DETECTION OF NEUROLOGICAL ABNORMALITIES IN ADOLESCENTS AND ADULTS WITH PHENYLKETONURIA

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LIST OF ABBREVIATIONS

PKU	phenylketonuria
MRC	Medical Research Council
Phe	phenylalanine
Hyperphe	hyperphenylalanine
MRI	magnetic resonance imaging
CT	computed tomography
IQ	intelligence quotient
IQ-sds	intelligence quotient standard deviation score
tyr	tyrosine
PAH	phenylalanine hydroxylase
DHPR	dihydropteridine reductase
VEP	visual evoked potential
SEP	sensory evoked potential
NCV	nerve conduction velocity
EEG	electroencephalogram

LIST OF ABBREVIATIONS CONT.

BAER	brainstem auditory evoked response
CMCT	central motor conducting time
CSCT	central sensory conducting time
CNS	central nervous system
PNS	peripheral nervous system
PLP	proteolipid protein
MBP	myelin basic protein
Gal-C	galactocerebrosidase-C
MS	multiple sclerosis
CI	confidence interval
δMRI	change in MRI
δphe	change in phe
δtime	change in time
PVL	periventricular leucomalacia

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DECLARATION

The work reported in this thesis results from a study I completed in the Willink Biochemical Genetics Unit in Manchester from April 1992 until April 1994.

The work was completed by myself with the exceptions described below.

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SUMMARY

Phenylketonuria (PKU) is an inborn error of amino acid metabolism. It occurs in approximately 1 in 10,000 of the United Kingdom population and follows an autosomal recessive pattern of inheritance. The disease results from a deficiency or defect of the enzyme phenylalanine hydroxylase which converts phenylalanine (phe) to tyrosine (tyr). In PKU there is an accumulation of phenylalanine that is toxic to the developing brain and causes mental retardation, spasticity and seizures. The treatment of PKU is the adherence to a special phe-restricted diet with amino acid, vitamin and mineral supplements. There are guidelines for the duration of dietary therapy in the United Kingdom produced by the Medical Research Council (MRC) Steering Committee on Phenylketonuria. These guidelines have been reviewed recently and lifelong diet is now recommended.

This study began prior to the publication of these recommendations at a time when many centres discontinued diet in early adolescence. The rationale for stopping diet in adolescence is that the majority of brain development is complete by the end of childhood. The risks to the brain of the subsequent rise in phe levels in acolescence and adulthood after diet cessation remain unclear. The impetus to this study was the emergence of reports of adults with PKU (who had discontinued diet after childhood) developing new neurologica signs such as spasticity of the limbs. Magnetic resonance imaging (MRI) of the brain in these patients showed an abnormal signal in the cerebral white matter and it was initially suggested that the MRI changes depicted the structural abnormalities causing the loss of function in these individuals. It was postulated by these authors that elevated phe levels in later life had a toxic effect on myelin and that adults with PKU were at risk of later onset neurological damage.

This study was designed to obtain more information about brair function in adults with PKU who had stopped dietary treatment and to attempt to ascertain whether the MRI abnormalities described in several adults had any clinical significance. It was initially designed in 2 stages with a period of observation between stage I and II. However, after the MRC PKU guidelines were

published during the study, the participants were given the opportunity to return to diet. Hence in the second stage of the study many patients were on some form of dietary treatment.

The initial study group was aged 14-48 years. They were patients with PKU who had discontinued dietary treatment at age 14 years. This group were examined for signs of neurological abnormalities, underwent comprehensive neurophysiological testing and MRI of the brain. The study was later extended to include MRI of a younger group of patients aged 10-14 years who were still on diet. This younger group were studied to see whether dietary therapy and therefore lower phe levels protected against the development of abnormal brain MR images seen in the older group. They did not participate in the neurophysiological investigations.

Clinical examination was normal in the majority of the patients. In 11 of the 58 examined, brisk reflexes were illicited. Five of these 11 patients had a fine tremor at rest. Neurophysiological testing showed a significant difference between the mean visual evoked potentials latency of the PKU group and a control group. There were no other significant differences between the groups on neurophysiological testing. Central motor conducting time was marginally prolonged in three individuals with PKU, visual evoked potentials in eight and peripheral nerve conduction velocities in five.

MRI brain abnormalities were present in 71 of the 74 patients scanned. The changes consisted of an increased T-2 weighted signal in the periventricular white matter indicating an increased water content of the myelin. A scoring system was used to assess the extent of abnormalities. The mildest changes involved only the occipito-parietal areas of the brain whereas more severe changes extended into the frontal lobes. The severity of the white matter appearance was unrelated to findings on clinical examination or the results of neurophysiological tests. The extent of MRI change was most significantly associated with phenylalanine levels around the time of MRI scan. There was no significant association between MRI severity and early exposure to phe.

Fifty-seven patients had repeat MRI scans after an interval ranging from 3 to 12 months. Sixteen of these were in the younger group and made no dietary alteration between scans. In the older age group who were on a normal diet at the first scan, five patients returned to a strict low-phe diet aiming for blood levels less than 400 μmol/l, 21 returned to a low phe diet and restarted amino acid supplement aiming for levels around 900 μmol/l and the other 15 made no dietary alterations and had blood phe levels around 1200 μmol/l.

The extent of white matter abnormalities on repeat scanning was again related to the phe level at the time of scanning. An improvement in the MRI appearance was recorded in those patients who had achieved lower phe levels. The greatest improvement was seen in those who had reduced phe levels to < 400 μmol/l. In the group who lowered phe levels slightly to <900 μmol/l, four scans showed a marginal improvement. There was little change in the patients who made no dietary changes. The extent of MRI improvement was significantly related to the degree of reduction in blood phe levels. Two adults underwent serial MRI scans before and after reducing phe levels to < 200 μmol/l. No change in scan appearance was seen after a period of four weeks.

Thirty of the adult group aged 14-49 years had repeat neurophysiological testing after one year. There were no significant changes in the results recorded from the previous year.

The main conclusions drawn from this work are that MRI white matter changes are a common finding in adolescents and young adults with PKU. Clinical or sub-clinical abnormalities are however unusual and their presence is unrelated to the changes observed on cerebral MRI. The MRI changes form a typical distribution in the periventricular area of the brain and their extent is most closely related to blood phe levels at the time of scan. The MRI abnormalities do not fluctuate on a daily basis. They are reversible when blood phe levels are reduced but changes take more than four weeks to disappear.

1. INTRODUCTION

1.1 Background

Phenylketonuria (PKU) is one of the most common inherited metabolic diseases in the United Kingdom occurring in approximately 1 in 10,000 births (1). In the majority of cases, it is due to a deficiency or defect of the enzyme phenylalanine hydroxylase (2,3). Patients with this disease are unable to metabolise the essential amino acid phenylalanine (phe) which accumulates in the body and is associated with damage to the developing brain. Without specific treatment the disease results in severe mental retardation, microcephaly and spasticity (4). Neonatal screening has been carried out for this disorder in the United Kingdom since the 1960s and children whose elevated phe levels meet certain diagnostic criteria are started on a phe-restricted diet (1). This diet includes artificial food products which provide other essential amino acids, vitamins and minerals whilst specifically restricting the quantities of phe. The diet is successful at preventing the severe complications associated with untreated PKU and is usually strictly followed at least throughout childhood (5-7). After childhood it had, until recently, been considered safe by many to discontinue or at least relax the diet, as the majority of brain development is thought to be complete in the first decade of life.

Recently there have been concerns about the development of neurological abnormalities in adults whose phenylalanine-restricted diet has been relaxed or discontinued (8-10). These reports added to the body of concerning evidence on the safety of diet cessation in PKU and led to the Medical Research Council (MRC) Steering Party on PKU recommending dietary treatment to be lifelong (11). This project aims to investigate several features in relation to the development of late neurological effects in a group of young adults.

1.2 Definition of the disorder

Hyperphenylalaninaemia is the inclusive term for a group of disorders that result in an elevated plasma phenylalanine greater than 120 μmol/l (12). Phenylalanine is an essential amino acid making up between 4 and 6% of the amino acid content of natural proteins and plasma concentrations are normally maintained within physiological limits by a balance between inflow from ingested protein, endogenous protein turnover and conversion of phenylalanine to tyrosine in the liver (13). The hyperphenylalaninaemias are a heterogeneous group caused by mutations at several different gene loci responsible for coding components of the hydroxylation reaction (14). They can be divided into phenylalanine hydroxylase (PAH) deficient and non-PAH deficient forms. Non-PAH forms result from errors in biopterin metabolism and present a different phenotype (13). PAH deficient hyperphenylalaninaemia itself includes a range of phenotypic presentations. It can be divided further according to the degree of hyperphenylalaninaemia that occurs after ingesting a normal protein diet (15). When the plasma phe is above 1200 μmol/l on a normal protein intake, the disease is sometimes referred to as "classical" PKU. It is this group of patients that are described in this thesis and the term PKU will be used hereafter for this condition.

1.3 Clinical description

In 1934, Dr. Asbjorn Folling investigated two mentally retarded sibilings, aged six and three years old, whose mother had noticed that the children exuded an unpleasant odour (16). Other than the mental retardation, there were no positive signs on examination. However, when Folling examined the urine he found that the addition of ferric chloride produced a green colour. The substance responsible was identified as phenylpyruvate and Folling called this disease 'imbecillitas phenylpyruvica.' On further studies it was confirmed to be a disorder of phenylalanine metabolism (2,3). As a result of screening children in mental institutions more patients were identified and the phenotype became recognised to consist of mental retardation with spasticity and epilepsy. Affected patients also have a mousy odour, are prone to eczema

and are hypopigmented with blonde hair and blue eyes. The name phenylketonuria was introduced by Penrose and Quastel (17) due to the elevated urinary excretion of phenylketones that occurs in affected patients. Treatment was first attempted by Bickel in 1953 (18,19) and Woolf in 1954 (20). In the initial description of the application of a low phenylalanine diet by Bickel (18), a two year old child with PKU who had marked developmental delay showed remarkable improvement in alertness and development within months of starting the diet. In an attempt to ensure this improvement was secondary to the diet, the child was fed phenylalanine. After the introduction of phe to the diet, the girl began to lose developmental skills again. The benefits of dietary treatment were thus established.

It is now realised that the phenotype of PKU is not uniform and that there are some individuals who despite not receiving treatment will not develop any of the classical features of the disorder (21). These people born prior to the introduction of newborn screening remain undiagnosed unless they conceive and produce offspring with the features of phenylalanine toxicity in utero such as microcephaly, mental retardation, growth retardation and congenital heart disease (22). The identification of such women of childbearing age remains a public health problem. Some regions have addressed this issue by adding blood phe testing at booking-in clinics in addition to the antenatal screening already established (23).

The major clinical feature resulting from PKU is neurological damage manifest as mental retardation, spasticity and epilepsy (4). Despite the increase in knowledge of the biochemistry of this condition, much of the neuropathology remains speculative. The current literature relating to the neuropathology of PKU is described later.

1.4 Diagnosis and treatment

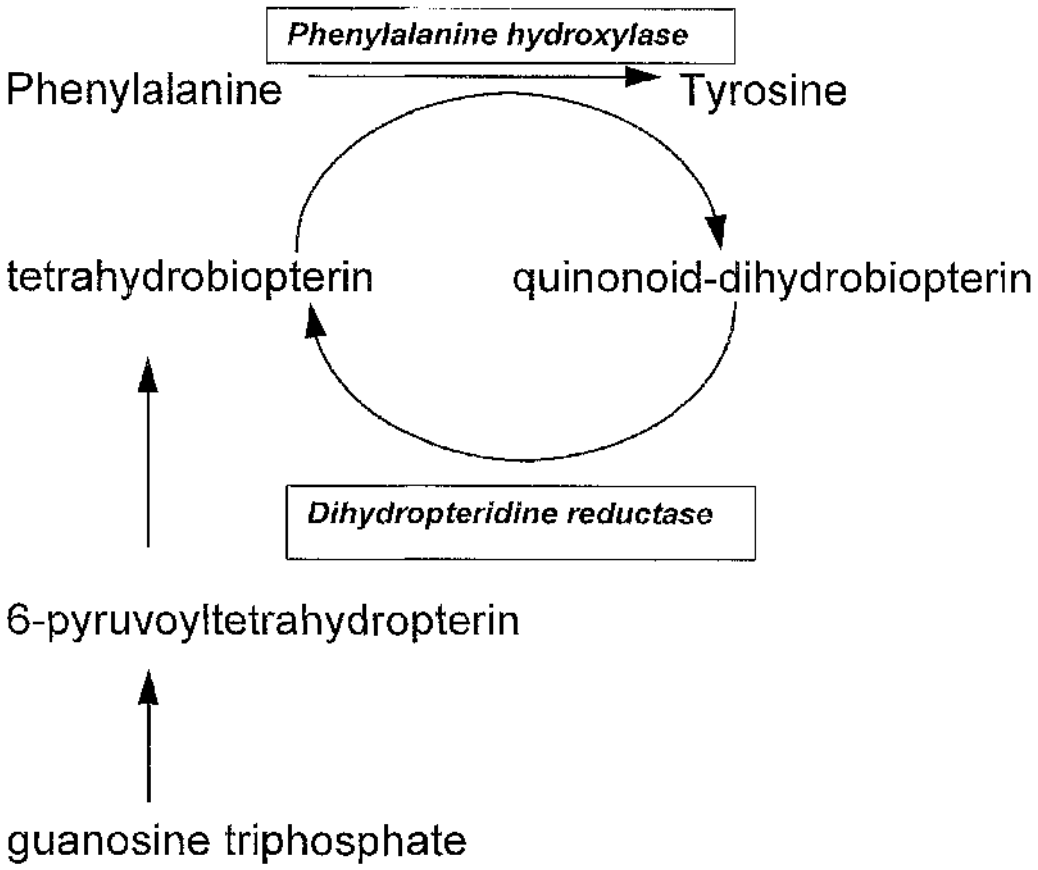
In order to detect inborn errors of phenylalanine metabolism, routine neonatal screening for PKU using whole blood obtained by heel-prick, is carried out in almost all countries with well-developed systems of health care (4). In the United Kingdom the chosen upper limit of normal varies from 180 to 240 μmol/l and the recommended time for testing is between 6 and 14 days after birth (24). Treatment consists of a reduction of natural protein intake to a minimum necessary to maintain phenylalanine in the required treatment range. This regime would not allow enough protein for growth; therefore a phenylalanine free protein substitute is given in sufficient quantities to provide the other amino acids necessary for normal development (25,26). Energy intake from carbohydrate and fat must be sufficient to avoid protein catabolism since this would lead to an endogenous elevation of blood phe levels. An adequate provision of minerals and vitamins must also be ensured (25).

1.5 Biochemistry

PKU results from an error in the phenylalanine hydroxylation pathway. The phe hydroxylation reaction is the rate-limiting step in the catabolic pathway that leads to the complete oxidation of phenylalanine to carbon dioxide and water (27). The body's supply of tyrosine is produced via this pathway and therefore in PKU tyrosine becomes an 'essential' amino acid since it cannot be manufactured from phenylalanine (27).

Phenylalanine hydroxylase activity in humans is largely restricted to the liver (27). Although PAH is detectable in reasonable amounts in the rat and guinea pig kidney, it is unclear whether it is present in appreciable levels in humans (28-32). The hepatic phenylalanine hydroxylating system contains three components: PAH, dihydropteridine reductase (DHPR) and the unconjugated pterin tetrahydrobiopterin. The reactions catalysed by PAH hydroxylase and DHPR in the presence of the pterin are shown in figure 1.

Figure 1: Biochemical pathway of Phenylalanine metabolism



1.6 Genetics

1.6.1 Phenylalanine hydroxylase gene

PKU is inherited as an autosomal recessive trait. Prior to the widespread use of molecular biological techniques, little was known concerning the structure and location of the gene encoding phenylalanine hydroxylase in man. In 1982 Robson et al. (33) synthesised cDNA from mRNA purified from rat liver by polysome immunoprecipitation. This clone was then used as a hybridisation probe to isolate several human PAH cDNA clones from a human liver cDNA library. The longest of these clones encoded a protein composed of 452 amino acids, with a predicted molecular weight of 51,862 (34). The amino acid composition of human PAH as deduced from the nucleotide sequence of the cloned DNA was very similar to that reported for the human protein (35). The PAH locus was assigned to chromosome 12 in 1984 (36). This localisation was achieved by hybridising human PAH cDNA probes with human/rodent cell hybrids containing different combinations of human chromosomes. The locus was localised further to band region q22-q24.1 region of chromosome 12 in 1985 (37).

The phenylalanine hydroxylase (PAH) gene is approximately 90 kilobases in length, contains 13 exons with intron sizes ranging from less than 1 to more than 20 kb, and encodes a mature messenger RNA of approximately 2.4 kb (38).

The PAH protein is a homopolymeric enzyme comprised of 52 kDa subunits. Comparisons of the primary structures of PAH proteins from different species (39) or of PAH and related hydroxylases in mammalian species (40) reveals a high degree of homology in regions of the protein encoded by exons 4 to 11 of the human PAH protein, with the highest degree of homology in the regions encoded by exons 7 to 9. This central region has been hypothesised to be important for the common functions of these proteins, namely binding of oxygen, cofactor and the catalytic conversion of substrate, while those features unique to individual proteins, such as

substrate specificity may lie cutside this region (27). The importance of this region is reflected in the large number of PKU-causing mutations found in this region in many ethnic groups.

1.6.2 Disease causing mutations in the PAH gene

The introduction of PCR-based mutation detection techniques has resulted in a rapid increase in knowledge of disease-causing mutations of the PAH gene. Large deletions of the PAH gene are not a frequent cause of PKU (27). The majority of other mutations are point mutations including nonsense, splicing and missense mutations. Many of the mutations occur in regions of the gene that are highly conserved. There are at least 25 missense mutations occurring at sites that are conserved in human PAH, rat tyrosine hydroxylase and rabbit tryptophan hydroxylase (40). The occurrence of disease-causing mutations at these regions suggests that these areas are extremely important for normal enzyme function. Exon 7 has the highest relative frequency of mutations associated with PKU but rather than being a particularly mutatable site it is thought that this exon confers an essential function for hydroxylation of phenylalanine (41). Using newer mutation detection methods it is now possible to characterise 99% of PKU disease causing mutations in some populations (42).

These newly developed detection methods will become extremely useful in characterising populations. In the English population, like many of the western European populations, the majority of individuals with PKU show compound heterozygosity for disease causing mutations (43). Unlike some countries where the majority of individuals share the same PKU mutation (44,45), there are several disease causing mutations implicated in England. Characterisation of the prevalent mutations cannot be performed by targeting techniques to detect specific mutations and gene-scanning techniques have to be used (46). Tyfield et al., (43) have used heteroduplex detection to identify mutations in the South West area of England and Scotland. Guldberg et al., (42) detected 99% of mutations in the Scandinavian population using direct gradient gel electrophoresis. Zschoke et al., (47) advocate a stepwise approach to mutation detection using a fluorescent multiplex assay for detection of three common mutations followed

by minihaplotype analysis to determine rare mutations which are confirmed by restriction enzyme analysis. The third step uses DGGE and sequencing to detect remaining mutations.

The most common mutations cited so far for the English population are R408W, I65T and IVS12nt (43). Mutations in the phe hydroxylase gene of the North Western population of England have recently been studied (Lesley Heptinstall, personal communication). This community shows a similar distribution to the previously quoted figures for England with R408W being the most frequently found mutation.

1.7 Efficacy of treatment

The efficacy of early treatment of PKU was shown in a study of 28 sibling pairs in whom one of each pair underwent dietary treatment (48). The index cases were late diagnosed children with PKU. Their IQ's were compared with those of an affected sibling who had been diagnosed and treated in the newborn period. The average IQ of the late diagnosed group was 45 (range 30 to 81) whereas in the early diagnosed group the IQ's were all above 80. A US sibling pair study compared IQ's of an early treated PKU child with their unaffected sibling in 36 sibling pairs (49). The PKU group average IQ was 94 and the unaffected group was 99. The North American Collaborative Study then undertook to evaluate the outcome of treatment by performing intelligence testing at regular intervals (50,51). Mean IQ at 4 years of age in 111 PKU children was 93; children treated in the first month had a higher mean score (95) than those treated between 31 and 65 days of age (85). This group was followed for several years. At age 8 years the PKU group mean IQ of 100 again verified the benefits of treatment. However, comparison with siblings showed that the PKU group had an IQ deficit. Furthermore, patients who had terminated the diet at an earlier age scored lower that those who had continued it longer (50,51). The UK MRC/DHSS PKU Register has followed up patients with PKU diagnosed by newborn screening in the UK since 1964. Data from this resource indicates that dietary treatment in PKU is successful in avoiding the brain damage seen in the majority of untreated cases. It has become apparent however, from the PKU register and other studies that some subtle changes in

intellect exist (52,53): PKU individuals have, on average, IQ's 0.5 standard deviations lower than their sibling's (54). In addition, several studies have identified other abnormalities in neuropsychological performance. Although all these studies differ slightly in the methodology, there is sufficient data to suggest that PKU can cause a variety of subtle abnormalities in performance. Abnormalities have been identified in simple reaction times (55), sustained attention (56), visual spatial motor function (57), perceptual motor function (58) and problem solving (59-61). In the UK, according to the PKU Register protocol, a behavioural assessment is completed by the schoolteacher at eight years. These assessments have identified abnormalities in behaviour in children with PKU consisting of hyperactivity, increased levels of anxiety and a tendency to solitude (62).

The overall merit of dietary treatment is widely accepted but the age at which it is safe to discontinue diet (if, in fact, it is safe at all) is not clear. There have been several studies aimed at assessing the effects on intellect after discontinuing dietary therapy. An early study followed a small group of American children for 2 years after the age of diet cessation aged 4 years (63). IQ dropped four points during that period. It was considered that this was insufficient evidence for diet continuation but that larger and longer scale trials were indicated. A larger trial between London, Warsaw and Heidelberg measured neuropsychological performance after termination of diet in two centres and relaxation in the third (5). Although IQ fell in all three groups the deficit was less in the group in Heidelberg who had not ceased diet completely. These findings were not however corroborated by the North American studies of Koff et al., (64) or Koch et al., (7). It is thought that differences in interpretation of neuropsychological testing may account for the lack of concurrence between these trials (65). In general the results were interpreted by many centres that diet cessation was detrimental early in childhood and could result in a fall in IQ score.

In the studies described above, the diet was generally terminated in early childhood, but recently it has been more common practice to continue diet at least until the age of ten years. There is less data available which measures the safety of stopping the diet after childhood. Beasley et al.

(66) report from the UK PKU registry on a group of patients detected by newborn screening between 1964 and 1971. It has been previously shown in this group that IQ is closely related to early phenylalanine control but that this effect was less pronounced after the age of eight-ten years (54). At age 18 years the group were re-tested. Twenty-three percent were on some form of relaxed PKU diet. After IQ was taken into account at age 14 years there was no further independent effect on IQ from phenylalanine levels after that time. Fisch et al. (67) assessed 19 early treated PKU patients who had been off diet for 12-28 years. Their IQ had not fallen greatly after diet cessation but five of the group had some form of mental illness and the authors concluded that IQ alone was not the prime consideration in whether it is safe to cease dietary treatment. Ris (68) reports on a group of adults with PKU who underwent a neuropsychological assessment aged over 18 years. Ten of the group were still on some form of diet, seven had stopped in adolescence and eight had discontinued it at various stages before adolescence. Those who had remained on the diet had better intellectual outcome. None of the group had overt neurological problems. Some clinics have maintained a diet for life policy (69) for many years. However, most centres have, until recently, allowed some relaxation of diet in adolescence in the knowledge that most of their patients have already stopped strictly adhering to the diet by that age.

1.8 Literature review on neurological problems

Interest in the question of the vulnerability of the adult brain to high phenylalanine levels has been renewed recently due to some reports of neurological deterioration in the older PKU population and the appearances of the PKU brain on magnetic resonance imaging.

In 1989, Villasara et al. (10) described 2 patients with hyperphenylalaninaemia. One of these patients, who had been diagnosed and started diet late at 3 years after investigation for mental retardation, developed spasticity aged 28 years. Diet had been discontinued at 12 years. MRI showed increased signal intensity around the centrum semi-ovale. Visual evoked potentials showed delayed latency as did sural nerve conduction. After re-commencement of a phe-

restricted diet, nerve conduction velocities returned to normal and clinical examination showed a reduction in muscle tone. Spasticity was still apparent and there was no report of a repeat MRI scan.

Villasana reported a second patient with HPA who was reviewed at 18 years after presenting with poor school performance (10). This patient had been diagnosed on newborn screening and treated neonatally, but had discontinued dietary treatment when aged 6 years. The only neurological abnormality clinically apparent was fine tremor of the outstretched hands. However, MRI again demonstrated increased signal in the periventricular area. Neurophysiological testing was normal and attempts at re-instituting diet were unsuccessful.

Following this in 1990, Thompson et al. (8) investigated seven PKU patients who had developed neurological abnormality. Four of this group had been diagnosed on newborn screening and three were diagnosed in childhood after presenting with developmental delay. Of the early treated patients, problems such as low IQ, poor growth and behavioural problems had developed in late childhood or early adolescence. Three of this group were thought to have good early control of diet. However, only one had an IQ above 90. All six patients who underwent MRI scanning had abnormal results with high signal areas in the cerebral white matter. Two patients with clinical abnormalities were followed serially with scans. One did not alter either on scan or clinically. Diet did not change in this two-year period. The other patient improved clinically after recommencement of diet. This was associated with a disappearance of some of the abnormalities on MRI.

