because of the non-specificity of the data available for twin selection. In larger hospitals, identical discharge dates means many infants are born and tested on the same day, with consecutive filter papers, and thus consecutive ID numbers. It is possible that common initials such as "C", "M", "S", and "T" were frequently represented among infants born on the same day, because only one initial was used for identification. Non-twin pairs may thus be responsible for some proportion of the apparent excess in the number of identified twin pairs, rather than a high incidence of actual twin pairs. Actual twin pairs may have been excluded from this dataset if they were born just before and just after midnight, giving them different birth dates or if they were screened on different days due to illness or complications. The heritability estimate of 12.0% is undoubtably lower than it would be for a similar analysis of confirmed twin pairs. False twin pairs caused by factors mentioned above dilute the heritability estimate measured in this data set. The heritability should therefore be considered to be a minimum estimate for the heritability of phenylalanine values in the newborn period.

C.2.8: Comparison of different test methods

Mean phenylalanine values recorded by the Newborn Screening Program were much higher than the previously reported 70 μmol/L population phenylalanine mean of adults (Scriver, 1985), so an experiment was performed to analyze the effect of two different methods of measuring phenylalanine. Blood samples were taken from 10 adult subjects at the Montréal Children's Hospital. One half of each blood sample was used to measure blood phenylalanine by elution column chromatography at the hospital. The other half of each sample was used to make blood spots on filter paper and was sent to the Newborn Screening Program for phenylalanine measurement by fluorometry. Paired comparisons were made on the resulting phenylalanine levels.

The experimental results on adult blood show a significant difference between the mean phenylalanine values for the two different methods (paired $t_9 = 18.5$, $p < 0.0001$). The mean phenylalanine value for blood spotted filter paper tested by fluorometry was 115 μmol/L, much higher than the mean phenylalanine value for the "gold standard" elution chromatography of 70 μmol/L (Table 12). These results agree with the results of Jew et al. (1987) that test different phenylalanine measurement methods. Their test of paired filter paper and serum fluorometry measurements of more than 30 newborn infants showed an average difference of 1.35 mg/dL (81 μmol/L) between serum and blood spot phenylalanine measurements. This compares favourably with the 56 μmol/L average difference between the two methods shown by this analysis. As Jew et al. (1987) measured both serum and blood spots by fluorometry, it would be reasonable to assume that the large difference in phenylalanine values measured in this experiment is primarily

Table 12. Mean phenylalanine values in adults assessed by whole blood chromatography and fluorometry of blood-spotted filter papers (May 1993), compared to the mean phenylalanine values from the Newborn Screening Program, 1973 to 1990

	Data from Newborn Screening Program	Elution column chromatography	Filter paper fluorometry
Mean (μmol/L)	127.7	70.3	115.3
Standard deviation (μmol/L)	--	13.3	11.5
Range (μmol/L)	111.1 to 150.3	--	--

attributable to differences between serum and filter paper testing, instead of the differences between fluorometry and elution column chromatography.

The consistently large difference in phenylalanine values obtained by different measurement methods should caution interpretation of phenylalanine values and threshold levels. If a recommended threshold level of 240 μmol/L is used, a screening program using fresh blood samples may not detect infants who, if screened by blood spotted filter paper, would have been detected as having slightly elevated phenylalanine values. Although the initial screening tests are done with filter paper analyzed fluorometrically, the difference in values resulting from the two methods is important in Québec because blood phenylalanine evaluation at the clinics may be primarily done by chromatography of fresh blood samples, and not spotted onto filter paper, although the same threshold is considered.

C.3: Analyses of individuals

C.3.1: Gender ratios

The gender ratio in all normal screened individuals over all years was 1.045 males per 1 female. The gender ratio of infants with phenylalanine values above the threshold, represented by 650 males and 578 females (and 2 with unknown gender), gave a ratio of 1.12, which did not differ significantly from the proportion of males in the screened population ($\chi^2_1 = 1.44$, $p > 0.1$).

C.3.2: Age at screening

The distributions of age for each reporting period differed significantly from a normal distribution by the Shapiro-Wilk test (for 1973) or the Kolmogorov-Smirnov goodness of fit test (for all other years). Non-normality indicated that the mean of the distributions may not be a good univariate descriptor but, as assigned age categories ranged from 0 to 7 days, comparing medians from each year gave little indication of the decrease in age at screening over time. The median age was 4 days of age from 1973 to 1982-83, and was 3 days of age in 1983-84, 1988-89 and 1989-90. The age at screening has decreased over time: 25% of newborn infants were screened at less than 4 days of age in 1973, whereas 66% were screened at less than 4 days of age in 1989-90.