There are difficulties in the interpretation of these findings. The early histories mention poor growth and low IQ's in some of the patients suggesting that the treatment of these patients was sub-optimal. Some of the patients whose phe levels were documented in the acceptable range even had poor growth and special learning needs. Although these reports were very important in that distinct neurological signs were documented in adults who had received some treatment for their PKU, it was difficult to know whether the patients formed an atypical group and whether the

work could predict outcome for teenagers who had maintained good biochemical control and were currently clinically normal.

An important feature of Thompson's paper (8) was the apparent reversibility of symptoms and MRI abnormalities on returning to diet in one case thus suggesting an effect possibly related to transiently elevated levels of phenylalanine.

Reversal of white matter changes was also reported in a single case by Battistini (70). An 18-year-old girl with PKU had developed depression and anxiety; neurological examination revealed hypotonia and weak abdominal reflexes and MRI of brain showed white matter abnormalities. Repeat MRI after two years on a low phe diet showed resolution of white matter changes.

In 1991, Bick (9) studied nine adolescents with PKU. This paper uses the term hyperphe rather than PKU and characterises the severity of the disorder according to Guttler's classification, which is based on phe levels on a protein intake of 2g/kg (15). In this categorisation, type I corresponds to classical PKU, type II to hyperphenylalaninaemia with phe levels > 600 μmol/l requiring diet and type III is hyperphe not requiring diet. Two of the group had type III hyperphe. Two boys with Type I ('classical' PKU) were late diagnosed at 1.8 years and 2 years and the other five children started diet by seven weeks. Two were still on diet aged 17 years at the time of study; one had stopped diet at 2.5 years, the others from 8-13 years. The group underwent VEPs, IQ and MRI. Clinical examination in the early treated or untreated children was normal. The two late treated children had neurological abnormalities consisting of mild spasticity of lower limbs and mental retardation. In all the patients with Type I or Type II hyperphe, marked changes in signal intensity in the periventricular white matter were seen on MRI scans. This is the same distribution as had been previously been documented. The scan was normal in the Type III patients. In the more severely affected patients, the lesions extended to the frontal and subcortical white matter. One of these patients who had continued diet from age six weeks and whose control was thought to be good also had cerebral atrophy. In addition his IQ was only 87. Visual evoked potentials were delayed in five of the eight patients tested; one of who had not

required treatment and had a normal MRI scan. There was no association between the MRI findings and the initiation, duration or quality of treatment or clinical neurological status.

Thompson further investigated this problem by scanning 25 asymptomatic PKU patients aged 7-25 years (71,72). Surprisingly, 23 of this group had increased signal intensity on MRI. Most of the group had achieved phenylalanine concentrations between 180 and 480 μmol/l during the first 8 years of life and seventeen of the group were still on diet. The subtler scan changes were seen in the younger patients who had never been exposed to long-term high phe levels. Patients who were known to have measurements of >1200 μmol/l for prolonged periods had the most extensive abnormalities. Neurological examination showed only hyperreflexia and tremor in 12 of the patients. Statistically significant associations were established between age, phe at time of scanning and phe in the period leading up to scan. This is the first paper that suggests a correlation between metabolic control and extent of MRI abnormality.

Shaw et al. (73,74) initially identified the white matter changes in a single case and then found a similar pattern in ten patients aged 13-27 years. They too recognised that the white matter abnormalities were less severe in those with satisfactory dietary control.

Pearsen performed MRI on fifteen patients with PKU (75). An attempt was made to correlate MRI scores with IQ and biochemical exposure. There was no positive association with either. It was noted that frontal lobe white matter change was present only in those with more severe white matter involvement.

Toma scanned 22 patients with PKU and found similar white matter changes in 9 of the group (76). Only those with good biochemical control had normal scans. The age range of this group is not reported.

A group of 16 well-controlled PKU children (6 of who did not warrant treatment) were examined in adolescence by Lou et al. (77) with neuropsychological testing, VEP, EEG and MRI. Two children had proton spectroscopy.

This is one of only 2 studies reporting proton spectroscopy results to date. Contrary to Ludolph's (78) and Bick's work (79), VEPs were normal. EEG abnormalities were frequent in the occipital lobe. MRI was abnormal only in 2 of the group with Type III hyperphe (not requiring diet). However all the others demonstrated some degree of MRI abnormality. Proton spectroscopy of an involved patch showed a normal proton spectrum, which indicated that there was no active demyelination at that site. Lou et al. (77) concluded that the abnormalities were more likely to relate to early exposure to high phe levels unlike the conclusions of Thompson et al. (72) who found that the degree of MRI abnormality related to later exposure to high phenylalanine levels. However, the results of proton spectroscopy in Lou et al.'s (77) report have added information by indicating that the lesion highlighted on MRI is not likely to be demyelination at least not in the sense of known demyelinating illnesses.

In Walter et al.'s study thirteen young adults with classical PKU underwent MRI (80). The number of individuals with abnormal scans is less in this group than in other reports. This is possibly due to the younger age range of the participants. Previous phenylalanine exposure was assessed in detail but no relationship could be established with present MRI abnormalities. Lack of correlation could relate however to the small numbers studied in this report.

Leuzzi et al. (81) investigated 22 early treated and 5 late treated PKU patients. Ten of the early treated group were still on diet and the other twelve had discontinued dietary treatment. Biochemical control was also assessed in detail in this study and positive correlations were found between MRI severity and phe levels in the year prior to scan. In the early treated group a significant correlation also existed between MRI severity and median monthly serum phe values for the whole period of treatment and for the period from age 4 years until MRI scanning. There was no significant association between MRI severity and age at scan, phe control until age 4

years or age at diet discontinuation. Soft clinical signs were observed in some of the group but there did not appear to be a significant association between the presence of clinical abnormalities and MRI scores. This paper was important in suggesting that MRI changes were related more to recent phe levels than early control although the number of patients studied in each group was small.

The most recent published work to emerge during the period of my study and is that of Bick et al. (79). In this report 10 adolescents and adults with PKU were investigated with MRI of brain, proton spectroscopy, T2 relaxometry and VEPs. This work adds support to the theory that the lesions seen on MRI are not indicative of active demyelination. Additionally T2 relaxometry suggests that the abnormalities represent free water in the myelin. There was no consistent relationship between MRI changes and IQ, time at diagnosis or initiation of therapy or VEP changes. MRI changes tended to be more severe in those with poor dietary control and high current phe levels. Reversal of changes was observed in 2 patients after strict diet was initiated. Bick concluded that the observed white matter changes probably represent reversible structural myelin changes rather than permanent demyelination.

The various clinical reports available are conflicting. Neurophysiological work has found normal and abnormal VEPs, EEGs, and NCVs. Neuropsychological tests have consistently documented PKU children to perform less well than their normal counterparts.

There is clear evidence that MRI changes exist in some young adults with PKU (8-10,70-73,75-77,79-81). The reports concur in the type of abnormal MRI signal produced in PKU and in the anatomical areas involved. The cause, duration and functional significance are not clear. There is a suggestion that levels of phe are important but it is unknown whether this relates to phe at time of scan or in the preceding lifetime. Reversibility has been noted in five patients (8,70,79).

The work to date highlights a problem: that adults with PKU, even those well treated from the neonatal period are at risk of developing abnormalities on MRI brain scans. A smaller number

may be at risk of developing florid neurological abnormalities. Studies to date have been on small numbers. The criteria for case selection are unclear in many of the studies and the population base is uniformly unstated. The following questions emerge from a review of the literature.

1) What is the nature of the abnormalities on MRI imaging or neurophysiological studies?

2) How long has it been there?

3) Is it progressive?

4) Is it clinically important?

5) Is it reversible with dietary manipulation?

6) Is it related to biochemical control of PKU?

7) Is there any relationship between MRI scan abnormality and other indicators of neurological status such as neurophysiological measurement?

8) Is the development of neurological abnormality related to specific genetic mutations?

1.9 Neuropathology of PKU

In order to plan a study of the possible neurological complications of PKU it is necessary to review the aetiology of the brain damage occurring in PKU and attempt to relate this to the normal process of myelination. Despite the expansion in knowledge of PKU, the mechanism of brain damage resulting from the disorder remains unknown. Although a certain amount of information is available on the effects of PKU through post mortem studies on humans which show the brain to be underweight and hypomyelinated (82-85) and dynamic studies on the rat model for PKU (86-89), there is no single process that seems to explain the brain abnormalities and it is surmised to be multifactorial (90).

Studies on animals are a major source of published data on the metabolic and chemical changes in brain associated with hyperphenylalaninaemia. Animals are rendered hyperphenylalaninaemic by phenylalanine infusions. However in animals the normal PAH activity

allows an elevation in tyrosine also. This activity has to be inhibited by infusing agents such as p-chlorophenylalanine and alpha-methylphenylalanine. These have their own secondary effects including inhibition of other amino acid hydroxylations and are therefore not ideal models (88). In 1990 however, an animal model, which is genetically manipulated to be PAH deficient, has been successfully produced. This mouse shows the phenotypic features similar to the untreated phenotype in man and in time will be of value in further animal experiments in PKU (91-93).

The possible causes of the brain pathology can be divided into three main theories:

1) Effect of phe on transport of amino acids,

2) Effect of phe on the synthesis of myelin and

3) Effects of phe on neurotransmitter availability

1) The appearances of the brain in PKU have been likened to that seen in malnutrition and it is thought that high phe levels, by inhibiting entry of other amino acids creates a state of protein deficiency in the PKU brain (94). Tyrosine is rendered an essential amino acid in PKU since it cannot be produced from the hydroxylation of phenylalanine and so it is hypothesised that a tyrosine deficient state exists which may jeopardise normal brain development. In Bessman's 'justification hypothesis' it is speculated that the homozygous PKU fetus is at risk of tyrosine deprivation for two reasons (95). Firstly, it is dependent upon the maternal metabolism, which in the heterozygote state cannot regulate tyrosine delivery to the fetus as effectively as the homozygous normal mother. Secondly, the PKU fetus cannot produce tyrosine itself due to inactivity of phe hydroxylation. This theory is unsupported however, by Scriver who demonstrated normal tyrosine in the cord blood of PKU infants (96). In addition, tyrosine supplementation without phe reduction in PKU does not prevent mental retardation whereas phe restriction by itself largely protects brain development (97). Untreated patients may show low levels of tyrosine but this is usually in conjunction with deficiencies of other amino acids and a more likely explanation for this is the competitive inhibition theory.

Phenylalanine crosses plasma membranes as it moves in and out of cells and organs. High intracellular levels of phe may interfere with the exit of other amino acids from the cell and parenchymal tissues contain high levels of amino acids in untreated PKU whereas plasma levels are low. In the brain however, the transport relationships are different and an excess of phe blocks the influx of amino acids. The brain is therefore deprived of its amino acid supply (94). Evidence of a competitive transport system exists between phe and tyrosine, tryptophan, valine, leucine and isoleucine and rat models have shown a reduction of these amino acids in the brain in the presence of high phe.

2) The exact nature of the insult on myelin resulting in structural changes visible at the microscopic level is not clear. Post-mortem examinations of the brain of PKU individuals shows reduced brain weight, increased water content and hypomyelination (82-85). Animal models of PKU have demonstrated several abnormalities related to myelin. There is evidence of a delay in initial myelination, an increased turnover rate of myelin early in life and of disturbed protein synthesis (86,87,98). In Prensky's work concentrating on specific myelin components a decrease in myelin basic protein, proteolipid protein, cholesterol and galactocerebrosides was found (99).

There are no reports of post-mortem findings in individuals with PKU who commenced treatment in the newborn period, in whom one would expect a more normal pattern of myelination. In an attempt to recreate this situation, rats rendered hyperphenylalaninaemic at 17 days of age were studied. This age corresponds to approximately 6-8 years in human beings. The rats were found to have an increased turnover of the fast component of myelin and a reduced amount of myelin protein (88). This work highlights the vulnerability of the brain to the toxic effects of phe beyond the childhood years. This instability of myelin may be due to the underlying effect of phe on ATP-sulphurylase (90,100). This enzyme is responsible for sulphate activation and may have a regulatory role in the uptake of sulphates by the brain. Elevated levels of phe inhibit this enzyme. The regional distribution of phe-sensitive ATP-sulphurylase in fetal calf brain correlates with the demyelinated areas of the central nervous system in untreated PKU patients (100). A decreased

content of sulphatides in the PKU brain has been demonstrated. It is thought that cerebroside sulphatides offer a protection against degradation and the relative lack of sulphatides due to reduced activity of ATP-sulphurylase may result in the increased tendency to degradation and rapid turnover of myelin seen in PKU models.

3) Several studies have focussed on the effects of elevated phe on neurotransmitter metabolism. Serotonin and catecholamine biosynthesis is inhibited by elevated phe. However, these effects seem reversible when phe levels are reduced (101). Taylor et al. (89) found a deficiency of serotonin in the brains of rats rendered hyperphe after the majority of brain development is complete thus implying a lifelong vulnerability of the brain to the effects of phe on neurotransmitters. Many studies contribute to the evidence that brain dopamine and serotonin levels are reduced in PKU (102-105). It is unlikely that such deficiencies cause the mental retardation observed in the untreated state. They may be the pathological correlate of neuropsychological and behavioural disturbances such as delayed reaction times or poor executive functioning but be unrelated to the structural abnormalities described in post-mortem reports (83,84).

An elevated phe level may exert different effects on the brain depending on the stage of brain development. It is thought that the infantile brain is at greater risk when exposed to a high phe than the brain of an older child in whom myelination is complete. Interruption of myelin synthesis may lead to severe cerebral difficulties whereas the brain of an adolescent may be relatively resistant to elevated phe. Because of this it was felt that relaxation of the diet in PKU was safe in adolescents and adults who were finding the constraints of a restricted diet difficult. However it is possible that that problems of neurotransmitter synthesis continue to be a problem at all ages and therefore that diet discontinuation may not be entirely without hazard.

1.10 Myelin

The process of myelination is briefly reviewed as an introduction to the consideration of the effects of elevated phenylalanine on myelin.

White matter is composed of myelin and non-myelin elements. Myelin makes up most of the substance of the white matter of the central nervous system (CNS), about 50-60% dry weight and this component accounts for the characteristic white appearance (106). Other elements of white matter are the neuroglia consisting of astrocytes, microglia and oligodendrocytes. Myelin is also present in large quantities in the peripheral nervous system (PNS). In both the CNS and the PNS myelin is essential for the normal functioning of the nerve fibres (107).

Myelin is produced from oligodendrocytes in the CNS (and from Schwann cells in the PNS). The oligodendrocyte cell becomes wrapped around the axon many times. As the wrapping occurs, the cytoplasm of the sheath cell retracts or is extruded so that the two layers of the sheath cells plasma membrane come together and fuse. As a result myelin develops a lamellar structure and this can be visualised using electron microscopy and X-ray diffraction (108). Each oligodendrocyte is connected to up to 30-60 axons and each axon may have a sheath containing around 160 compacted lamellae (106).

Myelin is a membranous structure composed predominantly of lipids and proteins. In addition to the lipid-protein core, myelin also contains carbohydrates. The lipids of myelin are composed of 25-28% cholesterol, 27-30% galactolipid and 40-45% phospholipid (106). Thus the molar concentration of cholesterol is greater than that of any other single myelin lipid. The most distinguishing feature of myelin lipid is the high content of galactolipids, especially cerebroside (107). These lipids are amphipathic. In an aqueous environment, the amphipathic nature of the lipids means that a molecular bilayer is formed in which the hydrophobic regions of the lipid are turned to the inside and thus shielded from water while the hydrophilic regions are turned to the outside and are immersed in water. This lipid bilayer allows interaction with the amphipathic proteins, which form an integral part of the membrane. Additional proteins are present on the

outside of the bilayer. Thus myelin membranes are bilayer structures coated with proteins on both sides and the basic repeating subunit is protein-lipid-protein-lipid-protein (106).

In comparison to other molecular bilayers, the myelin bilayer is unique in having a very high lipid content (containing 70% to 80% lipids by dry weight) and in containing chiefly saturated fatty acids with an exceptionally long chain length (108). This fatty acid composition also adds to a highly stable membrane structure.

The protein content of 20%-30% is relatively low (compared for example with liver cell membranes which are 60% protein) (107). The major proteins of myelin are proteolipid protein (PLP) and myelin basic protein (MBP). PLP is a transmembrane protein and makes up the largest component of myelin proteins. MBP is present mainly on the cytoplasmic surface of the myelin bilayer. Together these proteins are thought to be important for myelin stability and ordered compaction (106).

The process of myelination is a highly controlled phenomenon evolving with regular times and sequences. The initiating stimulus for the onset of myelination remains obscure although the axonal diameter is implicated and the presence of neuronal conduction along the nerve is also thought to be important. It is hypothesised that a chemical messenger secreted by neurons plays a role or that control is exerted by properties of the axonal membrane (109).

Myelination of the CNS takes place at different times in early development. Not only the rates of myelination appear to differ among tracts, but also there is a marked time change in topographic patterns of myelination throughout the last half of gestation and during the first postnatal years (110). During the last half of gestation and the first two postnatal years, the developing brain contains a series of ordered sequences of myelination, some starting early or late in gestation and rapidly attaining the maximal degree of myelination, others only slowly attaining the maximal degree of myelination (111).

Myelination in the nervous system is initiated in a caudocranial time sequence, following the order of phylogenetic development (110). In general the palaeotologically older structures are myelinated earlier than the newer structures and there is some evidence to suggest that the tracts in the nervous system become myelinated at the time they become functional (112). At birth, most of the structures and tracts are not fully myelinated. Large parts of the cerebral hemispheres still contain no myelin. The regions still completely or largely unmyelinated at birth are particularly those involved in higher level associative functions and sensory discrimination. After birth, myelination progresses but does not reach completion until adult life (113). During the first postnatal year myelin spreads throughout the entire brain. Progressively finer branching of the subcortical white matter continues until adult life. Post-mortem studies in which myelin is stained and then quantified have showed that in certain areas of the brain such as the hippocampus, myelination continues well into adulthood (113).

The introduction of MRI scanning has given more information on the timing of myelination in the normal subject (110,112). MRI studies show a rapid myelination in the first two to three years of life followed by slow progression until 20-30 years of age (111.114-116). It is thought that fibres in the areas around the lateral ventricles are amongst the slowest to myelinate. These areas are called the association areas of the brain and continue to myelinate throughout the first several decades of life (117).

After formation the myelin sheath and the axon remain mutually dependent (118). Since myelin once deposited is metabolically a relatively stable substance, it is relatively invulnerable to adverse external factors. The stability of myelin is not clearly understood. Sulphatide and galactocerebroside (GalC) are thought to play an important role in myelin stability. Supportive evidence for this theory arises from the study of genetically manipulated mice designed to be deficient in the enzymes responsible for synthesis of sulphatide and GalC (119). In these mice, myelin of normal ultrastructural appearance but thinner sheaths is formed and the myelin is rich in glucocerebroside instead of GalC. However, the neurological function of the mice is abnormal and they develop ataxia, and tremor. Conduction deficits consistent with abnormal myelin are

observed. The conclusion from this work is that GalC and its sulphated derivative, sulphatide is important for myelin stability and function.

Generalised vulnerability of myelin to noxious agents is likely to be confined to the period of active myelination since at this time, the large supply of precursors of myelin constituents necessary for myelin deposition can be quite readily restricted during their transport into the brain. It is reasonable to accept that stress factors will vary in their effect on myelin according to the process of myelination. The timing of the stress and its severity will also determine the characteristics of and the extent to which myelination can catch up once favourable circumstances have returned. The period in which the human infant is most vulnerable with regard to myelination probably lasts from about the seventh intrauterine month to the first few months or the first year of postnatal life. This theory is borne out by the effects seen in late treated PKU, where severe mental retardation is observed when dietary intervention is delayed beyond the first year of life.

In adults there is a continued synthesis of myelin and a slow turnover. Different components have different half-lives. There is conflicting data about the precise half-life of the various myelin lipids and proteins. Some components do turn over faster than others and all components show a slow and a fast turning over component. The data suggest that newly formed myelin is catabolized faster than old myelin (120).

Remyelination in the CNS is possible but usually incomplete. It often follows the pattern of myelination during development. As a general rule however, remyelination is only extensive when the lesion is small. Large areas of demyelination show remyelination at the margins only. Remyelinated fibres have thinner sheaths than normal (121).

1.11 Investigative techniques planned in the study

1.11.1 Introduction

Although the aetiology of the neurological problems recently described in PKU is unknown the MRI findings suggest a problem with myelin (8,9,79). To further investigate the neurological status of a group of patients with PKU it is important to perform tests that give information on the function and structure of the nervous system. The following sections discuss the background to the investigative tools that will be used in this study and describes their previous use in PKU.

Neurophysiological measurements may be a sensitive method of detecting sub-clinical abnormalities as they are known to be useful in other demyelinating disorders (122,123).

Electrophysiological studies of the nervous system may be considered an extension of the clinical examination. Neurophysiological studies can supplement the examination by providing precision, detail and objectivity and delineate a variety of pathological changes that are clinically obscure or undetectable. Neurophysiological techniques used in this study are described below.

1.11.2 Nerve conduction studies

Conduction velocity of motor fibres was popularised for study in humans by Hodes et al. (124) who recorded the mechanical response of a muscle. With improvement of recording apparatus, nerve conduction studies have become a simple and reliable test of peripheral nerve function. The method has become adequately standardised and is widely used as a means of objectively detecting an abnormality in nerve concuction.

The validity of the nerve conduction velocity calculation depends upon the accuracy in determining the latencies and the conduction distance. Sources of error in measuring latencies include unstable or incorrect triggering of the sweep; poorly defined take-off of the evoked response and inappropriate stimulus strength and inaccurate calibration (125,126). Errors in

measurement of the conduction distance by surface measurement result from uncertainty as to the exact site of stimulation and the exact course of the nerve trunk. Surface determination of the nerve length may be particularly imprecise when the nerve takes a non-linear path.

Because of these uncontrollable variables, the calculated velocities are not absolute values of nerve conduction (127). On repeated testing the values may vary as much as 10 m/s, because of the limitations inherent in the techniques. If standard procedures are adhered to however, the results are sufficiently reproducible. Since the range of normal is small, the use of nerve conduction studies as a clinical diagnostic test is justified. A number of factors such as temperature and age can alter motor and sensory conduction studies (127-129) and testing conditions must be controlled in the neurophysiology laboratory as well as collecting an age-appropriate control group. Adult values of nerve conduction are reached by age three to five years and remain in the same range until the fifth or sixth decade (127,129).

1.11.3 Magnetic stimulation of brain

Magnetic stimulation is a relatively new technique for stimulating nerve and cerebral cortex and is used to help quantify the characteristics of the nervous system, especially to measure conduction times. Its purpose is to create a pulsed electric current that will momentarily depolarise the nervous system. Magnetic stimulation of the cortex was first reported by d'Arsonval in 1896 (130) but commercial stimulators only became available in 1986. The advantage conferred by this new technique is that it provides the ability to introduce currents in previously inaccessible areas of the body. A changing magnetic field produced by the magnet induces an electrical field in the area tested. This causes electrical stimulation of the nerves in that area which produces recordable responses (130).

The first clinical results obtained with the stimulator were reported by Barker et al. in 1985 (131) who showed that there was a marked increase in conduction time in motor fibres between head and neck and head and lumbar region in patients with multiple sclerosis. These results were

subsequently confirmed by others (132-134). Barker also demonstrated a relationship between the degree of delayed conduction and the extent of clinical spasticity in patients with MS (131). Slowed conduction has since been recorded in other disorders such as cervical spondylosis (135). Jarratt et al. have reported a comparative study on visual evoked potentials and magnetic stimulation in the diagnosis of MS and conclude that the two investigations together increase the diagnostic accuracy in this condition (136).

As a neurophysiological tool, magnetic stimulation seems to be less sensitive at detecting sub-clinical lesions in the motor tract in MS than are VEP or SEP at detecting lesions in their tracts (137). Nevertheless abnormal responses to magnetic stimulation do correlate with the degree of neurological abnormality even if this is merely abnormal reflexes. Theoretically the application of magnetic stimulation is an important adjunct to the neurological investigation in PKU for two reasons: firstly it has proven benefit in detecting disorders of myelination and secondly it focuses the CNS investigation on the motor system which is the area of the nervous system affected in all the recent reports of neurological deterioration in young adults with PKU (8-10). It is therefore important to be able to assess motor function in detail.

1.11.4 Visual evoked potentials

Pattern reversal visual evoked potentials (VEPs) are a well-established technique for measuring nerve conduction along the visual nerve pathway (138). The technique records the cortical electric waveform using scalp electrodes produced in response to a rapidly alternating visual stimulus of a checkerboard pattern (139,140). The recorded waveform has been shown to be consistent between individuals (141). Several reproducible waves are identifiable. The most consistent is the major positive component (P100) and this is the measurement most often used for comparison between individuals. Abnormalities may occur both in the latency of the wave and the amplitude. It is generally thought that demyelination results mainly in a prolongation of the latency but can also affect the amplitude (142). This technique is useful in detection of

demyelination in multiple sclerosis (122). It is a robust technique if used carefully and control values established and is widely used in the diagnosis of neurological disease.

1.11.5 Somatosensory evoked potentials

A somatosensory evoked potential is a cortical or sub-cortical response recorded as a result of stimulating a peripheral nerve. The most widely used method of averaging responses was described in 1954 by Dawson (143). Using somatosensory testing it is possible to estimate the central sensory conducting time (CSCT). Following stimulation of a peripheral nerve a reproducible waveform is observed. There are several recording sites available. By placing cervical recording electrodes and scalp electrodes the latency of the evoked potential is monitored at several sites along the pathway. The CSCT is estimated by subtracting the total latency at the scalp electrode from the cervical latency. Again SEPs have found widespread use in the investigation of disorders of myelin and are abnormal in approximately 60 % of individuals with multiple sclerosis (123). In order to achieve consistency certain precautions have to be taken in the laboratory: tests must be performed with the limb temperature above 34 degrees centigrade and laboratory age controls should be established.

1.11.6 Neurophysiological studies previously reported in PKU

Since it is a relatively new technique, magnetic stimulation has only been previously reported in one previous PKU study by Ludolph et al. (78). Twenty-two adolescents with PKU underwent several neurophysiological investigations including magnetic stimulation of cortex. Although individual results were normal, the individuals with PKU had a prolonged mean central conduction time compared to a group of age matched controls.

There are several reports of the application of other more established neurophysiological techniques in PKU.

An early study by Schafer (144) showed that VEP latency could be shortened by decreasing blood phe or supplementing patients with L-Dopa or tyrosine thereby increasing indole and catecholamine precursors. Creel et al. in 1982 (145) detected abnormal VEPs in a small group of untreated PKU adults.

Pueschel et al. (146) studied 8 children with classical PKU aged 4-6 years through a period of diet cessation, measuring VEP, SEP and BAEP while on and off diet. There was no alteration in the early component of the waveforms of VEP, SEP or BAEP. The variation in the late component was inconclusive: although some patients had a prolongation of the late component of the SEP, in some, this developed prior to stopping diet and improved afterwards whereas in others the abnormality was detected after stopping diet.

Korinthenberg et al. (147) have studied the largest group to date. EEG, VEP and BAEP were carried out in 41 adolescents with PKU. Four had not required dietary treatment. The latency of the P100 peak of the VEP was prolonged in the patients with PKU types I or II who had been treated early. The latency was however normal in those with mild PKU and in those with type I or II who had been treated late which makes the interpretation difficult. There was a positive correlation between quality of treatment in the first 10 years and duration of VEP latency.

Ludolph et al. (78) in addition to using magnetic stimulation, investigated their group of 22 adolescents with NCV, VEP and SEP. They found subclinical neurophysiological abnormalities of statistical significance in the PKU group when compared with age-matched controls. There was no obvious clinical significance to these abnormalities. They were unable to correlate the findings with previous metabolic control. Indeed visual evoked potential latencies were more delayed in the mildest PKU group who had not required treatment and were normal in the more severe group. On the basis of their findings they hypothesised a 'morphological and pharmacological' basis to the late neurological problems implying that the elevated levels of phe cause disruption of neurotransmitters (pharmacological) and that there are also structural abnormalities (morphological) of myelin present.

Landi et al. (148) examined 14 PKU patients with EEG and VEP. They found abnormalities in VEP latency in six children with PKU and EEG abnormalities in three children. There was no correlation between the mean phe levels and the neurophysiological results.

The most recent studies reported are those of Bick et al., (79) and Jones et al., (149). Bick's study of eight children formed part of a MRI brain study. Four of the eight children tested had an abnormally prolonged VEP. In Jones's report, the 32 patients' MRI scan findings had been published separately. There were 27 patients aged 14-31 years and over 80% had an abnormality of VEP. Of the nine younger patients still on diet only one had an abnormal VEP. There were no differences between phe or tyr levels and VEP latency.

There are several studies of EEG changes in PKU. All suggest that there are frequently abnormalities in the EEG waveform in children with PKU, (56,77,78,145-147,150-153). Epileptiform activity is however, unusual in the treated PKU individual. Krause (153) reports that the abnormalities in EEG waveform are influenced by concurrent blood phe levels.

In this study we have used a neurophysiological assessment consisting of

1) magnetic stimulation of the cortex

2) somatosensory evoked potentials

3) peripheral nerve conduction velocities and

4) visual evoked potentials

The rationale for using these specific techniques is firstly to perform a comprehensive examination of the nervous system which looks for evidence of peripheral and central myelination abnormality, secondly to choose neurophysiological tests that focus on areas of the nervous system that are considered to be vulnerable in PKU and thirdly, to exploit knowledge of the advantages of neurophysiological techniques in other demyelinating disorders by using established techniques which are sensitive at detecting abnormal function of the myelin. Whilst

considering all these issues the examination had to be of a duration that we could reasonably expect patients to attend in addition to their clinic and MRI visits. The duration of the assessment was approximately 2 hours.

1.11.7 Magnetic Resonance Imaging

The introduction of MRI scanning to clinical practice has yielded new information on the brain appearance of young adults with PKU and suggests that they display abnorma MRI images (8-10,70-73,75-77,79-81).

MRI has been used extensively in medicine, especially to investigate disorders of the nervous system (154-157). The clarity of images of the nervous system far cutweighs that previously given by Computed Axial Tomography (CT) scanning (157). MRI has also been used to evaluate brain maturation in infants, children and adolescents (158-160). The diagnostic supremacy of MRI in neurological disorders is shown in multiple sclerosis where demyelinated plaques are clearly highlighted. In multiple sclerosis, MRI may detect lesions that are not clinically manifest nor have any subclinical effect that can be discerned by neurophysiological studies (161-164). It has been observed previously that a limitation of MRI may be its high sensitivity coupled with its lack of specificity. In serial studies several patients subsequently developed clinical deficit concomitant with the anatomical lesion first identified on MRI (163,164). The ability of MRI to herald neurological abnormality raises important issues in PKU. MRI abnormalities may develop clinical correlation in time or may have no clinical significance. In other disorders however such as multiple sclerosis a detailed neurophysiological examination usually highlights some dysfunction when MRI shows widespread abnormalities. Therefore in this study when MRI abnormalities are severe, it would be reasonable to expect concomitant neurophysiological abnormalities if there is permanent structural damage as a result of PKU.

1.12 Aims of study

This study aims to address some of the questions that arise from a review of the current literature. Specifically the null hypotheses to be tested are that:

1) adolescents and young adults with PKU do not have an increased incidence of overt clinical, neuroradiological or neurophysiological abnormalities

2) changes on MRI of the brain are not associated with any other marker of neurological disease

3) there is no association between the development of neurological abnormalities and the degree of dietary control in childhood

4) MRI changes are not associated with current levels of phenylalanine

5) neurological dysfunction is not progressive over the period of this study

6) MRI changes are not reversible with dietary manipulation

1.13 Design of the study

The study was a prospective examination and follow-up of a large group of adolescents and young adults with PKU. In stage I the neurological function of a group of patients who had discontinued dietary treatment was established by clinical examination and detailed neurophysiological testing. This group also had MRI of brain and the relationship between brain white matter abnormalities; neurological status and biochemical control was measured statistically. The MRI white matter changes were graded using a scoring system, which allowed statistical analysis of the results. A younger group of patients with PKU were also recruited to have MRI of brain alone. These patients were still on diet and they were studied when it became clear that all the adults had MRI brain changes in order to see whether the abnormalities developed even in younger patients on diet.

In stage II, patients made a decision to return to a diet or remain off diet and the examination, neurophysiological studies and MRI scans were repeated. The results of stage II were compared

statistically with those of stage I. The second stage of the study was performed after approximately one year.

2. METHODOLOGY

2.1 Patients

The study population was adolescents and adults with PKU who attended the Willink Biochemical Genetics Unit based at the Royal Manchester Children's Hospital, Manchester. The age at diagnosis, diagnostic phe levels and current age of the study population is given in Table 1.

Patients aged over 14 years who had discontinued dietary therapy were invited to attend for

1) clinical neurological examination,

2) neurophysiological assessment

3) MRI of brain

4) blood phe level

Patients aged 10-14 years were invited to attend for MRI of brain and blood phe only. After a period of observation, which ranged from six to twelve months, clinical examination, neurophysiological assessment and MRI of brain were repeated in the 14 years and over age group and MRI alone was repeated in the 10-14 year olds.

Invitations with tear-off slips were sent to 99 adolescents and adults who regularly attended the clinic. Patients who were pregnant were not considered appropriate for this study. If no response was received, a second slip was sent. No further action was taken if a negative reply was received or if there was no response to two slips.

The Ethics Committees of Salford and Central Manchester Health Authorities gave ethical approval.

Table 1: Details of PKU patients participating in study, their age at start of study, age at which treatment commenced, diagnostic phe level and age at diet discontinuation

number	age (years)	age treatment commenced (days)	diag phe (µmol/l)	diet discontinued (years)
1	16	12	1280	14
2	17	19	4394	14
3	35	240	NK	4
4	30	14	>1200	5
5	14	14	1748	14
6	18	14	4029	14
7	15	15	NK	14
8	19	17	4120	14
9	14	17	3000	14
10	13	14	NK	CONT
11	15	14	3410	14
12	21	17	3600	14
13	18	11	1200	14
14	15	16	3240	14
15	22	16	>1200	14
16	15	20	3760	14
17	13	10	3260	CONT
18	10	15	>1200	CONT
19	26	60	1340	19
20	24	90	2500	14
21	23	16	>1200	14
22	10	15	1402	CONT
23	22	4 YEARS	2489	15
24	23	17	4411	16
25	21	15	3000	11
26	22	20	1200	16
27	21	22	4960	11
28	23	60	1411	17
29	19	8	2328	13
30	25	20	>1200	14
31	12	31	4190	CONT
32	13	10	2590	CONT
33	13	15	2400	CONT

NK: not known
CONT: diet continuing
preg: treated only in pregnancy

number	age (years)	age treatment commenced (days)	diag phe (µmol/l)	diet discontinued (years)
34	12	13	1200	CONT
35	24	12	1568	6
36	23	17	>1200	16
37	18	15	3300	14
38	29	14	2100	9
39	13	16	2260	CONT
40	19	17	3255	14
41	13	16	4580	CONT
42	20	11	1200	14
43	17	10	3500	14
44	21	14	1200	14
45	30	3 months	1302	14
46	26	6 weeks	>1200	14
47	13	14	2910	CONT
48	10	17	1200	CONT
49	22	26	1800	4
50	48	preg	1647	----
51	20	6	1200	14
52	31	preg	1200	----
53	14	15	1500	14
54	17	2 years	1500	14
55	16	25	1322	14
56	19	10	2500	14
57	19	18	2812	14
58	30	5 months	1320	16
59	20	15	1200	14
60	21	23	1958	14
61	23	36	2400	14
62	20	10	2914	14
63	24	11 weeks	1650	14
64	13	21	4590	CONT
65	25	32	3900	14
66	18	15	1200	14
67	16	11	3476	14
68	10	13	2065	CONT
69	10	15	2150	CONT
70	10	14	2370	CONT
71	11	15	2093	CONT
72	13	19	3300	CONT
73	17	22	4308	14
74	25	8 weeks	2605	14
75	21	25	2400	14
76	17	15	5503	14
77	10	19	1830	CONT
78	16	15	3500	10

NK: not known
CONT: diet continuing
preg: treated only in pregnancy

2.2 Clinical examination

The assessment of the nervous system consisted of a clinical history and the following examination:

i) inspection of posture, observation for muscle wasting or fasciculation, voluntary and involuntary movements such as tremor

ii) examination of muscle tone

iii) assessment of power in the limbs using isotonic method of testing,

iv) tendon reflexes assessed as normal, brisk or very brisk,

iv) testing of co-ordination using finger-nose test and rapidly reversing palm-dorsum movements of hand test.

The methods used were that of McLeod's Clinical Examination (165).

These examinations were performed at the time of neurophysiological testing with the patients semi-recumbent on a testing couch in the neurophysiology department or in the consulting room of the clinic.

2.3 Intelligence Quotients

It has been shown previously that the intelligence quotients of the United Kingdom population have increased over the years since the population norms were established (166). To counteract this effect when looking at IQ results from a group tested in different years, Smith et al. (167) suggest converting IQ results to IQ standard deviation scores (IQ sds) and this technique is used in this thesis.

2.3 Neurophysiological tests

2.3.1 Magnetic stimulation of cortex and cervical spine

Magnetic stimulation of cortex and cervical spinal cord was performed using a Dantec SC120 magnet (Dantec*) of maximum output 1.4 Tesla. Fifty-five subjects were tested in a semi-recumbent position using the technique described by Ingram et al. (133). The resulting action potential to cortical and cervical magnetic stimulation was recorded using Ag/Ag Cl electrodes affixed to the skin overlying the tendon and belly of abductor pollicis brevis (APB). Stimulator strength was increased to a level where the maximum action potential was observed. In the majority of cases this was around 70-75 % of maximum output. Readings were taken with a reasonable voluntary contraction of APB. Four reproducible latencies were recorded and the shortest latency was used for further analysis. Amplitudes were noted from the recordings. A group of 45 healthy volunteers (age range 17 to 34 years) was similarly tested. Central motor conducting time (CMCT) was derived using 2 methods: from the latency difference between cortically-stimulated and cervically-stimulated action potentials and from the latency difference between the cortically-stimulated action potential and the peripheral latency as measured by the F wave technique (168). The shortest latency of 8 F waves was measured following electrical stimulation at the wrist. The peripheral component of the cortically derived conduction time is then calculated as F+(M-1)/2 and this value is subtracted from the latency of the response evoked by magnetic stimulation of the cortex.

2.3.2 Peripheral nerve conduction velocities

56 patients had peripheral motor nerve conduction velocities measured for the median nerve by recording over APB following stimulation at the wrist and elbow and for the common peroneal nerve by recording over extensor digitorum brevis following stimulation at the ankle and at the fibular head. Index to wrist median and antidromic sural sensory potentials were also recorded by averaging responses. Surface electrodes were used for recording and stimulation and supramaximal stimuli were used. A Counterpoint electromyography machine (Dantec*) was used in all recordings and 20 healthy volunteers (age range 19 to 40 years) were similarly tested to provide control data.

2.3.3 Visual evoked potentials (VEPs)

Pattern-reversal stimulation was performed in 48 patients by 2/s black and white checkerboard pattern reversal. The total field subtended 15 degrees at the eye, each square subtending 72 minutes. Each eye was stimulated separately on whole-field stimulation and patients were asked to fixate on the centre of the screen. With a sweep time of 256 ms, 100 sweeps were averaged by a Concerto Brain Mapper (Dantec*). The process was repeated to ensure reproducibility of the responses. Recording was by a horizontal occipital chain of three Ag/AgCl electrodes (referred to Fz) at 5 cm intervals, with the central electrode on the midline 2 cm above the inion. Readings were made of P100 latencies and amplitude was assessed as the peak to peak amplitude of P75 to P100. Control data were recorded from 20 volunteers age range 18 to 40 years.

2.3.4 Somatosensory evoked potentials (SEPs)

SEPs were recorded using Ag/AgCl disc electrodes over the ipsilateral Erb's point, C7 and C2 spinous processes and over the contralateral sensory cortex (C3 and C4 using the international 10-20 system) following 2/s median nerve stimulation at the wrist. The stimulus intensity was

sufficient to produce a reasonable twitch in the APB. 256 responses were averaged using a Concerto Brain Mapper (Dantec*) and the recordings were repeated to ensure reproducibility. Peaks N9, N11, N13 and N20 latency and amplitude were measured. Central sensory conducting time (CSCT) was derived by subtracting N13 from N20 latency. Forty-eight individuals with PKU were tested. Control data was available from 25 healthy adults (age range 18-42 years).

2.4 Magnetic Resonance Imaging

Magnetic Resonance Imaging of the head was performed with patients awake at the Department of Radiology at Manchester Royal Infirmary. MRI of the head was performed using a 0.5 Tesla (GE) MR system, obtaining sagittal T_1 weighted spin echo and transverse intermediate and T_2 weighted sequences through the brain, with a section thickness of 5 mm. Diazepam sedation was available if patients felt claustrophobic.

The scans were graded according to severity and extent of white matter changes (see Table 2). A score of zero represented a normal scan. The highest possible score for each anatomical area was 5 bilaterally and there were 6 anatomical areas scored thus giving a total possible score of 60. The scans were scored by a single observer (JPRJ), a consultant neuroradiologist who was aware that the patients had PKU but not of their clinical or biochemical data.

Table 2: MRI grading system.

Grade	Scan appearance
0	Normal
1	< 10% white matter involved
2	10-30% white matter involved
3	30-50% white matter involved
4	50-75% white matter involved
5	> 75 % white matter involved

Scores of 0-5 are given for each of the following anatomical regions: frontal, parietal, occipital, temporal, brainstem and other areas. A score of 0 represents a normal scan; a score of 60 is the most severely affected possible.

2.5 Phenylalanine measurement

Blood phe was measured by amino acid analyser (Waters HPLC system using a lithium cation exchange column, Pickering Lab Inc, California) or thin layer chromatography. All serial blood phe concentrations, from diagnosis until the date of MRI, as obtained for routine monitoring, were entered onto computer for each patient. Data were analysed to give the average yearly phe concentration (area under the phe curve as plotted against time, divided by the age at MRI) and the aggregated times that the blood phe concentration remained below a) 120 µmol/l, above b) 400 µmol/l, above c) 800 µmol/l and d) above 1200 µmol/l. Early control was assessed from the average yearly phe concentration in the first 4 years of life and more recent control from the average yearly phe concentration over the five years prior to imaging (80).

2.6 Control data

Controls were tested for the neurological techniques of magnetic stimulation, peripheral nerve conduction velocities, visual evoked potentials and sensory evoked potentials.

There were no control subjects for MRI scans, as normal age-related data were considered sufficient.

Healthy volunteers were recruited from doctors, medical students, nurses and technicians working in Royal Manchester Children's Hospital or Hope Hospital. Recruitment was by inviting colleagues or asking student groups. Nursing staff at Hope Hospital were recruited by a general invitation to the wards in close proximity to the Department of Neurophysiology. The only exclusion criteria used were epilepsy, pregnancy, presence of a metal implant for magnetic stimulation and trauma to the limbs being tested for nerve conduction studies. There was no financial incentive for participating.

2.7 Statistical analysis

All variables were tested for normality using the Shapiro-Wilk test. 95% confidence intervals were calculated for the difference in the means obtained between the PKU group and a control group for each neurophysiological test and for the difference in mean MRI grades for the younger and older patients. Individual results were designated as abnormal if the result was greater than 2.5 standard deviations outside the mean of the control group. On repeat neurophysiological testing the Student paired 't' test was used to discern changes over time in an individual's results.

The association between MRI severity and phenylalanine control and neurophysiological result was assessed using Pearson's correlation. Stepwise multiple regression was used to further investigate these relationships.

3. RESULTS

3.1 Introduction

The results of the two stages of the study are presented followed by the comparison between the findings at each stage.

3.2 Stage I: Initial assessments of PKU group

3.2.1 Clinical examination

Clinical neurological examination was performed in 58 PKU patients of age 14 to 49 years. Of this group 11 individuals had abnormal neurological signs. Six patients had brisk tendon reflexes alone and five had a tremor at rest and brisk tendon reflexes. No patients had an abnormal gait or spasticity of the limbs. The median age of this neurologically abnormal group was 19.4 years compared with 20.0 years for the normal group. There were no significant differences between the two groups in factors relating to biochemical exposure to phe as shown in Table 3. Median MRI grades were similar between the groups, clinically abnormal group median score 12.5, normal group 12, $p = 0.9$.

Table 3: Comparison of age, biochemical control and MRI score of PKU patients with normal and abnormal clinical examination

	Clinically normal n=47	Clinically abnormal n=11	p value
age (years)	20	19.4	0.9
exp (μmol/l/year)	779	864	0.3
time<120 μmol/l (years)	0.4	0.6	0.9
time>1200 μmol/l (years)	2.7	5.3	0.2
phemax (μmol/l)	2914	3500	0.6
first 4 years(μmol/l)	1693	1734	0.4
last 5 years(μmol/l)	1102	1304	0.4
phe at time of scan (μmol/l)	1350	1500	0.9
MRI score	12	12.5	0.5

all values given are median values

exp: average lifetime exposure to phe

time <120 μmol/l: duration of time that blood phe was below 120 μmol/l

time >1200 μmol/l: duration of time that blood phe was above 1200 μmol/l

phe max: maximum recorded blood phe

first 4 years: average blood phe over the first 4 years of life

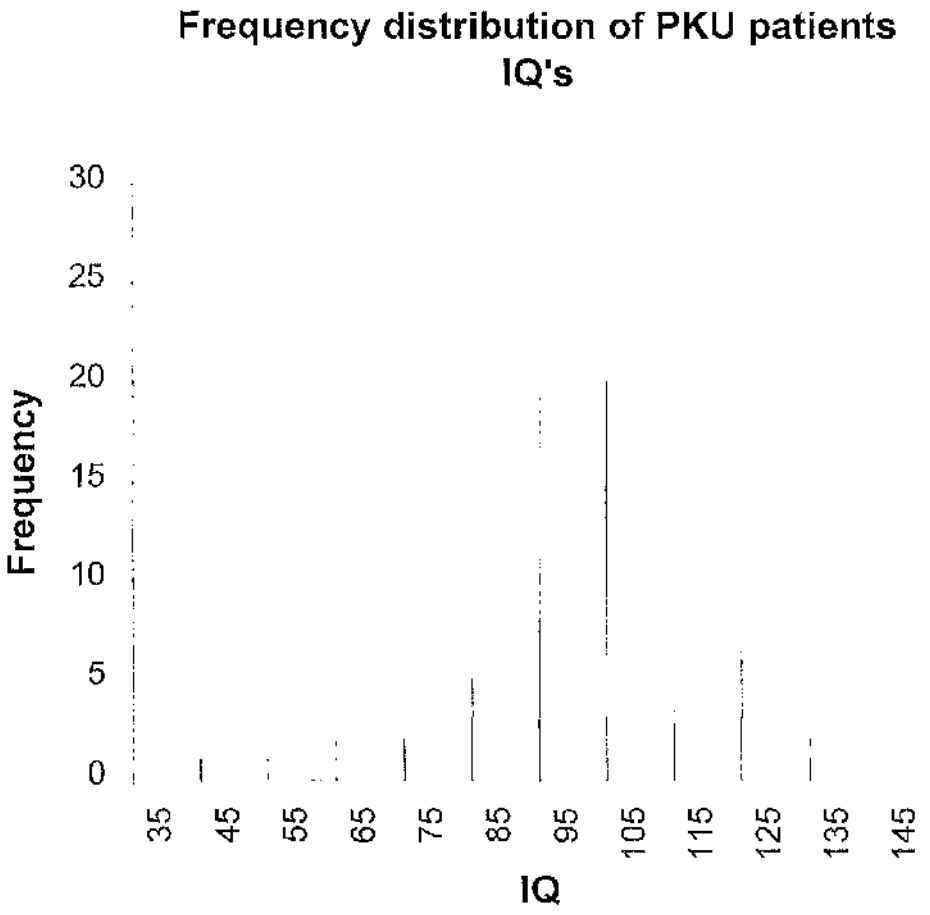
last 5 years: average blood phe over 5 years leading up to start of study

p value: probability value

3.2.2 Intelligence quotients

IQs were normally distributed with a mean of 97 and standard deviation of 17 (figure 2). IQ-SDS ranged from -4.6 to 1.77 with a mean of -0.7. There was no correlation between IQ-SDS and MRI grades ($r = -0.62$, $p = 0.3$).

Figure 2: Frequency distribution of PKU patients IQ's



3.2.3 Neurophysiological testing

The results of all neurophysiological tests are tabulated with patient age, IQ and clinical examination result in Table 4.