Despite their unsuitability, means are used in further calculations to demonstrate trends. A simple regression of mean age over reporting periods showed that this mean has changed significantly ($F^{1}_{11} = 177.8$, $p < 0.0001$) since 1973, decreasing from 4.15 days in 1973 to 3.42 days in 1989. The distribution of ages for infants with phenylalanine values above the threshold (Figure 26) was similar to that of individuals with PKU or non-PKU HPA (Figure 13). There was a nearly significant ($F^{1}_{11} = 3.8$, $p < 0.08$) change in age at screening over time that paralleled the trend seen in the normal population, decreasing from 4.76 days in 1973 to 3.82 days in 1989-90. A comparison of the mean age at screening of the infants with phenylalanine values above the threshold and normal infants by year indicated that the mean age for infants with phenylalanine values above the threshold was significantly higher than that of the normal infants ($t_{13} = 3.07$, $p < 0.01$) when matched by reporting period. Infants with phenylalanine values above the threshold

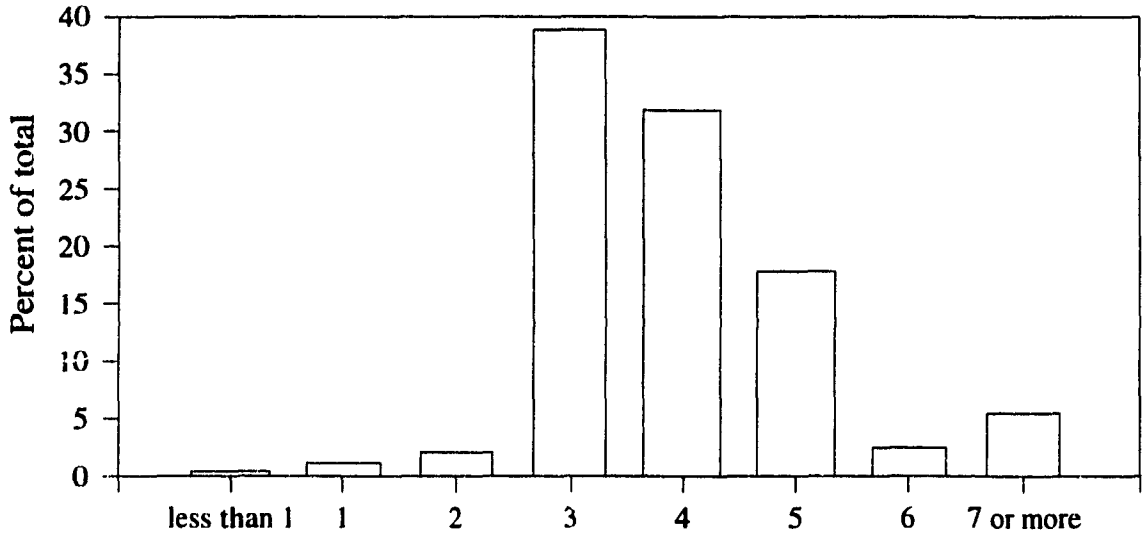

Figure 26. The distribution of the age, in days, at which the screening test was done for infants with phenylalanine values above the threshold.

were older at the time of testing than normal infants by an average of 0.3 days. The only three reporting periods in which the infants with phenylalanine values above the threshold had a lower mean age at first testing than normal infants were reporting periods during which there were a large number of infants with phenylalanine values above the threshold in comparison to other reporting periods.

C.4: Summary

These results show general trends in the data available for newborn infants who were screened by the Québec Newborn Screening Program. The bias caused by thick blood spots is not estimable in these datasets and remains a barrier to determining the accurate estimators of phenylalanine values in the newborn population of screened infants. Two sources of bias are known: recording bias and thick blood spot bias, but cannot be quantified. Other sources of bias which are not evident from the selected data obtained for this study may affect these datasets as well. These analyses serve as suggestions should a more complete and adequate dataset of newborn screening data become available.

If the data were reanalysed, several better approaches to analysis could be used. Thorough testing of the assumptions of normality and homoscedasticity would be necessary. These tests would be used to choose the statistical test that would be best for the data distributions. A weighted analysis of records, instead of a random sampling, would better represent the wide range of phenylalanine values in the datasets. Another approach would be to match records of individuals with PKU or non-PKU HPA with

randomly drawn samples of records of normal infants or infants with phenylalanine values above the threshold and match on demographic variables to examine effects of interest. Such an approach would compare the characteristics of individuals with PKU or non-PKU HPA directly to a matched group of unaffected individuals.

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