Table 4: Results of 58 PKU patients aged 14-49 years (discontinued diet at time of testing): age, clinical examination, IQ and neurophysiological testing measurements

patient	age (years)	clin exam	IQ	cmct (ms)	vep (ms)	csct (ms)	med mot (m/s)	med sen (m/s)	lat pop (m/s)	sural (m/s)
1	16	N	111	6.2	101	4.5	55	46	50	42
2	17	N	109	7.1	109	5.5	51	50	52	42
3	35	N	87	6.8	NT	NT	60	70	NT	NT
4	30	N	NT	9.5	102	4.8	56	43	57	37
5	14	N	85	6.9	113	4.5	57	46	50	45
6	18	N	98	7.1	NT	NT	56	44	NT	NT
7	15	N	127	4	108	6	54	42	48	50
8	19	ABN	109	7.6	102	4.9	55	46	66	51
9	14	N	105	5.5	102	6	70	58	48	48
11	15	N	93	6.4	109	5.6	59	45	50	46
12	21	N	80	7.0	104	4.7	53	UT	44	43
13	18	N	113	7.5	111	5.3	45	43	54	58
14	15	N	82	6.8	88	5.4	52	51	49	48
15	22	N	70	8.2	94	5.6	68	55	62	52
16	15	N	111	5.1	98	4.0	61	45	52	38
19	26	N	94	5.2	105	5.6	62	48	57	60
20	24	ABN	83	UT	106	UT	61	45	50	45
21	23	ABN	92	5.1	144	4.7	59	53	41	45
24	23	ABN	117	9.3	109	5.3	62	52	53	43
25	21	N	94	10.8	100	5.2	59	41	50	44
26	22	N	106	5.5	NT	NT	63	37	48	53
27	21	N	87	5.6	NT	NT	66	50	48	33
28	23	ABN	90	7.3	110	3.5	55	42	42	43
29	19	N	107	7.0	109	5.5	58	48	54	46
36	23	ABN	94	5.1	99	5.2	74	53	65	46

N: normal clinical examination

ABN: abnormal neurological examination

NT: not tested

UT: uninterpretable trace

Table 4 continued

patient	age (years)	clin exam	IQ	cmct (ms)	vep (ms)	csct (ms)	med mot (m/s)	med sen (m/s)	lat pop (m/s)	sural (m/s)
37	18	N	102	7.1	108	4.9	68	55	63	45
38	29	ABN	100	8.3	94	5.1	65	48	52	38
40	19	N	97	8.2	109	6.1	35	UT	42	47
42	20	N	101	6.5	102	6.0	53	42	46	63
43	17	ABN	72	7.0	110	6.7	58	60	46	44
44	21	N	106	UT	104	5.2	60	38	44	38
45	30	N	69	5.4	100	4.7	64	46	54	53
46	26	ABN	90	6.3	107	4.9	65	51	48	47
49	22	N	33	NT	104	NT	NT	NT	NT	NT
50	48	N	61	5.6	102	4.7	58	50	55	50
51	20	N	77	5.8	123	4.7	65	55	50	42
52	30	N	105	6.3	105	5.8	63	43	51	47
53	14	N	96	7.0	111	5.6	70	54	58	50
55	16	N	92	4.0	104	5.5	61	46	48	64
56	19	N	106	6.8	94	5.5	58	54	46	UT
57	19	N	105	8.2	NT	NT	55	55	51	43
58	30	N	71	5.5	107	5.2	60	55	40	47
59	20	N	105	5.7	106	3.9	58	48	42	42
60	21	N	121	6.9	NT	NT	61	50	57	45
61	23	N	125	7.1	103	5.0	57	50	46	UT
62	20	N	138	5.7	NT	5.0	58	41	46	43
63	24	ABN	128	7.2	104	6.0	53	50	UT	36
65	25	N	110	6.1	102	5.6	60	55	50	42
66	18	N	122	6.8	99	5.3	61	45	50	47
67	16	N	91	9.6	108	4.3	59	48	48	41
73	17	N	119	7.8	107	5.3	52	35	54	34
74	25	N	94	7.1	102	4.2	62	52	50	43
75	21	ABN	89	6.8	98	5.5	59	52	53	41
76	17	N	6.9	5.8	97	60	46	52	UT	53
23	22	N	100	6.2	NT	NT	62	50	48	52
54	23	N	84	NT	NT	NT	59	51	49	51
78	16	N	90	NT	NT	NT	NT	NT	NT	NT
30	17	N	110	NT	NT	NT	NT	NT	NT	NT

N: normal clinical examination

ABN: abnormal neurological examination

NT: not tested

UT: uninterpretable trace

3.2.3.1 Magnetic stimulation of the cortex

Results are reported from both recording techniques for determining an estimation of CMCT.

F wave method CMCT

Using the F wave method, the mean latency of the central motor conducting time was 6.8 ms (95% CI: 6.42 to 7.18) for the PKU group and 6.6 ms (95% CI: 6.24 to 6.96) for the control group. This difference did not reach statistical significance (CI for difference in means -0.32 to 0.72). Individually, there were 3 patients whose CMCT lay outside the upper limit of normal (mean + 2.5 s.d.= 9.6, PKU values 9.8, 10.2 and 10.3 ms). These patients' details are given in table 5. A scatter diagram of the results is in Figure 3.

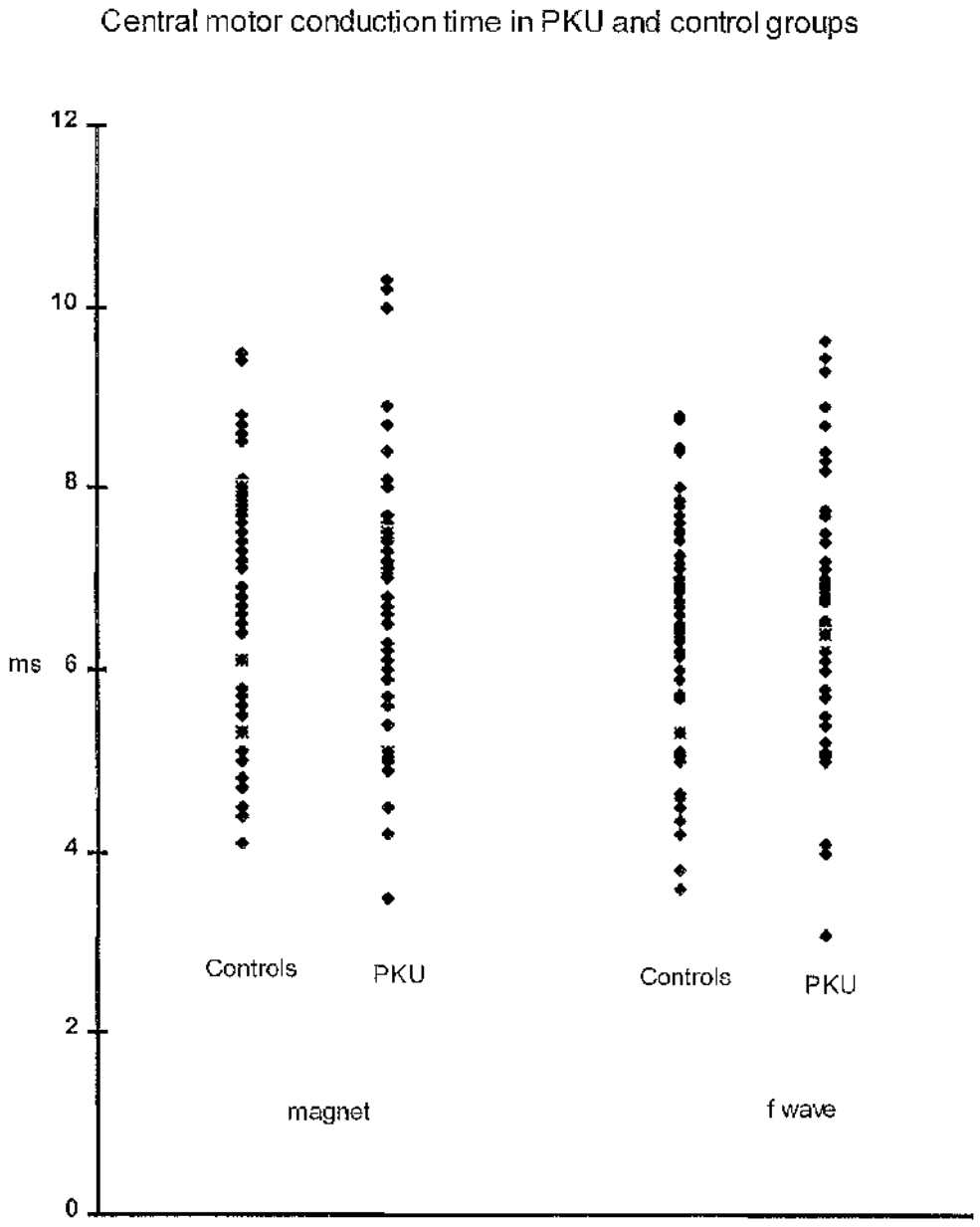
Cervical magnetic stimulation method CMCT

Following cervical magnetic stimulation, the mean CMCT latency was 6.9 ms (6.50 to 7.30) for the PKU group and 6.7 ms (6.34 to 7.06) for controls. This difference is not statistically significant (CI for difference in means -0.36 to 0.76). The same individuals had CMCT outside the normal limits.

Table 5: PKU patients with abnormal CMCT results

Patient	age (years)	years off diet	current phe (µmol/l)
24	23	6	1650
25	21	6	1000
67	16	5	1000

Figure 3: Scatter diagram of central motor conducting times in PKU group and controls



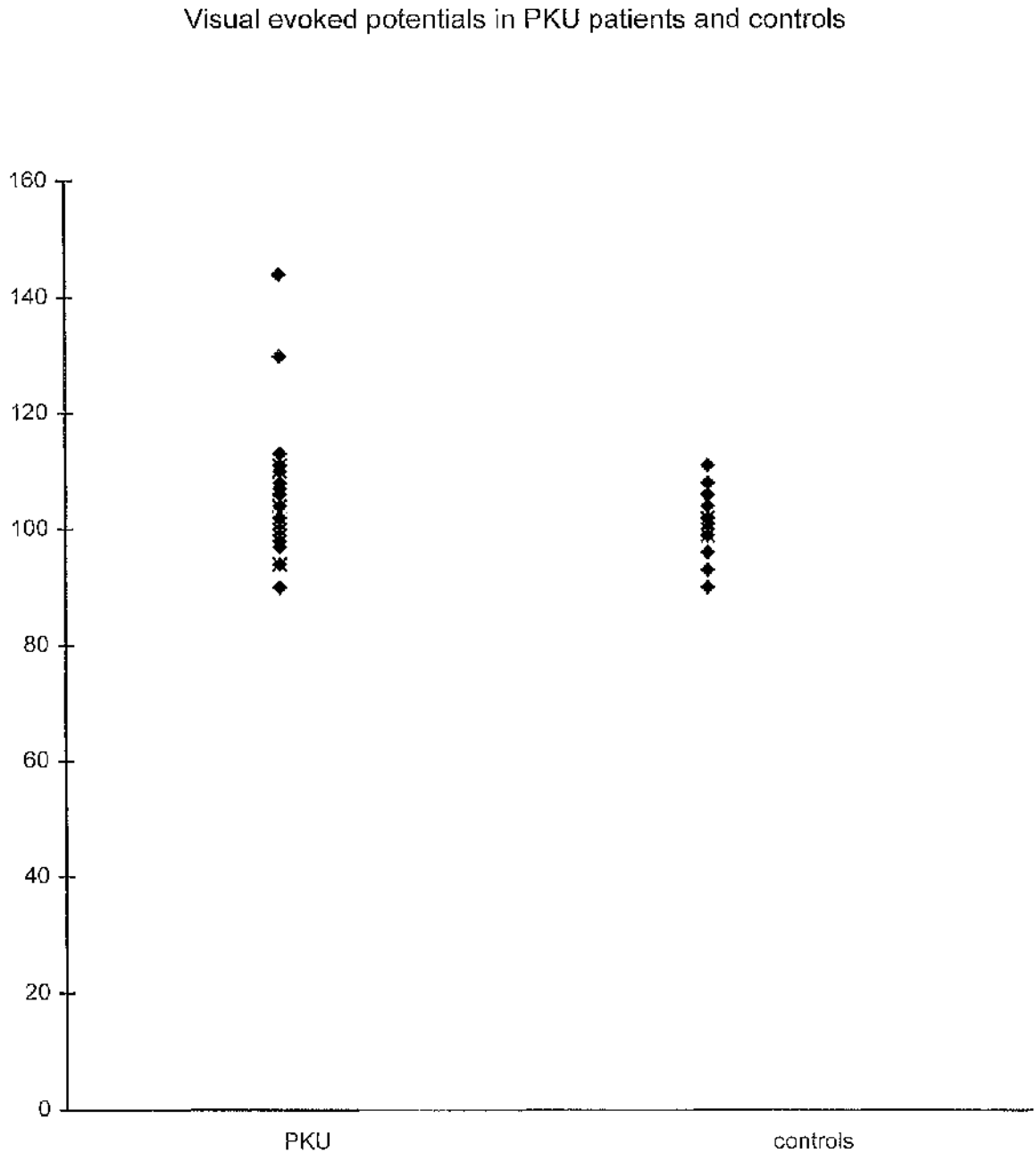
3.2.3.2 Visual Evoked Potentials

The mean latency of the major positive P100 peak of the VEP was 105.3 ms (CI 102.7 to 107.8) in the PKU group, and 100.5 ms (CI 98.4 to 102.5) in the controls, which was significantly different (95% CI 1.6-8.0). There were 8 individuals whose latencies were above the upper limit of normal (mean + 2.5 s.d.= 111.6 ms). Two were markedly delayed at 133 ms and 123 ms whereas the others ranged from 112-114 ms. The details of these individuals are given in table 6. Mean amplitude for the PKU group was 10 milliV (s.d.7) and 15 milliV (s.d.5) for the control group. This difference was not statistically significant, CI for difference in means: -0.68 to 10.6. Figure 4 is a scatter diagram of the results.

Table 6: PKU patients with abnormal VEP results

Patient	age (years)	years off diet	current phe (μmol/l)
2	17	3	1350
5	14	0.5	1200
21	23	9	1730
43	17	3	1700
46	26	12	1880
51	20	7	700
53	16	2	1200
74	25	11	1700

Figure 4: Scatter diagram of visual evoked potentials results for PKU group and controls



3.2.3.3 Somatosensory evoked potentials

Reproducible waveforms were recorded in 46 of 48 individuals tested. Central sensory conducting time (CSCT) was not statistically different from controls, CSCT PKU = 5.2 ms (CI 5.0 to 5.4), controls = 5.3 ms (CI 4.9 to 5.7), CI for difference in means: -0.57 to 0.30. There were no individual abnormally prolonged results.

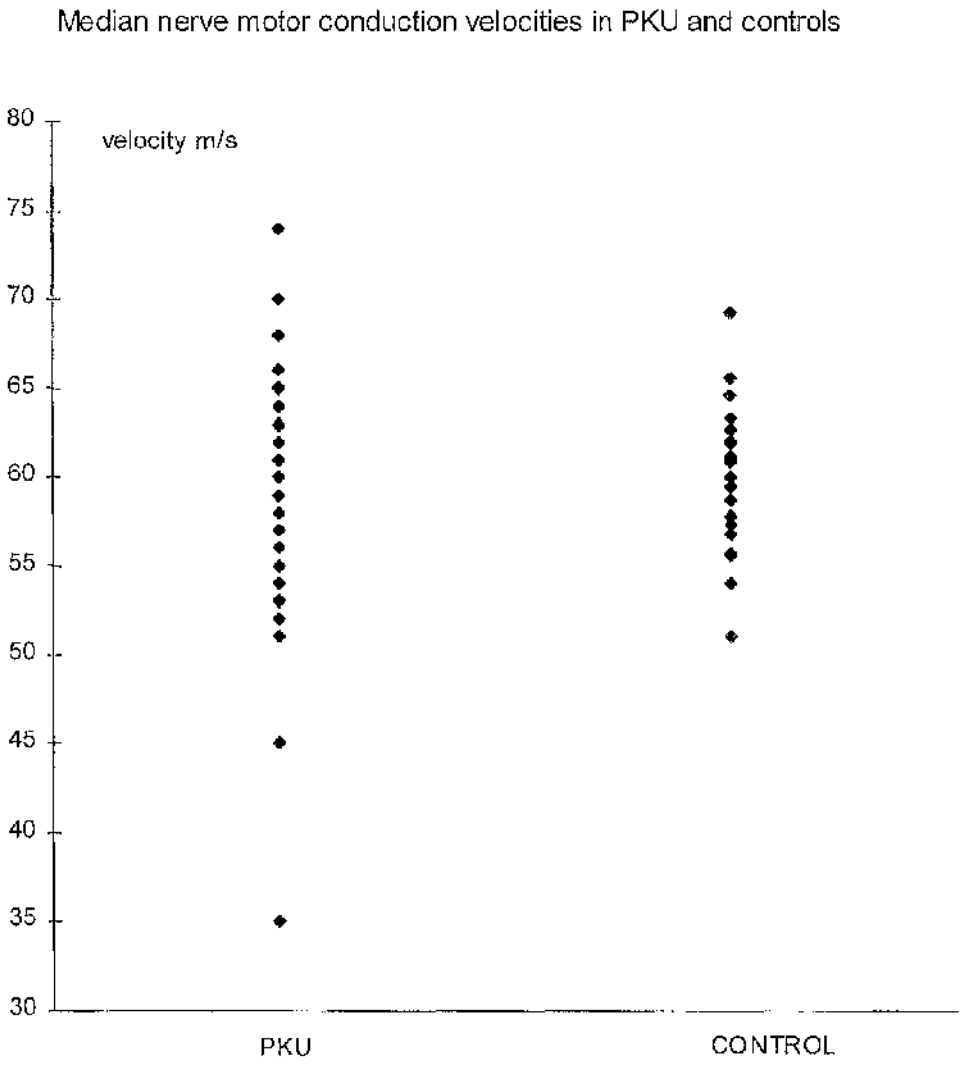
3.2.3.4 Peripheral nerve conduction velocities

Median nerve motor and sensory nerve conduction velocities were similar to controls. Mean median nerve motor conduction velocity was 59 m/s (57.2 to 60.9) in the PKU group and 60 m/s (58.4 to 62.0) for controls, CI for difference -3.7 to 1.33). One patient recorded a median nerve conduction velocity of 45 m/s which was outside the lower limit of normal (mean - 2.5 s.d = 50 m/s. Median nerve sensory conduction velocity was 49 m/s (46.8 to 50.4) in the PKU group and 50 m/s (47.7 to 52.4) for controls, CI for difference -4.44 to 1.4. One abnormal value (lower limit of normal 37m/s) was recorded in the PKU group of 35 m/s. In the lower limbs, common peroneal motor conduction velocity was 50.5 m/s (48.7 to 52.2) in the PKU group and 51.5 m/s (48.0 to 55.0) for controls, CI for difference -4.85 to 2.8. There were no individuals with results outside the normal range. Mean sural conduction velocity was 46 m/s (43.9 to 47.8) in PKU and 46m/s (44.4 to 48.4) in controls, CI for difference -3.35 to 2.17. Individually there were 4 abnormal results of sural nerve conduction velocity in the PKU group (mean - 2.5 s.d.=35.4 m/s) and details are in Table 7. These values ranged from 27 m/s to 35 m/s. Figure 5 is a scatter diagram of the results for median nerve motor conduction velocity.

Table 7: PKU patients with abnormal NCV results

patient	abnormal NCV	age (years)	years off diet	current phe (μmol/l)
13	median nerve motor	18	4	1500
73	median nerve sensory/ sural nerve	17	3	1300
2	sural nerve	16	2	1000
27	sural nerve	21	10	1800
67	sural nerve	16	2	1000

Figure 5: Scatter diagram of results of median nerve motor conduction velocities in PKU group and controls



3.3 Magnetic resonance imaging

Three individuals did not tolerate scanning due to claustrophobia. Only one child had a normal scan and two others had equivocal scans. The other 71 scans showed some degree of abnormality. This consisted of an increased intensity of the MR signal on the T2 weighted image in the white matter. The areas affected most commonly were the posterior occipito-parietal lobes (affected in all patients with abnormal scans) and the frontal lobes (involved in 90% of affected scans). Occasional patients had temporal lobe or basal ganglia abnormalities. Examples of the images obtained are shown in figures 6-8. In the majority of patients the abnormal signal was uniform throughout the affected areas but in 16 patients there were also discrete punctate lesions of stronger signal in certain areas. The scores ranged from 0 to 25, with a mean and median of 12. There was a significant difference in the mean MRI score between the younger group still on diet and the older patients, mean score 10-14 years = 9 (CI 7.2 to 10.8), mean score 14+ years = 12 (CI 10.9 to 13.1), 95% CI for difference in means 2.48 to 3.52. Figure 9 is a frequency distribution of the MRI grades.

Figure 6: Photograph of T-2 weighted MRI of head showing a normal scan

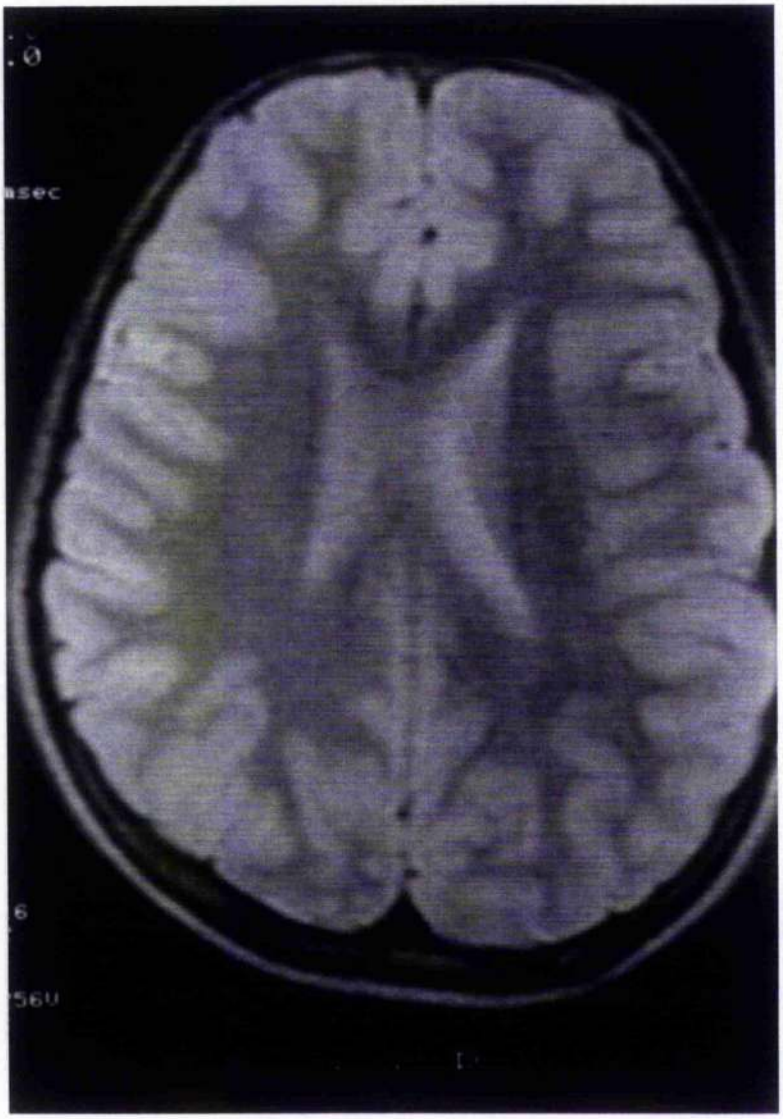


Figure 7: Photograph of T-2 weighted MRI of head showing cerebral white matter abnormalities scored 12.

The arrow highlights the abnormal white matter.

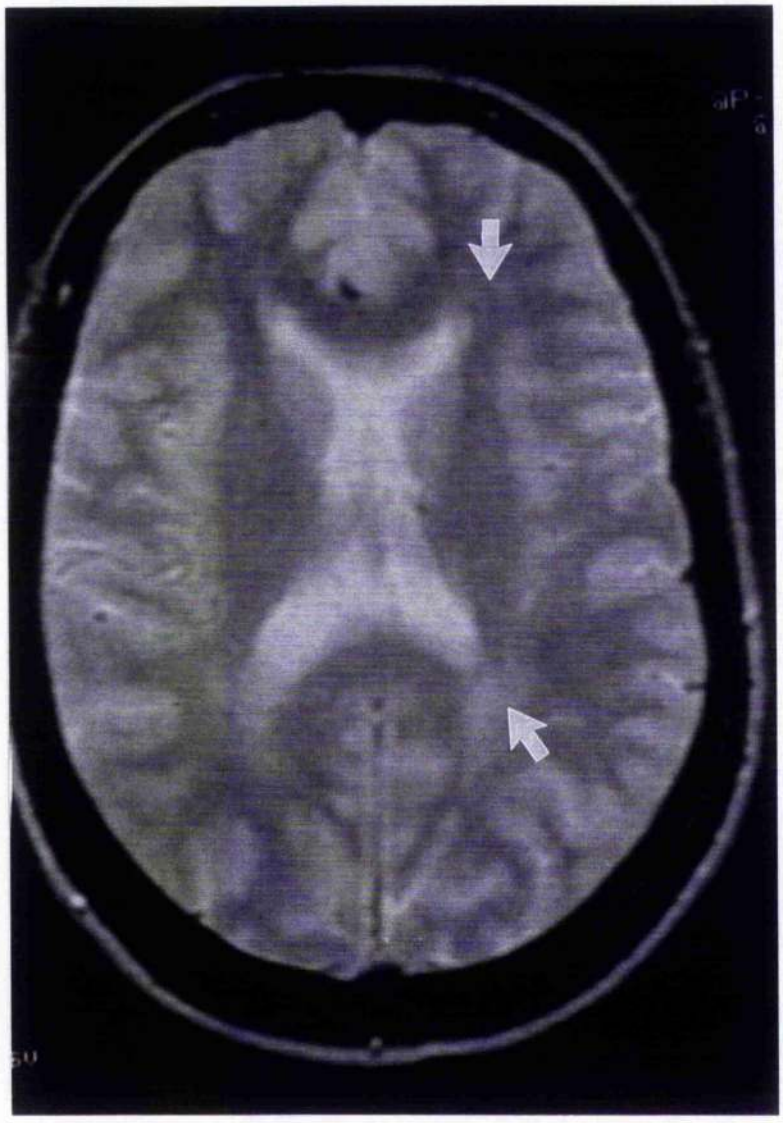


Figure 8: Photograph of T-2 weighted MRI of head showing cerebral white matter abnormalities scored 25.

The arrows highlight the abnormal white matter.

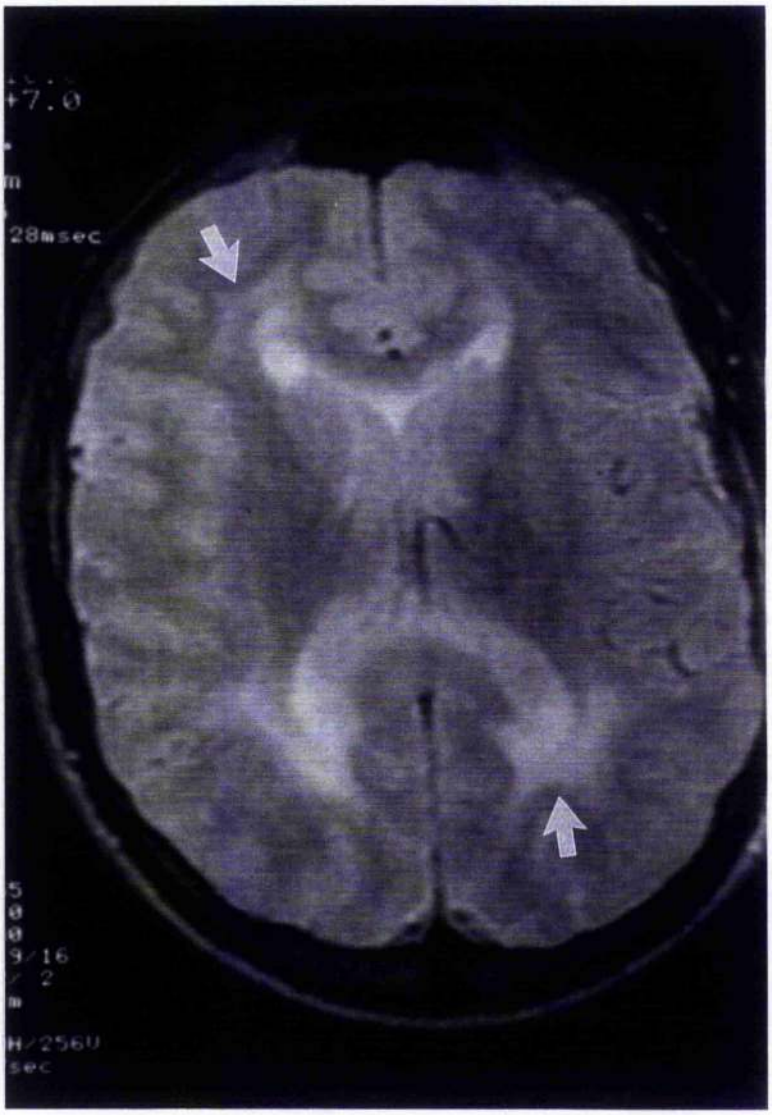
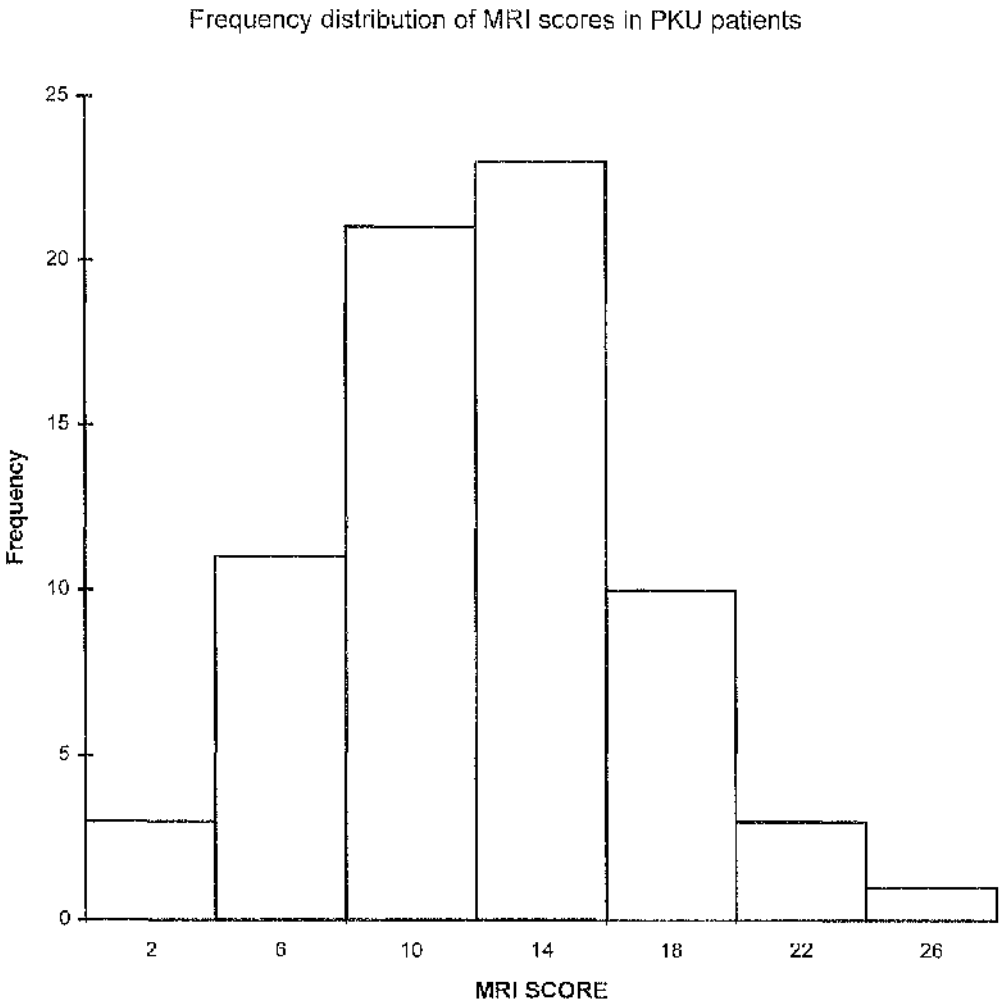


Figure 9: Frequency distribution of MRI grades in 71 PKU patients



3.4 Relationship of MRI with age and IQ of PKU patients

The results of Pearson's correlation between MRI grade and age and IQ are shown in Table 8.

Table 8: Correlation between MRI grade and age and IQ

	r	p
Age at MRI scan	0.24	0.08
IQ	-0.62	0.28

r: correlation coefficient, p: probability value

3.5 Relationship of MRI with neurophysiological abnormalities

The results of Pearson's correlation between MRI grade and neurophysiological results are shown in Table 9. There was no significant correlation between MRI grading and any of the neurophysiological findings. The plots of these relationships are in Appendices 1-7.

Table 9: Correlation between MRI grading and neurophysiological investigations

Neurophysiological test	r	p
Central motor conducting time (CMCT)	0.03	0.78
Visual evoked potentials (VEPs)	0.04	0.86
Central sensory conducting time (CSCT)	-0.23	0.16
Sural nerve conduction velocity (NCV)	-0.08	0.6

r: correlation coefficient, p: probability value

3.6 Relationship of MRI with biochemical control

Correlation analysis of MRI grade and biochemical control is shown in Table 10. There was a significant correlation ($p<0.05$) between MRI abnormality and average life phe exposure, phe in the last 5 years and phe at time of scanning. There was no association between MRI grade and time phe >1200 μmol/l, time phe <120 μmol/l, first 4 years exposure to phe or maximum phe. The strongest correlation was between severity of scan abnormalities and phe at the time of scanning. Stepwise multiple regression analysis showed phe at the time of scan to be the strongest association. When this variable was accounted for, no other factor showed a significant association. The least squares linear regression equation for this association was MRI grade=0.00514 x phe level + 5.67. The plots for the significant associations are shown in Figures 10-12. The other plots are in Appendices 8-11.

Table 10: Correlation of MRI grade and biochemical control

	r	p
Time blood phe <120 μmol/l	-0.21	0.13
Time blood phe >1200 μmol/l	0.26	0.06
Mean blood phe throughout life	0.38	**0.0043** *
Mean blood phe in first 4 years	0.16	0.27
Mean blood phe in last 5 years	0.40	**0.0025** *
Maximum blood phe	0.21	0.12
Blood phe at time of MRI scan	0.58	**0.0002** *

r: correlation coefficient, p: probability value

* denotes the statistically significant results

Figure 10: Plot of MRI grade and blood phe at time of scan

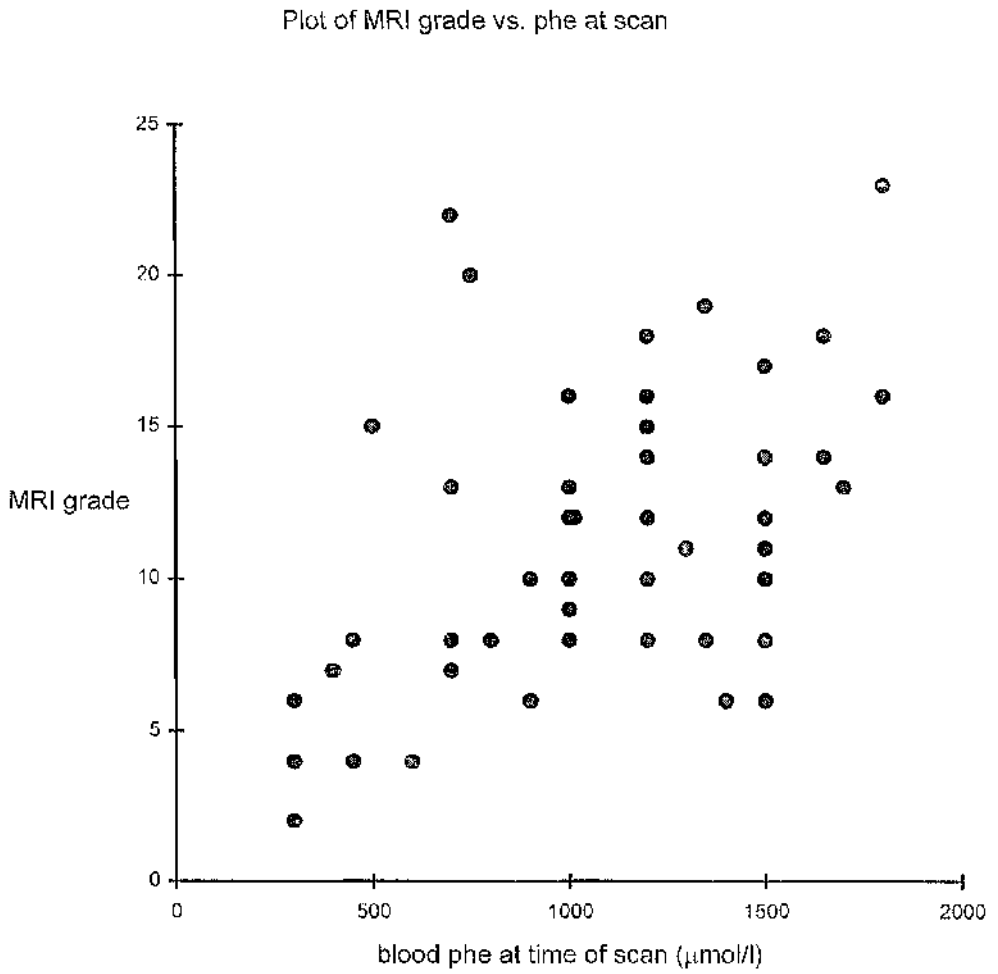


Figure 11: Plot of MRI grade and average exposure to phe

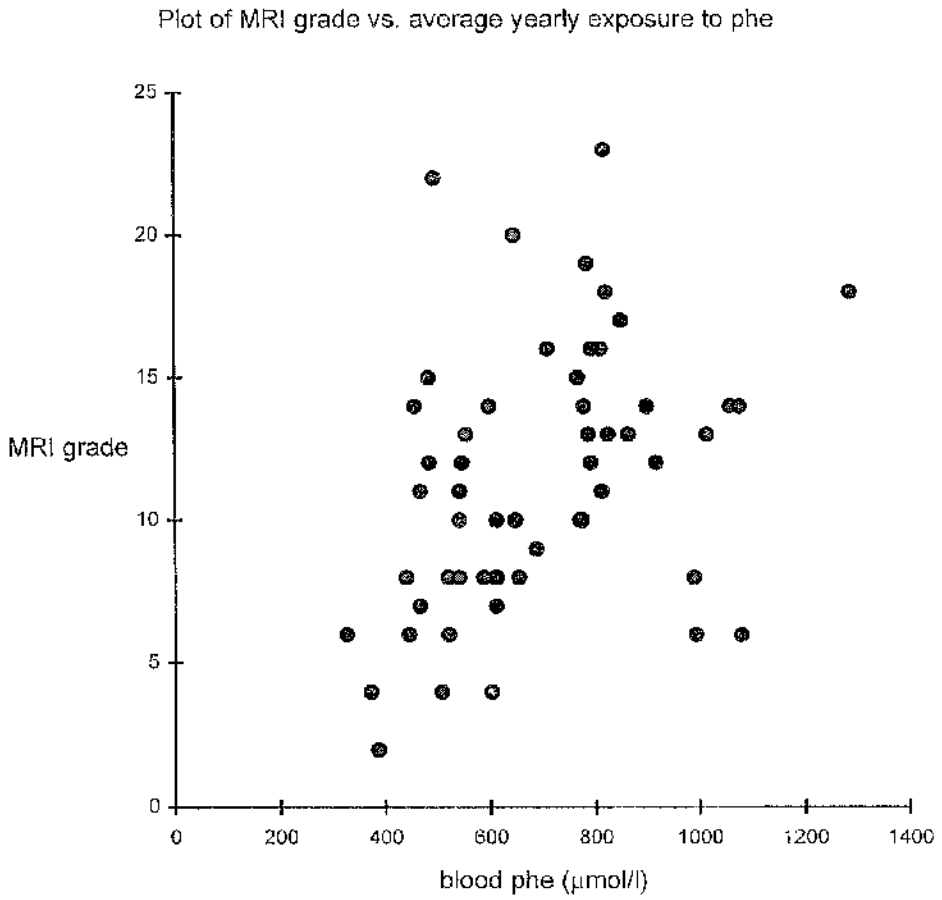
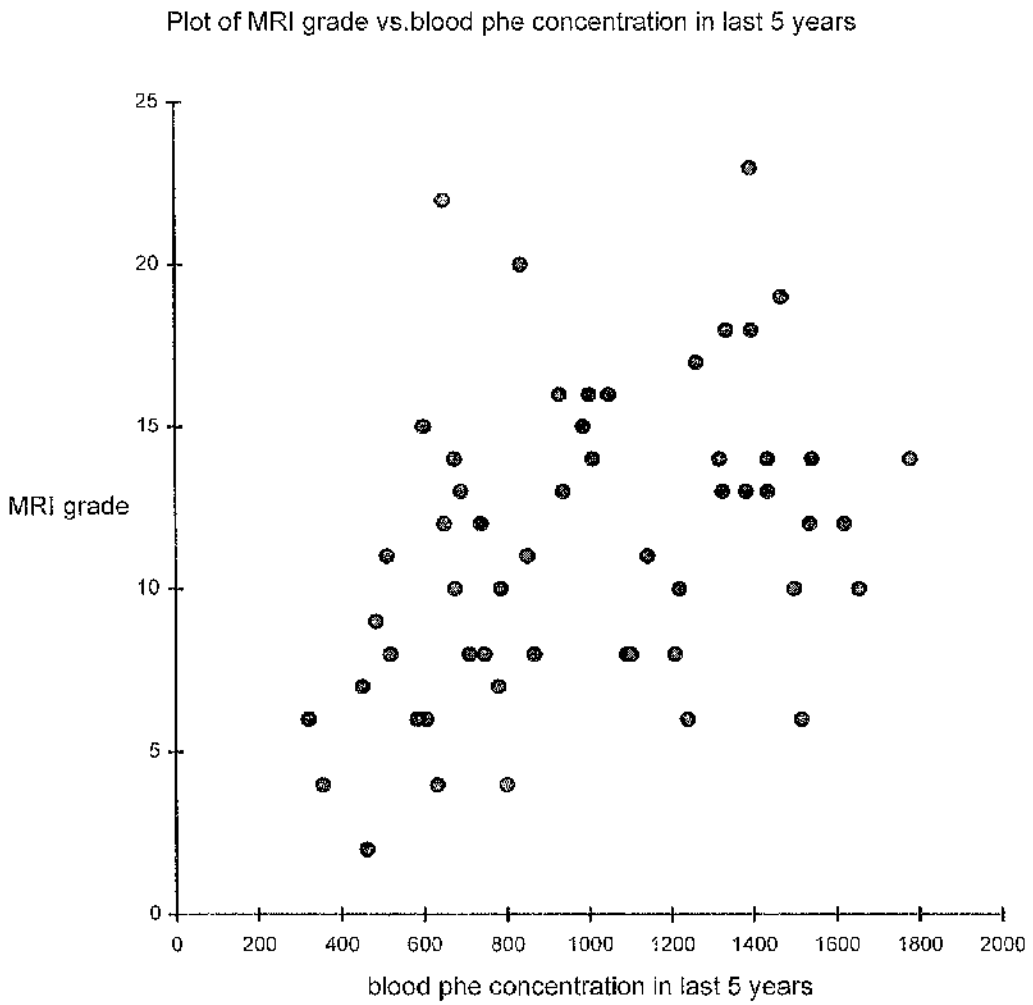


Figure 12: Plot of MRI grade and average blood phe in the five years leading up to scan



3.7 Stage II results

3.7.1 Introduction

In this section, the results of repeat clinical examination, neurophysiological tests and MRI scans in the second stage of the study are presented. The results of stage I were explained to the patients. At this time the Medical Research Council published guidelines for the treatment of PKU including a recommendation for diet for life (11). On this basis the patient study group made a decision regarding their diet. There was no attempt to randomise the patients.

3.7.2 Details of patients dietary status in stage II

Forty-one of the original 58 patients on a relaxed diet were re-scanned. Of this group, five patients decided to return to a strict phe restricted diet, re-starting amino acid supplement and aiming to reduce the phe level to 400 μmol/l. Twenty-one patients wanted to restrict the phe intake to a certain degree and restart supplement but felt they could not achieve levels as low as 400 μmol/l. This group aimed for levels around 900 μmol/l. The remaining patients (totalling 15) did not wish to make any alteration to diet and their levels remained around 1200-1500 μmol/l. The younger patients did not make any dietary alteration between first and second scans: they maintained a phe restricted diet and amino acid supplement aiming for blood phe levels <700 μ mol/l. Tables 11-13 show the different groups of patients studied in the second stage of the project.

Table 11: Patients on strict diet in second stage of project: ages, phe levels and time interval between scans

patient	age at first scan (years)	phe at stage I (μmol/l)	phe at stage II (μmol/l)	δphe	δtime (months)
6	18	1200	500	-700	6
5	15	1500	100	-1400	3
9	14	1200	900	-300	6
19	26	1350	400	-950	12
74	25	1700	300	-1400	4

δphe: change in blood phe levels between stage I and stage II of the study at time of repeat MRI scanning

δtime: time interval between first and second stage investigations

Table 12: Patients on low protein diet in second stage of project: ages, phe levels and time interval between scans

patient	age (years)	phe at stage I (μmol/l)	phe at stage II (μmol/l)	δphe	δtime (months)
1	16	1000	700	-300	6
2	17	1350	1200	-150	6
5	14	1200	500	-700	8
14	15	1500	800	-700	12
13	18	1500	1350	-150	12
15	15	1500	1000	-500	6
23	22	1500	1300	-200	9
24	23	1650	1500	-150	6
25	21	1800	900	-900	12
36	23	1650	1200	-450	12
28	23	1500	900	-600	6
37	18	1800	1500	-300	14
42	20	900	900	0	12
46	26	1700	1200	-500	9
44	21	1000	600	-400	13
45	30	1000	800	-200	13
55	16	1300	900	-400	6
59	20	1200	750	-450	10
66	18	1500	800	-700	9
67	16	1000	750	-250	8
73	16	700	700	0	7

δphe: change in blood phe levels between stages I and II of study

δtime: time interval between stages I and II of the study

Table 13: Patients who made no dietary alteration between stages I and II: ages, phe levels and time interval between scans

patient	age (years)	phe at stage I (μmol/l)	phe at stage II (μmol/l)	δphe (μmol/l)	δtime (months)
8	19	1500	1500	0	12
12	21	1200	1200	0	13
20	24	1350	1500	150	6
26	22	1350	900	-450	6
38	29	1400	1200	-200	7
40	19	1200	1200	0	7
50	48	1500	1000	-500	12
51	20	700	750	50	12
53	14	1200	1500	300	12
56	19	1500	1500	0	12
57	19	1500	900	-600	6
60	21	300	600	300	6
61	23	1700	1500	-200	14
62	20	1500	900	-600	9
65	25	1500	1500	0	12

δphe: change in blood phe levels between stages I and II of study

δtime: time interval between stages I and II of the study

3.7.3 Results of repeat clinical examination

30 patients returned for repeat neurophysiological testing. On repeat testing those patients with abnormal signs exhibited similar signs. Two patients previously considered to be normal were noted to have brisk reflexes. These were patient 40 aged 19 years and patient 37 aged 18 years.

3.8 Results of repeat neurophysiology

3.8.1 Magnetic stimulation of cortex

Repeat tests were performed on 30 patients. This group which was retested were a similar group to those who declined retesting in terms of age and biochemical severity. There was no difference in CMCT between stage I and stage II of the study. Only the F-wave method is reported. The results are given in Tables 14 and 15.

Table 14: CMCT results of 30 PKU patients re-tested in stage II of study

Mean CMCT (ms)	Confidence Interval
6.3	5.7-6.8

F wave method n=30

Table 15: Comparison of CMCT's between stages I and II of study in PKU patients

Mean CMCT stage I	6.5
Mean CMCT stage II	6.3
Confidence interval for difference in means	-0.68 to 0.25

n=27

3.8.2 Visual evoked potentials

Repeat tests were performed in 30 patients. The difference between the groups fell just outside the 95% confidence limits, the mean latency of the group studied in stage II being slightly shorter than in the initial study. When this difference was examined more closely the improvements had occurred in the group who had returned to a strict diet. The results are shown in Tables 16 and 17.

Table 16: VEP results of 30 PKU patients re-tested in stage II of study

Mean VEP (ms)	Confidence Interval (ms)
104	101 to 107

Table 17: Comparison of VEPs between stages I and II in PKU patients

Mean VEP stage I (ms)	105
Mean VEP stage II (ms)	104
CI for difference in means (ms)	-0.02 to 3.94

n=27

3.8.3 Peripheral nerve conduction velocities

Repeat nerve conduction velocities were performed in 30 individuals. There were no significant changes in results between first and second testing. The results are given in Table 18 and 19.

Table 18: NCV results of 30 PKU patients re-tested in stage II of study

Nerve tested	mean NCV (m/s)	Confidence interval of mean
Median nerve motor	59.0	56.8 to 61.2
Median nerve sensory	51.3	48.1 to 54.7
Lateral popliteal nerve	52.0	48.8 to 55.1
Sural nerve	44.4	40.7 to 48.1

Table 19: Comparison of NCVs between stages I and II of study in PKU patients

Nerve tested	CI for difference in means
Median motor nerve	-2.0 to 2.8
Median sensory nerve	-7.6 to 1.1
Lateral popliteal nerve	-7.0 to 2.3
Sural nerve	-2.3 to 8.6

n=30

3.9 Results of repeat MRI scanning

41 patients attended for repeat scanning from the 58 older patients who had been on a relaxed diet at the time of first MRI scan. Two of the group were pregnant at the time of second scan, the remaining 15 did not wish to be rescanned. Seventeen of the 19 patients aged 10-14 years were rescanned. They had made no dietary alteration during the year.

Of the group of 41 patients, 5 patients returned to a strict phenylalanine (phe) restricted diet with amino acid supplement (Maxamum Scientific Hospital Supplies Ltd, Liverpool) reducing plasma phe levels to < 400 µmol/l. Twenty-one patients reduced protein intake and recommenced amino acid supplement, aiming to reduce plasma phe levels to < 900 µmol/l. The remaining 15 patients made no dietary alteration. Mean plasma phe of this group was 1200, range 900 to 1500 µmol/l. Tables 11-13 shown previously give the patient details in each group.

The results on repeat scanning of the younger and older groups are analysed separately. Table 20 shows the mean grades before and after dietary changes in each sub-group. A graph depicting the grades at first and second scans is shown in figure 13. All scans improved in the group who had returned to a strict diet (mean change in MRI grade: -5, range -4 to -6). In the group who had reduced protein intake and commenced amino acid supplement, six scans improved, nine remained the same and two worsened slightly. In the group who made no dietary alteration, most scans were unaltered. Two patients' scans improved, in these patients the phe level was in fact lower at the time of second scan.

Table 20: MRI grades at first and second scans in each dietary group

		MRI grade at 1st scan	MRI grade at 2nd scan	δMRI
strict diet	n=5	13 (10 to 17)	9 (5 to 13)	-4 (-6 to-2)
low protein diet	n=21	13 (11 to 15)	13 (11 to 15)	0 (-1 to 0)
no change	n=15	12 (9 to 15)	12 (9 to 15)	0 (-1 to +1)

MRI grades are mean grades for the group with confidence intervals given in parenthesis

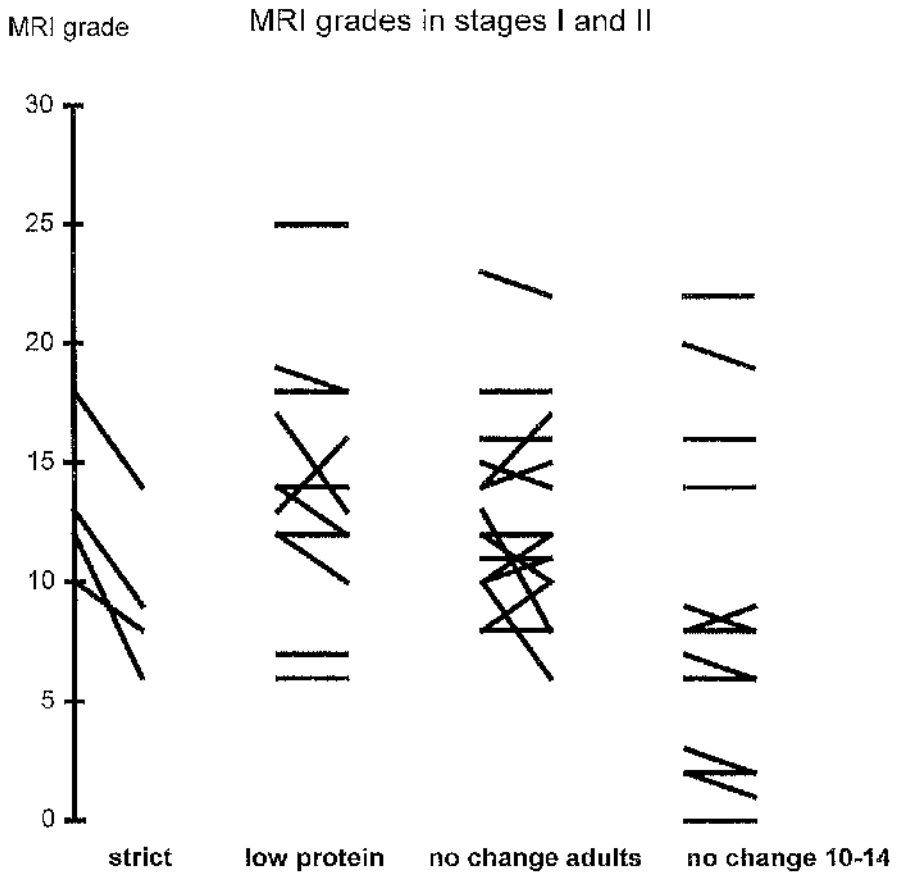
δMRI: represents the change in MRI grade between first and second scans

The association between the change in plasma phe levels between scans and the change in MRI score is shown in Figure 14. There was a highly significant association between the change in MRI grade and the change in the plasma phe level (Pearson's correlation: r = 0.57, p = <0.001). The least squares regression equation for this association was MRI grade = 0.003 phe + 0.32.

The association between the change in MRI scan and phe levels at second scan is shown in Figure 15. The association is significant. It can also be seen from this plot that scan improvement was nearly always in those whose phe levels were below 900 μmol/l. Figure 16 shows MRI scans on a patient whose scan improved three months after reducing phe levels from 1200 μmol/l to 300 μmol/l.

For the younger group of patients aged 10-14 years, repeat scanning was performed after a period of one year during which no changes were made to their diet. The MRI scores were not significantly changed over the period of observation (mean δMRI 0) nor was there a significant alteration in blood phe levels (δPHE 70). The plot of the association between δMRI and δphe is shown in Appendix 12.

Figure 13: Depicts the MRI grades in stages I and II of the study in each different dietary group



3.10 Relationship between MRI scanning and neurophysiology

As in the first stage of the study there was no statistical relationship between MRI scores and results of neurophysiological testing on repeat examination see Table 21.

Table 21: Correlation between neurophysiological investigations and MRI grading in stage II of study

Neurophysiological test	r	p
Central motor conducting time (CMCT)	0.25	0.21
Visual evoked potentials (VEPs)	0.28	0.19
Sural nerve conduction velocity (NCV)	-0.26	0.27

r: correlation coefficient

p:probability value

Figure 14: Plot of change in MRI grade (δMRI) against change in phe level (δphe) between stage I and II of study for the older group (14-49 years) of PKU patients

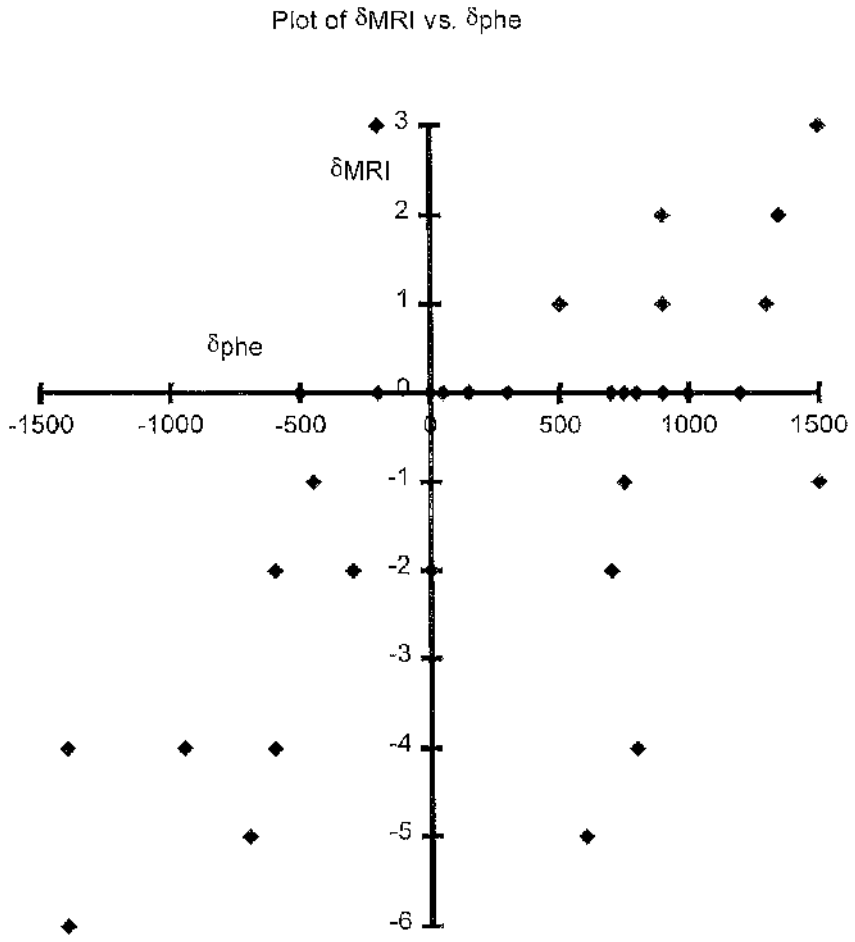


Figure 15: Plot of change in MRI grade (δMRI) between stages I and II of the study against actual phe level at stage II for the older group (14-49 years) of PKU patients

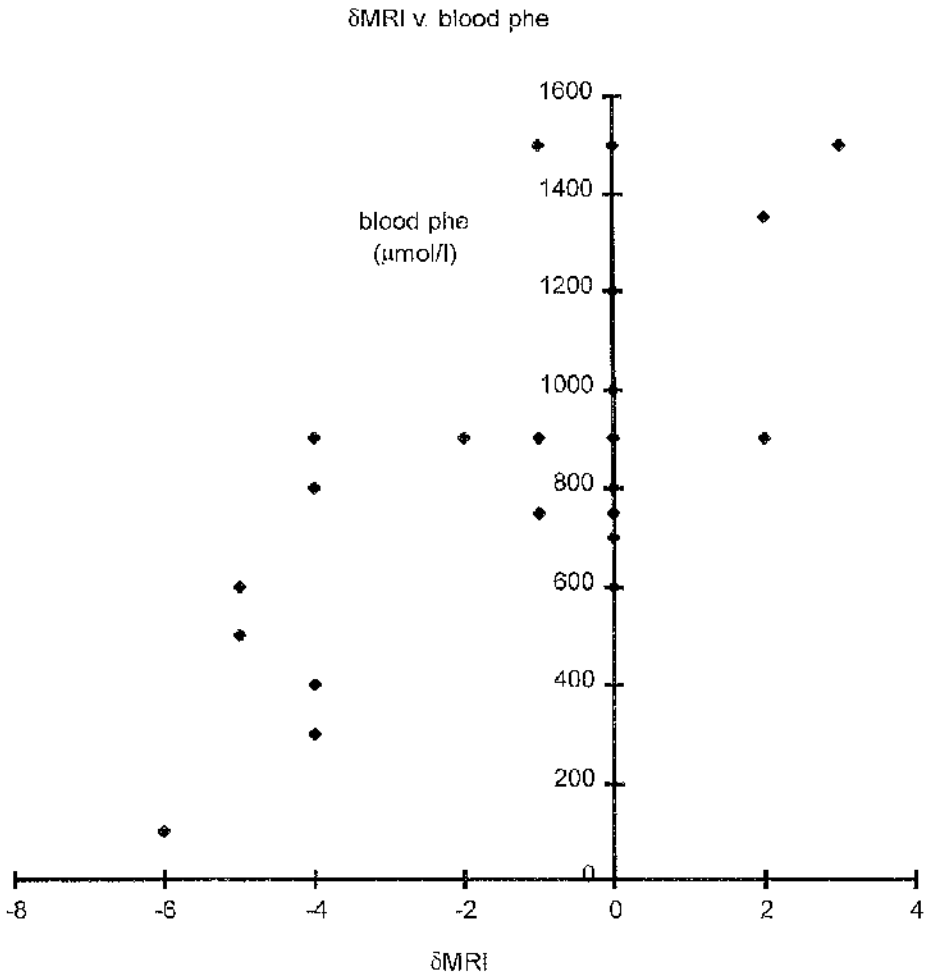
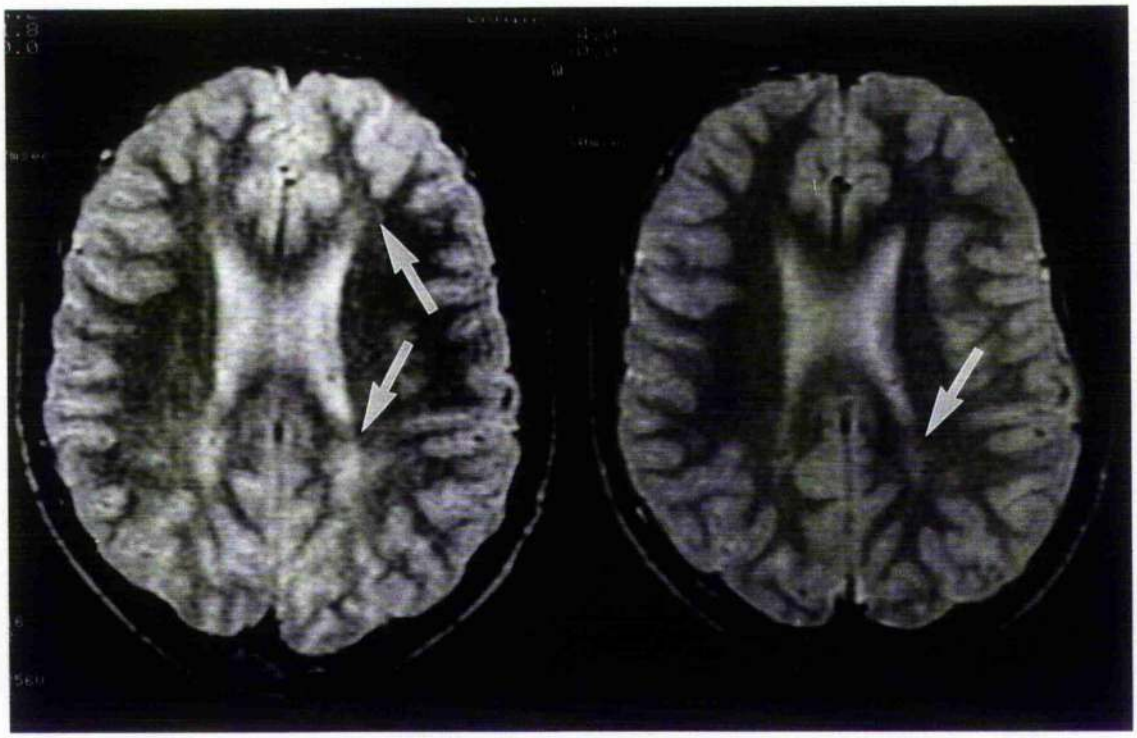


Figure 16: MRI of head in PKU patient showing improvement in scan appearance after reducing phe levels with dietary therapy

The arrows highlight the abnormal white matter which is more extensive on the scan on the left performed before phe levels were reduced.



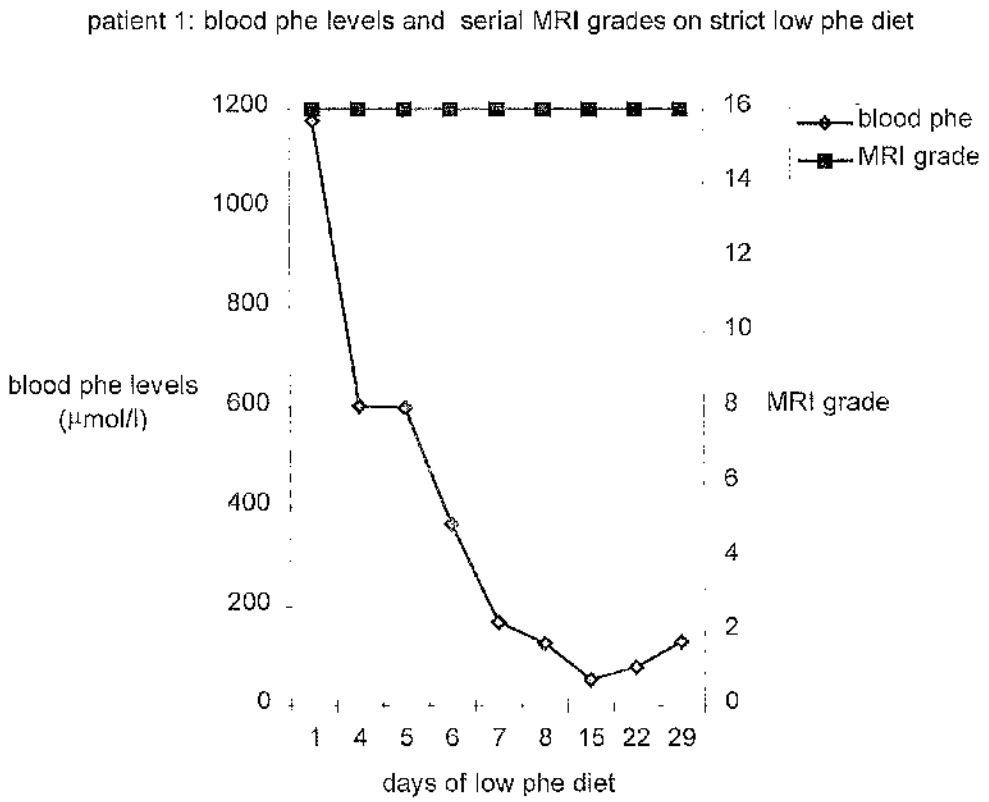
3.11 Serial cranial MRI on two siblings

The two siblings who returned to a strict low phe diet on a trial basis achieved phe levels in the desired range (<200 μmol/l) as shown in Table 22. Phe levels were reduced to < 200 μmol/l by days 6-7 of diet. MRI grades were 16 in sibling 1 and 13 in sibling 2. No change was observed in the MRI appearances and after 3 weeks of strict dietary restriction of phe, the brothers relaxed their diet. These results are shown in Figure 17 and Appendix 12.

Table 22: Blood phe levels in two adults who returned to strict diet after scan on day 1

days	SIBLING 1 phe levels (μmol/l)	SIBLING 2 phe level (μmol/l)
1	1177	1081
4	603	418
5	599	264
6	367	148
7	170	57
8	126	129
15	54	96
22	79	140
29	130	170

Figure 17: Plot of serial MRI grades and blood phe on sibling I on returning to strict low phe diet



4. DISCUSSION

4.1 Introduction

In this chapter the results of the studies and their significance are discussed. The discussion is divided into the following topics: study design, clinical and neurophysiological findings and their relationship to MRI changes, relation of MRI abnormality to biochemical control and an interpretation of the MRI changes.

4.2 Study design

4.2.1 Study group

The patients studied all attend the PKU clinic at the Willink Biochemical Genetics Unit in Manchester. For the majority, their treatment has solely been with this clinic, occasional patients have entered the clinic at a later age. The patients have 'classical' PKU, that is phe level >1200 µmol/l on a normal diet with a protein intake of 2g/kg (15). 78 patients were studied in total. 59 of these were over 14 years and had discontinued strict diet (around the age 14 years) and were currently taking a low protein diet (1g/kg) or normal diet without amino acid supplement. Fifty-eight of this group were studied for evidence of clinical and neurophysiological abnormality as well as MRI scanning. The younger group (those less than 14 years) only underwent MRI scanning. 59 adults represents 80% of the adult group whom still attend the clinic. Some adults have been lost to follow-up over the years and it was considered whether these patients were likely to form a particular subset of patients with a milder form of PKU or a less well treated group with a poorer neurological outcome. Whilst this remains theoretically possible, it is unlikely since in terms of their diagnostic phe, IQ and duration of treatment they did not differ from the study group.

The North West region of England is a large geographical area and patients studied here are likely to be representative of patients with PKU in UK. The one area in which this group may differ from the general PKU population in UK is that dietary treatment has been continued in Manchester until age 14 years. This is longer than many centres in UK and Europe. If there are hazards after dietary discontinuation causing the development of neurological illness, then this group may not show problems for a number of years. The age of the group studied was 14-32 years (except one woman aged 49 years). Other patients with neurological problems have been aged 18 years and been off diet for around 10 years. Hence it would be expected that this group is sufficiently old and large for emerging neurological difficulties to be observed.

4.2.2 Control group

The age range of participants for control neurophysiological studies was 16-40 years, average age 21 years. After adolescence, nerve conduction measurements stabilise at adult values until around 60 years when nerve conduction slows (129) and so this is a suitable age range to use as control subjects.

All controls were either employed or students and are likely to be representative of an socio-economic group I, II or III. However, there are no reports of nerve conduction being related to socioeconomic groups and although one could speculate that such groups will have better early nutrition, the results we obtained from testing these groups agree with published results in the literature and they were considered suitable as control data.

A control group was not considered necessary for MRI since similar abnormalities have been reported in PKU by several groups and MRI of brain is a standard procedure with established age related normal appearances.

4.2.3 Study methods

The study was designed to recruit a large number of patients with classical PKU that could be considered representative of patients with PKU. Recruitment was invitation by letter to which a tear-off reply was attached. Only 5 patients replied with a negative answer but some others did not answer or after initially indicating a willingness to participate declined to attend appointments. Some of these were due to travel difficulties and others were cancelled, as the patients had become pregnant in the interim. The method of recruitment seemed successful and a group accounting for 80% of the possible total was recruited.

A comprehensive neurophysiological assessment was performed which yielded important information. However, on repeat testing it proved difficult to get this group to return. This may partly have been due to the nature of the tests or it may have been the length of the assessment. In order to reduce the duration of the tests, the SEPs were omitted from the repeat assessment since they were all normal on initial testing. The outcome of the first stage coincided with the MRC recommendations on lifelong treatment for PKU (11). The initial study design included a period of observation between scans without dietary manipulation. However, after hearing results and MRC recommendations some patients wished to return to diet. Although this gave three study groups to study on repeat scanning, there was no attempt at randomisation and the resulting groups were of varying size. In particular, the group who opted for strict low phe diet consisted only of five patients.

4.3 Clinical examination

In this study 58 patients were examined. The clinical examination consisted of assessment of power, tone and reflexes of the limbs. Rest tremor and intention tremor was noted. Cerebellar integrity was tested by alternating rapid hand movements and finger-nose test. Abnormalities were observed in 11 of the 58 patients. Brisk reflexes were present in all these patients. Additionally rest tremor was observed in 6 of the 11 patients. The tremor did not worsen on intentional actions.

The nature of the clinical examination was chosen on the basis of previous reports of adult neurological deterioration in PKU (8-10,70). In these reports several individuals with PKU had developed definite neurological signs. Villasana et al. (10) found spasticity of the lower limbs and an abnormal gait in two young adults aged 28 and 18 years. Battistini (70) noted hypotonia and weak abdominal reflexes in an 18 year old girl. Thompson et al. (8) have seen 7 patients with a combination of neurological signs: pyramidal weakness of the lower limbs and brisk reflexes, dystonia and cerebellar signs. Bick et al. (79) have noted normal neurological examination in a group of 10 adolescents. Likewise the group examined by Lou (77) were normal. However, Thompson et al. (71,72) in their later study of asymptomatic patients identified brisk reflexes and tremor in 12 of 26 patients examined. Taking these studies overall it seems that the older patients are the most likely to show mild neurological signs of tremor and brisk reflexes. The more specific stronger neurological abnormalities are observed in a smaller number of individuals. It is possible that the early treatment of some of these patients was suboptimal. Several of Thompson's patients had reduced intellectual function and poor growth at a young age. (8). However, four of the seven in the group had been diagnosed by newborn screening and maintained acceptable phe levels in the early years and so there is no particular reason to suspect that their treatment was suboptimal. Another consideration is their nutritional status. Serum folate and vitamin B12 deficiencies have been reported in PKU patients and may cause neurological decline (169). Folate and B12 status is documented in only one of these reports by Villasana (10) and was normal. It is possible that the major neurological decline is seen in

patients either with poor early treatment or late nutritional inadequacies. The patients studied in my group who were well treated from birth did not show any symptomatic neurological decline. The patients here, although representative of PKU in the North West Region, have continued dietary therapy until 14 years. This factor may have some bearing on their wellbeing, as myelination is known to continue at least throughout the first decade.

The clinical signs seen in this group and many other groups of treated PKU patients are considered soft neurological signs. Tremor at rest is seen in Parkinsonism and other subcortical dementias (165). It is also observed in anxiety states. Cerebellar tremor is usually an intention tremor. Brisk reflexes can be a sign of pyramidal tract involvement. In pyramidal tract abnormality increased tone of the muscles is usually observed. The areas of the brain that could cause these signs are the motor cortex positioned in the precentral area of the frontal cortex or the basal ganglia. Neither of these areas are the regions of the brain especially noted as being abnormal on MRI scanning in PKU. It is therefore not unexpected that there is no correlation between clinical abnormality and the extent of white matter involvement in this group of patients. An alternative theory is that disturbances in neurotransmitter availability are responsible for the neurological abnormalities described in PKU. Deficiencies in neurotransmitter availability are known to occur in PKU. Dopamine, serotonin and noradrenaline availability is markedly reduced when phe levels are high (102-104). This form of neurological abnormality would not be highlighted on MRI scanning. The number of patients with neurological signs was small, however, in this study and any statistical significance between MRI severity and clinical abnormality would possibly be missed. After one year's observation, there was little change in physical examination in the group. Patients with neurological abnormalities displayed similar signs on repeat examination. Two patients previously thought to be normal had developed brisk reflexes. The development of new signs did not relate to worsening of MRI appearances or to alteration in diet.

4.4 Neurophysiological investigation

A comprehensive neurophysiological examination was performed in 56 adults with PKU aged 14-49 years the majority of whom had discontinued diet at the age of 14 years. The neurophysiological studies were specifically chosen having reviewed the current literature in PKU in which it was suggested that the MRI white matter changes were a result of demyelination (8-10). Additionally previous neurophysiological studies had reported abnormalities in VEPs and NCV in PKU (10,77). Magnetic stimulation had not been previously reported at the outset of this study, being a relatively new technique but a report emerged during this study of CMCT in 22 treated adolescents with PKU (78). We were particularly interested in the results of magnetic stimulation since the measurement can indicate delay in nerve transmissions in the pyramidal tract and this was the region reported by Thompson and others as being abnormal in PKU (8).

Although only 6 individuals had prolonged P100 wave on VEP, the mean P100 was statistically longer in the PKU group than in controls. This finding may represent a subtle change in VEP in PKU. There was no correlation between VEP latency and MRI scores, a finding corroborated by others. It is of interest that the main abnormality found in the neurophysiological assessment was in VEPs as this region of the brain was the area most commonly noted to show white matter pathology. Two individuals had marked prolongation of the P100 wave with poorly formed waveforms. These were aged 23 and 20 years and had been on dietary therapy until the age of 14 years. However, their biochemical control had never been good. IQ's were 92 and 77 respectively.

Other individuals had abnormal results on CMCT and NCV. There were no abnormal results for SEP. These results are not in agreement with some other published data on neurophysiology in PKU (78,79,146-149). This lack of agreement in neurophysiological results is probably due to several considerations: different patient groups ie, different severity of PKU, and different laboratory techniques.

If demyelination was present in PKU it is likely that a greater degree of neurophysiological abnormality would have been observed. In MS, neurophysiological studies can highlight clinically undetectable abnormalities in nerve function although MRI is far more sensitive at detecting demyelinated plaques than any neurophysiological technique (132,137,163). However, when widespread disease is obvious on MRI then some degree of abnormality is found on neurophysiological testing. Also it has been observed that the combination of neurophysiological techniques improves the detection of areas affected by demyelination (136). In several studies, magnetic stimulation of the cortex has been found to be highly sensitive at detecting demyelination (132-134). From the relatively normal results of this assessment, it is possible to conclude that the MRI abnormalities are unlikely to be related to demyelination.

The lack of correlation between MRI score and any neurophysiological test is of interest. Perhaps the only test that seems to correlate with the distribution of MRI abnormalities is the VEP and other investigators have suggested that an 'occipital syndrome' may exist. However, Lou (77) and Bick (79) have not found an association between the VEP and extent of MRI lesions.

The functional tests performed in our study have failed to detect any major disturbance in neurological wellbeing although some minor findings of uncertain significance have been detected. It appears that the abnormalities resulting in the abnormal neurophysiological findings are unrelated to the white matter changes seen on MRI of brain.

4.5 MRI abnormalities

4.5.1 Frequency of MRI abnormalities

MR brain scans showed abnormalities in the T2 weighted images of the white matter in 71 of the 74 patients scanned (three patients could not tolerate scanning due to claustrophobia). The three patients who had normal scans were: one girl aged 11 years who was diagnosed and treated at 20 days who always had good biochemical control (current phe level at time of scan was 100) and two other younger children had equivocal changes on scan (scoring 1 bilaterally i.e. the radiologist could not discern whether the scans were abnormal or not). These boys were aged 11, diagnosed and treated from the newborn period and their phe levels 300 and 450 μ mol/l at time of scanning.

All the other patients showed some abnormalities on MR scanning. The abnormalities were similar throughout the group although they varied in degree of severity.

4.5.2 Pattern of the MRI abnormalities

The areas of the brain affected were the occipital, parietal, temporal and frontal cortices. Lesions in the basal ganglia were observed in two patients. No changes were observed in the cerebellum or brain stem.

The abnormalities consisted of a hyperintensity of the signal of the cerebral white matter on T2 weighted images. In the majority of cases this was a diffuse homogeneous finding but in some patients (16 of the total group) discrete focal lesions were observed. There was a gradation in the severity of the abnormalities: in patients with the least change white matter abnormalities were seen only in the occipito-parietal region. With increasing severity white matter abnormalities extended into the frontal lobe and then the temporal lobe. This gradation in involvement of the white matter has only been suggested previously by one other investigator. Pearsen et al. (75) noted that the more severely affected scans involved the frontal lobes.

However, published results suggest that this phenomenon was occurring in other centres. Lou et al. (77) who studied a young population of adolescents with PKU report white matter change only in the occipitoparietal region. This study also found frequent EEG abnormalities in the occipital cortex and on this basis describe an 'occipito-parietal syndrome' occurring in PKU. It is possible that frontal lobe changes would have been observed had older patients or individuals with higher phe been studied.

The abnormalities were symmetrically distributed and this was reflected in the scoring. Although the scoring system allowed for a discrepancy between left and right by scoring each hemisphere separately, there was never more than one point difference between each side. The focal discrete lesions however, when they occurred, were asymmetrically distributed. No account of these was taken when scoring the scans but they were noted in a written report accompanying the scan.

4.5.3 Relationship of MRI scores to biochemical control

Several parameters relating to biochemical exposure to phe were examined in this study. The technique used was designed by Walter et al. and applied to a study of 11 Bristol schoolchildren with PKU (80). In that study only 4 scans were abnormal showing the characteristic appearances of white matter changes. There were no statistically significant associations between MRI severity and biochemical control but a suggestion that the children with the worst scans had the worst biochemical control. The study did not measure blood phe at the time of MRI. However, the number of patients studied was small and since they were a younger group they tended to have blood phe levels in the lower range. Statistical significance may have been reached in a larger group. We used the same computer programme in this study and found several factors relating to biochemical control to show statistical significance: mean blood phe throughout life ($p=0.0043$), mean blood phe in past 5 years ($p=0.0025$) and blood phe at the time of scan ($p=0.0002$). Stepwise multiple regression analysis showed that when phe at time of scan was accounted for, no other measure of biochemical control showed a significant association

with the MRI grade. Thompson et al. (72) and Bick et al. (79) were able to establish a relationship between the degree of white matter change and biochemical severity. Thompson in particular demonstrated that recent phe levels were strongly associated with MRI severity (72). Their data was analysed using ordinal statistics as the results were not normally distributed. In our larger study the MRI did approximate to the normal distribution allowing the use of parametric analysis. Bick was unable to detect statistically significant associations but noticed the trend that MRI severity seemed to relate to recent phe exposure rather than early exposure (79). In my study there was no relationship between early phe exposure in the first 4 years and MRI score ($p=0.27$) nor was there a significant association between IQ and MRI score. This last fact is of interest as it corroborates the lack of association with MRI and early control since early phe control is known to be associated with final IQ.

The strong association between MRI grade and blood phe levels allows speculation on the cause and nature of the abnormal appearances of cerebral white matter on MRI in PKU. There is no information to prove a causal effect of blood phe in the production of this abnormal white matter appearance. However, one can speculate a causal effect based on current knowledge of the effects of elevated blood phe on the central nervous system and this is discussed further later.

Further evidence of the role of blood phe in changing white matter appearance on MRI is given in the second part of this study when repeat scans were performed. In the second stage of the study the older patients underwent scanning after a period of 3 to 12 months (median 9 months) and the younger group after a period of 1 year. The groups were analysed separately since the younger group were on diet and made no dietary alteration between scans, whereas some of the older patients had elected to restart some form of dietary therapy. Within the older group, some reduction in the extent of the white matter abnormalities was observed in those whose phe levels were substantially lower at repeat scan. There was a significant association between the change in blood phe between scans and the change in MRI score ($p=0.0002$). There was also a strong inverse correlation between blood phe at second scan and the change in MRI score ($p<0.0001$).

The scans that showed reduction in extent of changes were generally in those in whom phe levels were <700 μmol/l at second scan. Patients who reduced phe levels from 1800 μmol/l to 700-900 μmol/l although achieving a large δphe tended not to show any improvement. It therefore seemed that the actual phe attained was more important than the degree of change in phe level. The correlation found in stage I of the study was still present i.e. phe at time of study was still significantly associated with MRI score. The reduction in severity of MRI score was a reflection of reduction in white matter change throughout the brain. There was a tendency to improve firstly in the frontal lobes and then the occipitoparietal lobes, the reverse of the way in which the abnormalities started. This part of the study adds weight to the theory that blood phe plays an important role, either directly or indirectly, in the generation of the white change seen as hyperintensity of the T2 images on MRI. The strength of the association of blood phe and MRI severity led to the question of whether blood phe could alter MRI appearances on a day to day or week to week basis. The further work with two adults who underwent serial scanning was planned to answer this question. In this small study it was observed that despite stringent reduction of blood phe, which was maintained for three weeks, no alteration was seen in daily or weekly MRI scores.

4.6 Interpretation of MRI abnormalities

4.6.1 Introduction

Although MRI is an extremely sensitive tool for investigating the brain, the images obtained are not specific (170). It is the best investigation available for detecting changes in the white matter but the interpretation of these alterations presents difficulties. Many different disorders can result in similar MR appearances (118). Disorders of myelin encompasses a range of problems. When myelin is lost from the CNS, it can be a primary or secondary defect. Primary defects are those where myelin is abnormally produced in a form that renders it more susceptible to breakdown or where an external toxin 'attacks' the myelin sheath. Secondary myelin loss can occur when the axon is damaged (118). The interdependency of the axon and myelin sheath means that loss of

myelin will ultimately lead to axonal loss and vice versa. There has been some work indicating axonal loss in the brains of PKU patients (171). However, the majority of current research suggests that PKU primarily affects myelin. Additionally the apparent reversal of MR pathology on re-institution of diet argues against an absence of axons with secondary non-myelination as the explanation of the MR findings. The interpretation of the MR abnormalities is discussed further in the context of a pathological effect on myelin.

4.6.2 MRI pattern in PKU compared with other white matter disorders

It is sometimes advocated that the intensity and distribution of MR changes is useful in suggesting the nature of the underlying neurological condition and that such 'pattern recognition' is important in interpretation of the underlying nature of the abnormalities (118). Some interpretation of the nature of the changes seen in PKU is discussed below in relation to the pattern observed.

The reason for the symmetrical distribution, the specific areas affected or the gradation in severity of changes remains unknown. Symmetry of MR changes suggests a systemic effect from raised phe but some regions of the brain seem particularly susceptible and of these some are affected prior to others.

In order to attempt to interpret abnormal signals it is necessary to consider their signal intensity and distribution. The comparison between the changes seen in PKU and and many other causes of hyperintense T-2 weighted signals on MRI of the brain is discussed.

Firstly, symmetry of white matter changes: in demyelinating diseases the white matter plaques are usually asymmetrical in their distribution (172). Symmetry of abnormal signals is seen in some of the metabolic dysmyelination disorders such as metachromatic leucodystrophy (arylsulfatase A deficiency) but with that disorder the lesions begin in the frontal lobe and progress towards the occipital lobe (173). In Alexander's disease an autosomal recessive

disorder due to aspartyl acyclase deficiency, the lesions tend to be symmetrically distributed (174). Symmetrical distribution seems to occur where there is a generalised toxic process. Demyelination seems to be more characterised by patchy involvement of the brain.

Secondly, the distribution of the PKU changes is predictable and stereotypic occurring in the periventricular areas of the occipital, frontal and temporal lobes. These areas of the brain are involved in several metabolic disorders such as Alexander's disease and mucolipidosis (156). However, the intensity of the signal and the homogeneity of the abnormalities in PKU gives it a different appearance from these disorders.

Thirdly, the gradation of involvement according to severity of the white matter changes. This feature seems to be fairly typical of PKU and is not described in other de- or dysmyelinating disorders.

4.6.3 Are the MR changes pathological?

MRI demonstrates changes in signal intensity that correlate well with histological demonstration of myelination (110). Myelination begins during the fifth fetal month and continues throughout life in a predictable pattern proceeding from caudal to rostral direction (175). It proceeds rapidly up to 24 months of age but slows markedly after this age. It is thought that fibres in the areas around the lateral ventricles are amongst the slowest to myelinate. These areas are called the association areas of the brain and continue to myelinate throughout the first several decades of life (175). The late myelination of these areas is thought to be responsible for areas of high signal intensity persisting in the white matter dorsal and superior to the trigones of the lateral ventricles on the long TR/TE images. These regions are homogeneous with indistinct margins. They gradually disappear usually by the end of the first decade. These images are normal findings and they can be differentiated from damaged brain such as periventricular leucomalacia (PVL) by looking for evidence of tissue loss that is seen as enlargement of the ventricles or atrophy at the periphery of the brain in damaged brain (117). In addition, damaged brain will

normally show abnormal signal intensity on the first echo of a long TR sequence whereas unmyelinated regions do not and normal brain contains a layer of myelinated white matter between the trigone of the ventricle and the homogeneous high signal.

The signal intensity correlates with the degree of myelination (158,159). This implies that MRI can differentiate immature white matter, myelinated white matter and gray matter. The water content is different in each of these and this factor probably accounts for at least some of these differences in signal. It has further been suggested that shortening of the T2 relaxation time in maturing white matter is related to protein, cholesterol or glycolipid content of the myelin sheath (174). The lipid proteins of myelin do not directly contribute to the MRI signal intensity. It appears likely that the myelin membrane is involved in the restriction of water proton motion (156,176).

It is interesting that the area of the brain most commonly affected in PKU is the white matter of the peritrigonal areas. In our scans there was no evidence of a strip of normal white matter as is seen in normal brain and there was frequently evidence of involvement in other areas of brain with cortical atrophy in some patients. These factors suggest that the findings in PKU represent pathological changes. The frequent involvement of this region may be related to the later completion of myelination of these association areas, as it is possible that this younger myelin is more liable to the effects of hyperphe.

4.6.4 Possible effects of phe on cerebral white matter leading to production of MR signal

4.6.4.1 Introduction

It is reasonable to assume that phe either directly or indirectly is responsible for the abnormalities seen on MR imaging in these patients since there is a strong association with phe levels and the severity of the abnormalities and an association between reversal of the abnormal signal and reduction of blood phe levels. The aetiology of the abnormal appearance of the white

matter in the PKU brain are now discussed in relation to the present knowledge of the pathological effects of raised phe on white matter.

4.6.4.2 Origin of the MR signal of white matter

The origin of the MR signal is complex but depends primarily on the proton content of tissues. Protons in myelin are so tightly bound that no perceptible signal is emitted from them. The main contributor to the T2 signal is protons of water in the white matter (156,177). Hyperintensity of the T2 weighted signal in the MR images results from increased water content of the white matter in these areas (156).

4.6.4.3 Development of the myelin sheath

Figures 18 and 19 are illustrations showing the development of the myelin sheath and highlighting the production of the lamellar structure. Myelin is produced by the wrapping of oligodendrocyte extensions around the axon. As the surfaces of the membrane become apposed the cytoplasm is extruded and the membranes adhere (107). The myelin sheath thus formed is composed of proteins, lipids and some carbohydrates. Myelin has a hydrophobic centre formed mainly by the lipid component and a hydrophilic coating that interacts with the aqueous environment. This arrangement creates a stable membrane relatively resistant to external toxins. There is a repeating protein-lipid-protein-lipid-protein unit. The major protein is proteolipid protein (PLP) which stretches across the hydrophilic and hydrophobic areas. The other main protein is myelin basic protein (MBP) which is located in the cytoplasmic membrane (107)

Figure 18: Diagram of the oligodendrocyte extensions wrapping around the axons

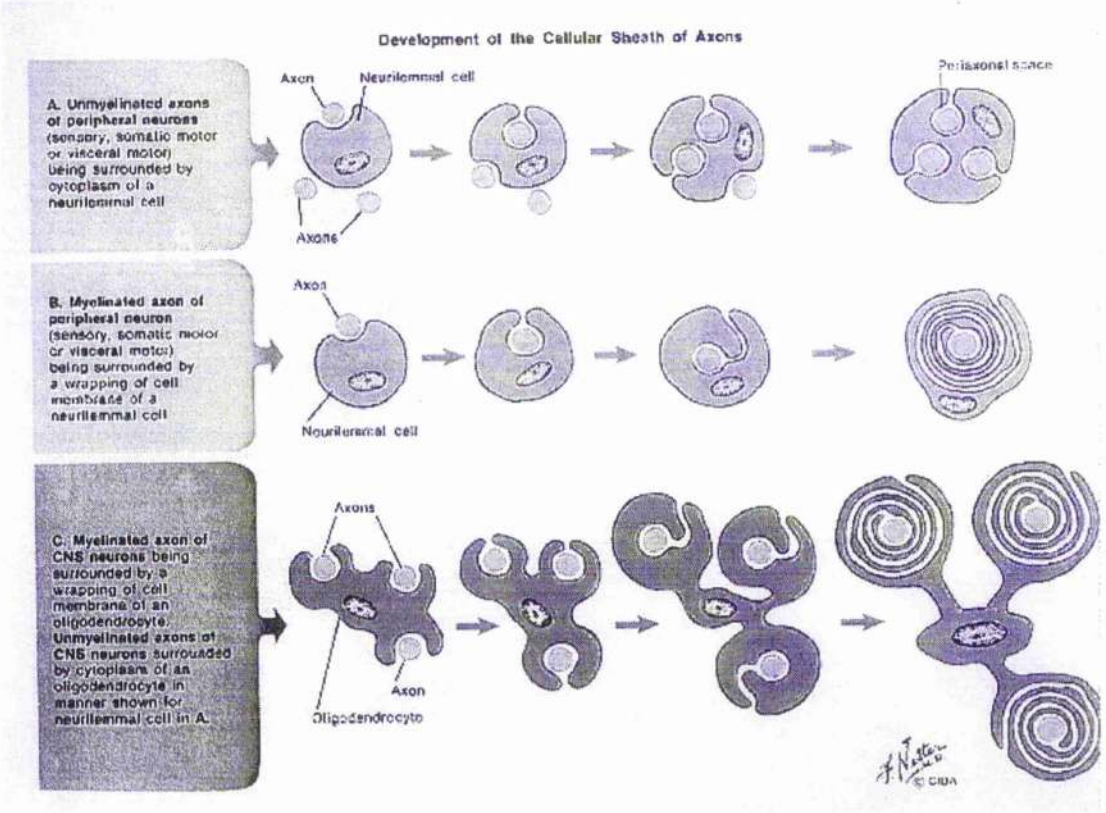


Figure 19: Illustrative diagram of the microscopic structure of the myelin sheath showing lamellar structure

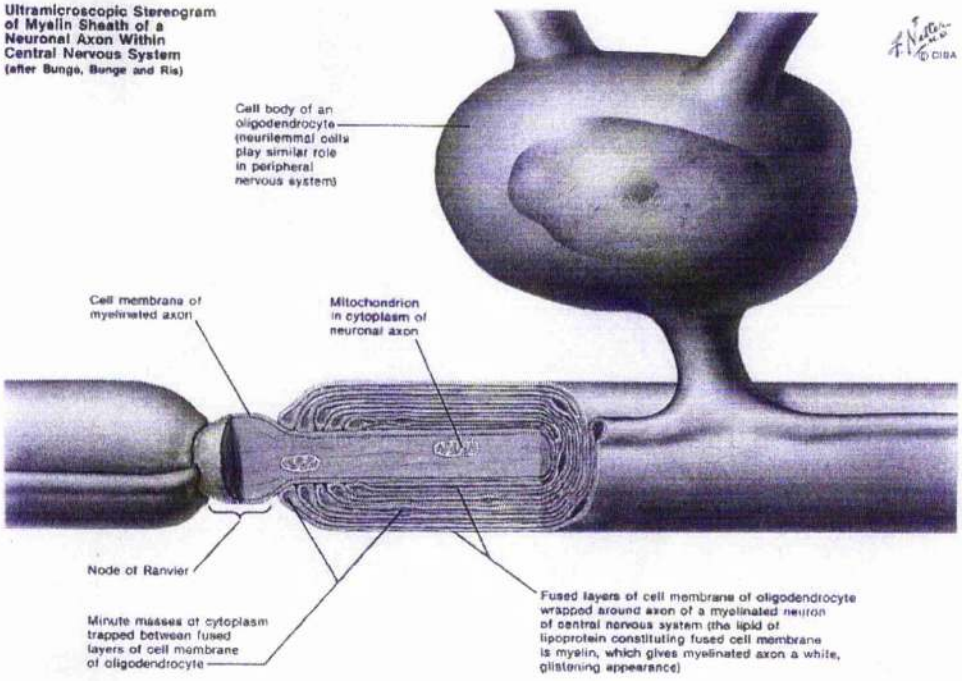
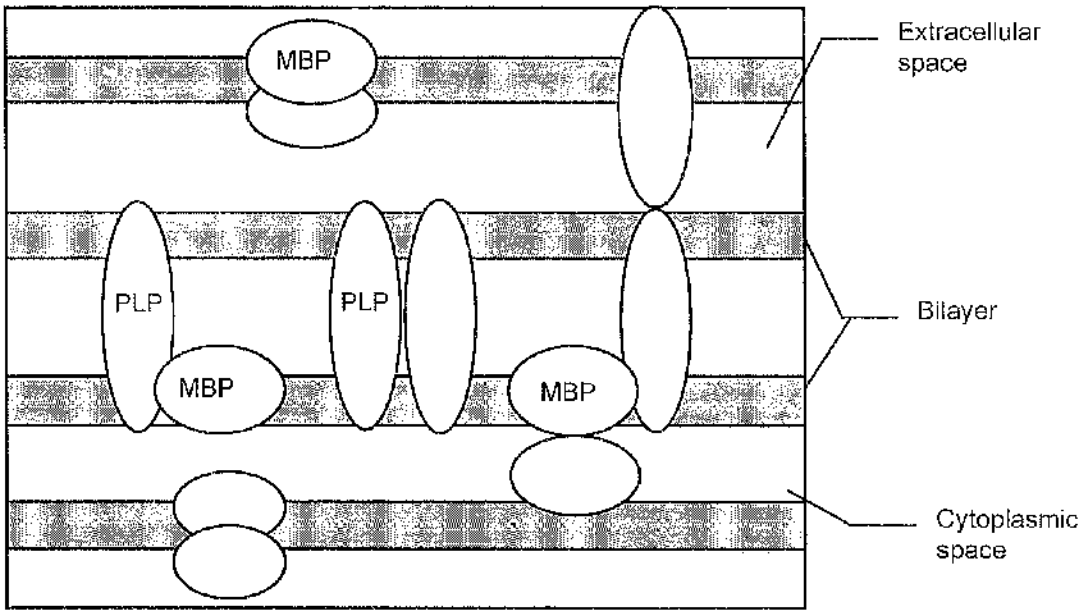


Figure 20: Illustration of the components of the myelin sheath



PLP: proteolipid protein, MBP: myelin basic protein

4.6.4.4 Possible sites of water accumulation considered in relation to MR signal and effects of raised phe on white matter

Theoretically there are three possible sites of an increased water content of white matter: an accumulation of extracellular fluid around the myelin sheath due to loss of myelin as in MS, extracellular water accumulation between the lamellae of the myelin sheath (vacuolating myelopathy) or an increase in the intracellular water compartment of the myelin sheath. The arguments for each of these is discussed further below in relation to the MR pattern seen in this study and the known pathological effects of phe on white matter.

Raised phe levels have been observed to have several effects on brain development. The majority of this work relates to postmortem findings on untreated adults and on animal models equivalent to the untreated patient (82-85,99,104). There is also a body of work performed on the rat model rendered hyperphenylalaninemic at 25 days, approximately comparable with the treated individual who discontinues diet aged eight years (88). There are no PM studies on

treated individuals. There is no unifying theory from these studies although certain facts emerge regarding the effects of elevated phe on the central nervous system.

Elevated phe levels have an effect on protein synthesis in the CNS. In PKU there is a relative deficiency of certain other amino acids due to competitive inhibition of phe for transport pathways. The deficiency of these amino acids may be a rate-limiting factor in protein synthesis. Low levels of both PLP and MBP, the major proteins in myelin, have been measured (99). In their study on rats with late-onset hyperphe, Taylor and Hommes found that myelin turnover was increased and that myelin was less stable (88). The patients studied in my project had continued treatment longer than 8 years, usually until aged around 14 years. Nevertheless, these animal studies suggest that phe continues to be toxic to myelin even after the rapid phase of myelination is completed. Phe was thought to exert this effect by interfering with the proteins necessary for the synthesis of myelin. An interesting feature of this work was that the effects were only seen at relatively high levels of phe: a threshold seemed to exist for the interrupting effects of elevated phe. Another theory of phe toxicity related to its effect on ATP sulphurylase. It is thought that sulphatide protects myelin and contributes to its stability (88,90,100). ATP sulphurylase has been shown to be inhibited by elevated phe levels in the brain thereby causing a reduction in levels of sulphatides. It is hypothesised that this reduction in sulphatide level may render myelin more vulnerable to proteolytic degradation and thereby increase myelin turnover.

Phe also has an effect on brain lipids. Early work identified reduced amounts of myelin lipids especially cholesterol in the brains of patients with untreated PKU (99). More recently low levels of essential fatty acids has been found in treated individuals with PKU (178,179). It is now recognised that dietary treatment in PKU reduces the levels of some polyunsaturated fatty acids (PUFAs) since it restricts the intake of animal fats that are rich in protein (179). As these PUFAs are components of structural lipids in cell membranes, they are fundamental to the normal development of the central nervous system. The importance of fatty acids in the diet of children for the normal development of the infantile cortex has been recognised (180). Specifically, levels of arachidonic, eicosapentaenoic and docosahexaenoic acids are reduced in circulating plasma

lipids in treated PKU (179). Levels of linoleic and oleic acids are higher in children on a PKU diet. It is speculated that these derangements of fatty acids could have a detrimental effect on intellectual outcome in treated PKU. The degree of phe control inversely correlates with levels of PUFAs i.e. those individuals with low phe levels had more abnormal levels of PUFAs. Since the MR abnormalities in this study were more extensive in those with high phe it is unlikely that low PUFAs explains the MR findings. Also the MR abnormalities were more prominent in those who had discontinued diet whereas the low PUFA levels are a concern for those on dietary treatment. Although they probably do not relate to the MR images, fatty acid levels in PKU may contribute to the clinical problems and slightly reduced IQ's observed in the treated PKU population.

From the work to date it is possible to speculate that raised phe in the mature PKU brain can cause disruption to myelin, by one or more of the explanations mentioned above and this leads to proteolytic breakdown and increased turnover of myelin. However, the myelin now produced is no longer normal since it is being continually renewed in the face of hyperphe and relative tyrosine deficiency. These problems could produce a poorer quality of myelin sheath. If the myelin was deficient predominantly in PLP, which is present on the outer layer, then it is possible that an increased amount of extracellular water could penetrate between the lamellae due to instability of the outer layer of the sheath. This would cause a picture of vacuolating myelopathy. If, however, there were deficiencies or alterations in ratios of lipids and/or MBP then disruption of the hydrophobic core or cytoplasmic membrane could ensue. This would give rise to an accumulation of intracellular water. If the predominant effect of raised phe was myelin loss similar to that observed in the demyelination of MS then water would accumulate as the extracellular fluid fills up the spaces left by myelin destruction. Any of these explanations could give similar MRI pictures. Factors which give weight to the theory that raised phe is responsible for myelin disruption and that is causing the abnormal MR appearances are as follows:

1) MRI is detecting some pathological process in the white matter

2) Animal model evidence is strong that phe can damage mature myelin

3) MRI changes show a strong association with phe levels

4) Reversal of changes is observed after reducing phe; recovery of myelinogenesis is reported on reducing phe levels in rats with myelin damage.

5) A suggestion of a threshold effect was observed in repeat MRI studies: only after phe levels were below 700 μmol/l was reversal observed. A similar effect was seen in animal studies of myelin disruption.

However, if the MR appearances resulted from extensive demyelination, one would expect some abnormality in function of the nervous system that is not supported by the neurophysiological or clinical studies. Intracellular water usually does not contribute greatly to the MR signal since intracellular water is bound to many components of the cytoplasm. It is unclear but doubtful whether an increase in this compartment would cause the MR findings observed in this study. An increase in interlamellar water seems a better explanation presently for our findings. In hyperphe rats the earliest pathological change observed is a disintegration of myelin with disruption of the myelin sheath (171). Accumulation of extracellular fluid between the lamellae could be a reversible feature when blood phe levels are restored to lower levels as new myelin is synthesised with a more normal constituency. The time taken for reversal of changes would support this theory, as it would approximate to the turnover times of myelin. This explanation is based on an interpretation of the current knowledge but remains merely speculative.

4.7 Further work

Although this study did not identify functional neurological problems in PKU to any large degree it would now be important to look for more subtle evidence of neurological problems. There is some evidence to suggest that white matter changes as observed on cerebral MRI in PKU can have neuropsychological consequences (181,182). There is a growing body of evidence that neuropsychological abnormalities do exist in PKU particularly in executive task performance (59-61). These deficits have been shown to be related to current blood phe levels and neuropsychological performance is improved when blood phe levels are lowered. This work has been reported in a small group of adolescents with PKU. An important study to follow the results

presented in this thesis would be to perform detailed neuropsychological examination in the large group of patients studied in addition to MRI brain scans to seek a correlation between higher brain performance and MRI white matter lesions.

Although there is no suggestion of progression of abnormalities on MRI over a time period of one year, this study should continue by performing annual serial MRI scans in the same group of patients.

The explanation for the neurological decline observed in a small group of adults whose problems triggered this study remains unclear. It is possible that severity of neurological outcome is related to underlying genetic factors, which are as yet undetermined. It is possible that there is another gene implicated in the neurological phenotype of PKU in addition to phenylalanine hydroxylase deficiency (Dr Susan Ramus, personal communication). The adults who have shown specific neurological decline should be studied in more detail. Specifically it would be of interest to attempt to establish genotypes of that group and compare it with the many other adults with PKU whom remain neurologically well.

5. Conclusions

The conclusions that can be drawn from this work are as follows:

1) Cerebral white matter changes are common in adolescents and adults with PKU who were treated from the newborn period,

2) The white matter abnormalities are present even if still on dietary therapy,

3) The extent of white matter changes strongly relates to the blood phe levels at the time of scanning,

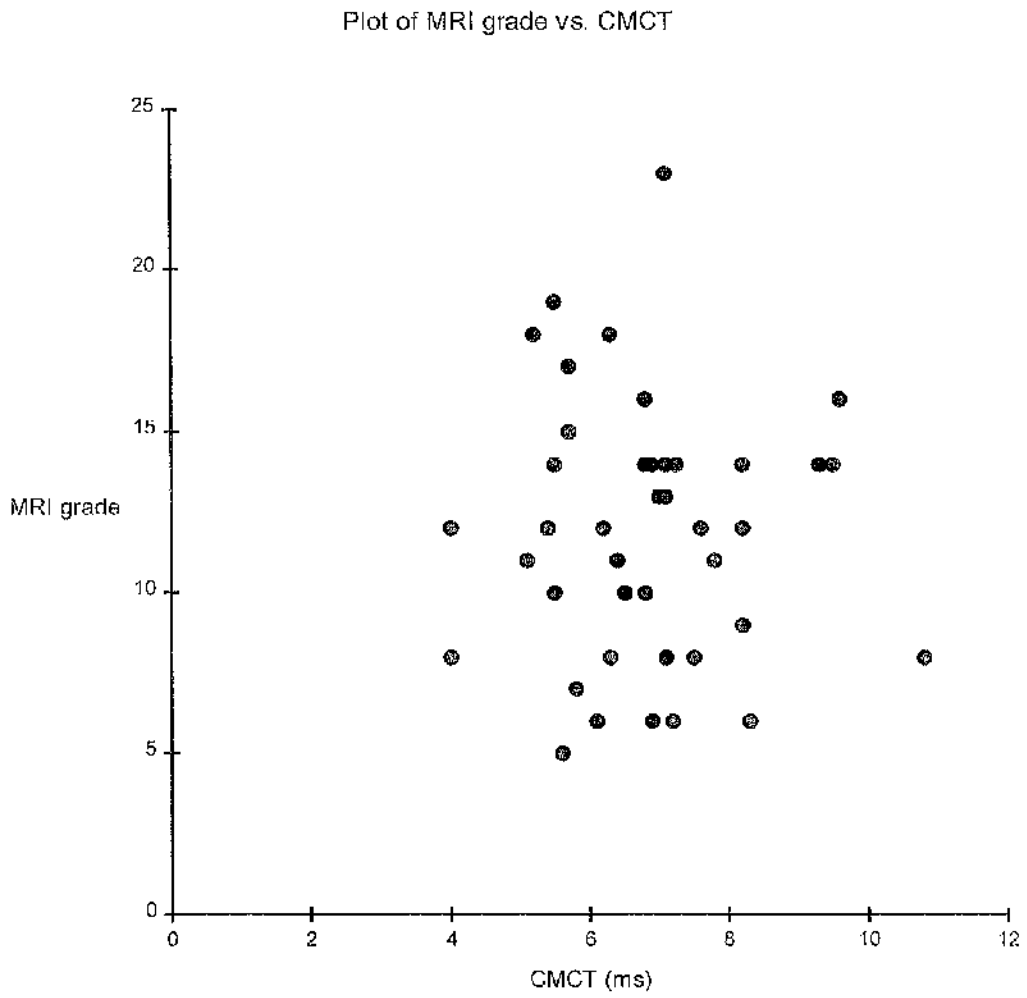
4) The white matter changes are not associated with early biochemical control,

5) The extent of white matter change is unrelated to clinical or neurophysiological abnormalities and

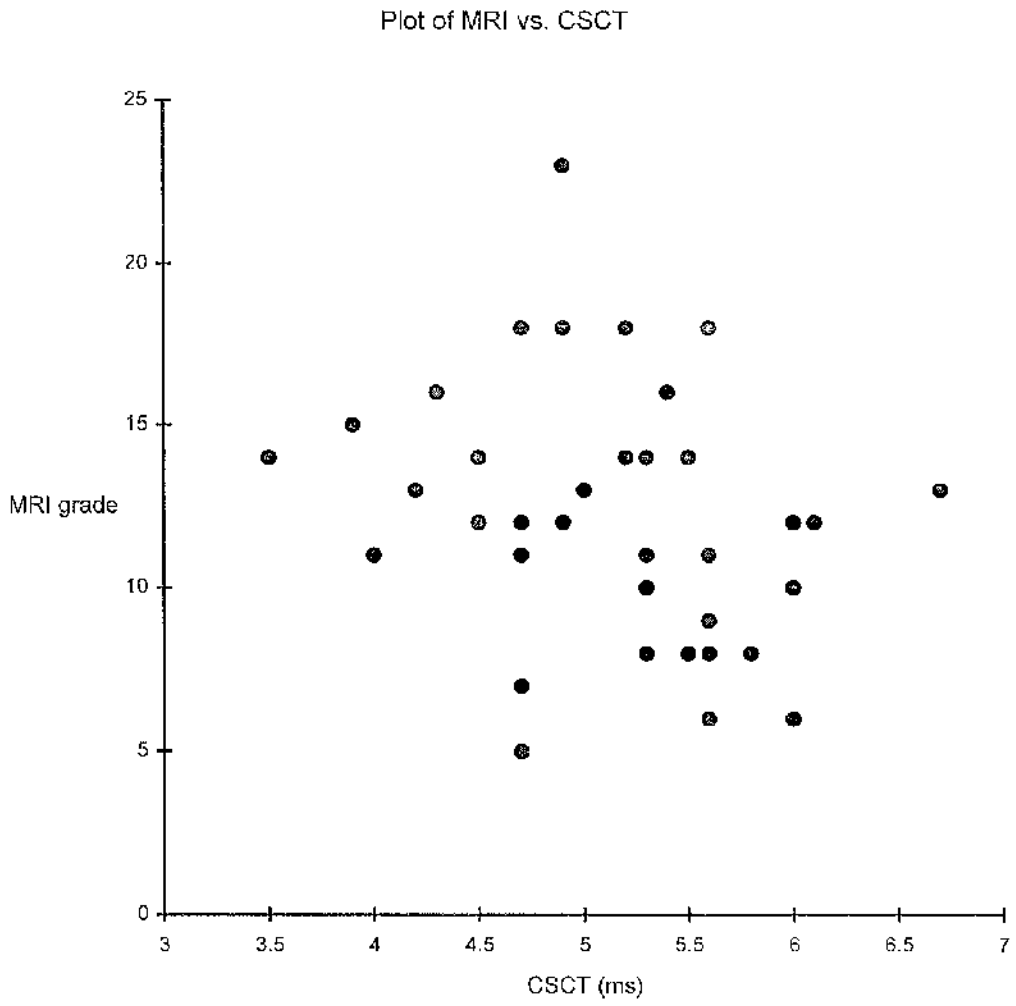
6) The abnormal images maybe reversible when blood phe is reduced by dietary therapy.

APPENDICES

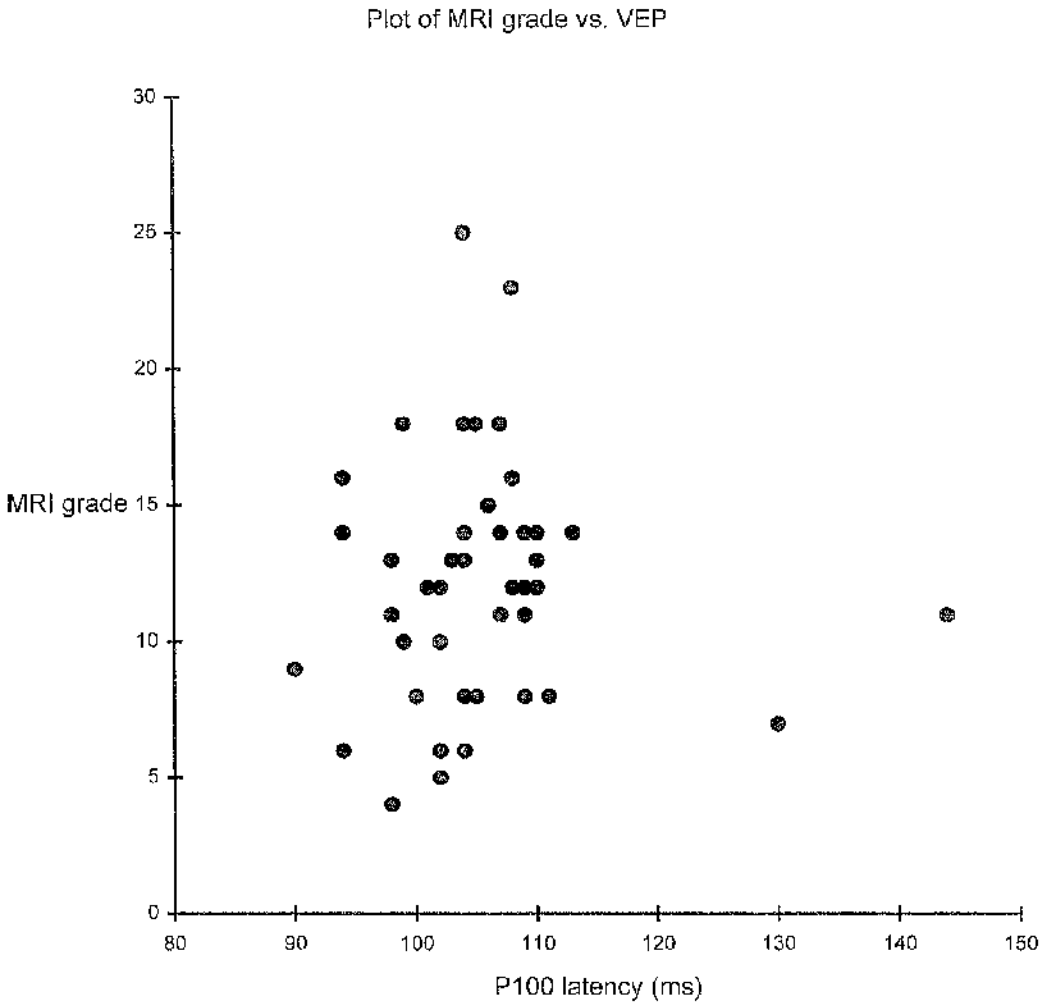
Appendix 1: Plot of MRI grade against central motor conducting time (CMCT)



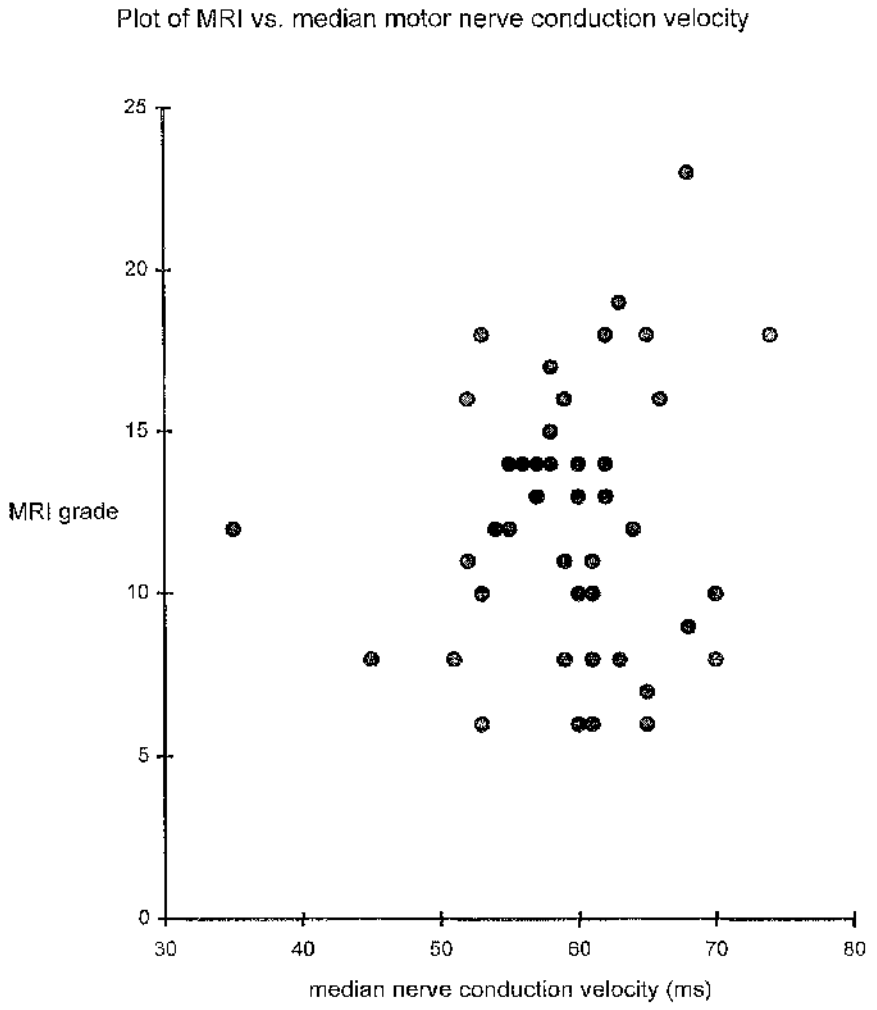
Appendix 2: Plot of MRI grade against central sensory conducting time (CSCT)



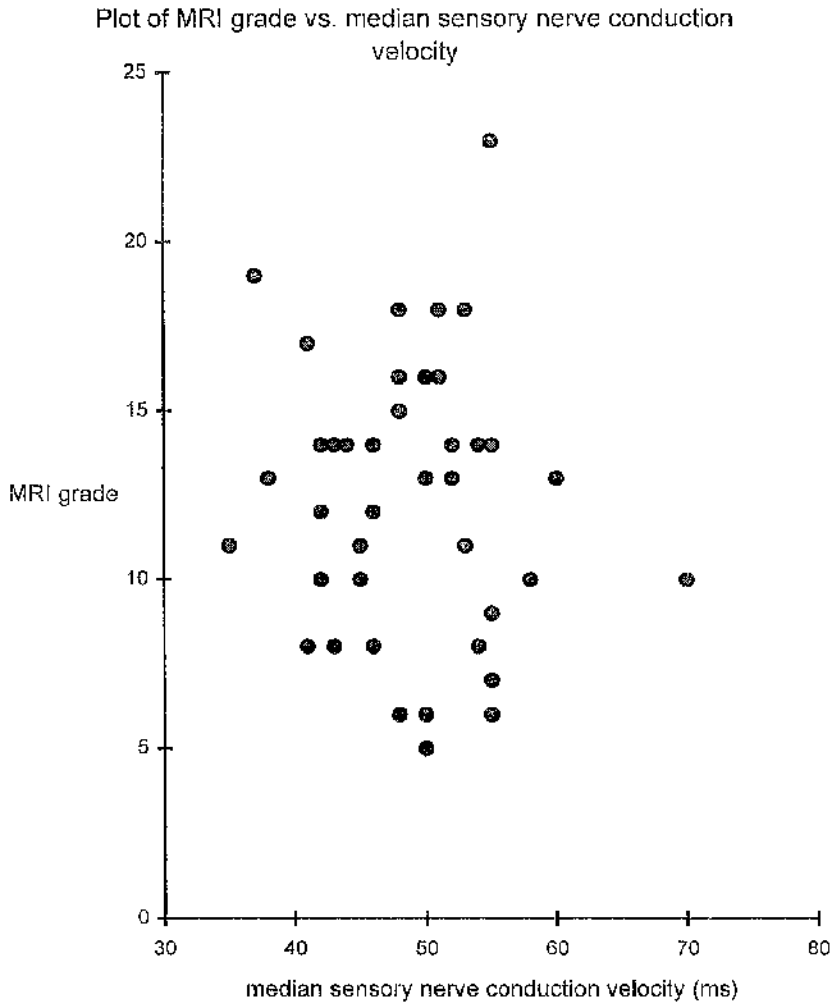
Appendix 3: Plot of MRI grade against visual evoked potential latency (VEP)



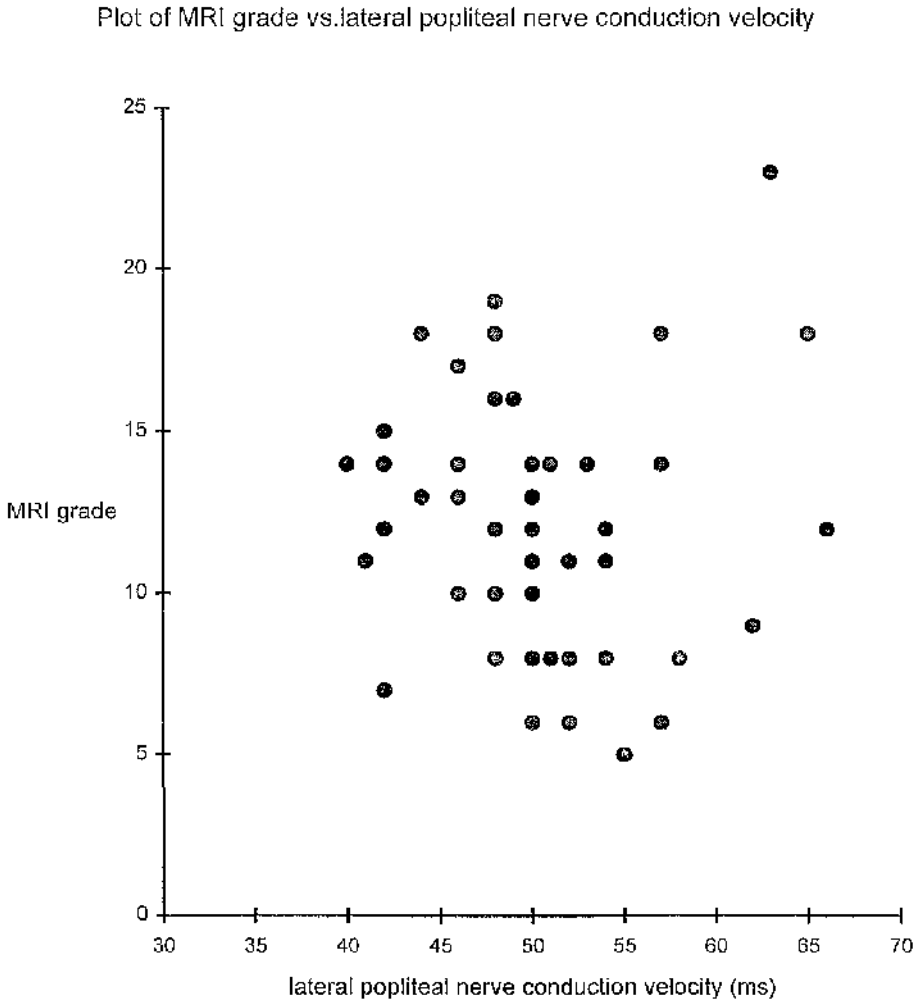
Appendix 4: Plot of MRI grade against median nerve motor conduction velocity



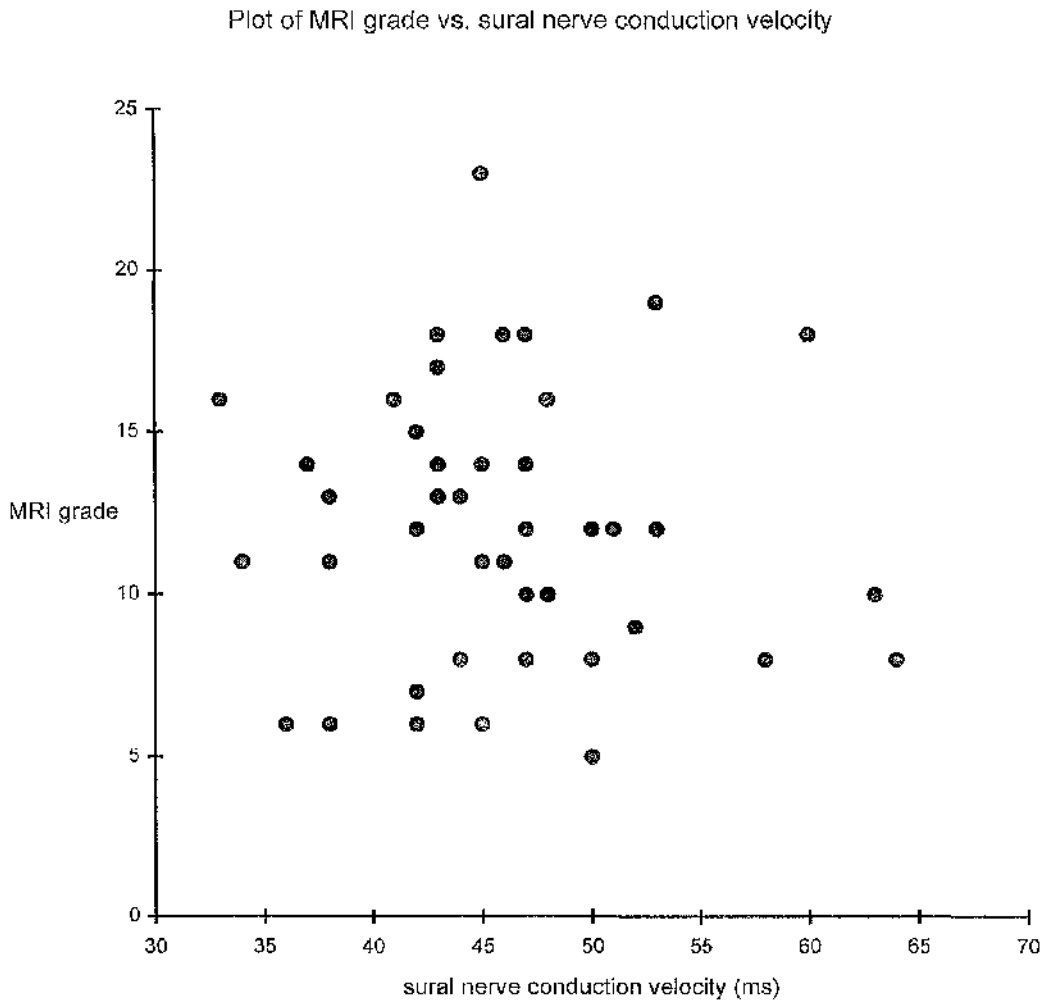
Appendix 5: Plot of MRI grade against median sensory nerve conduction velocity



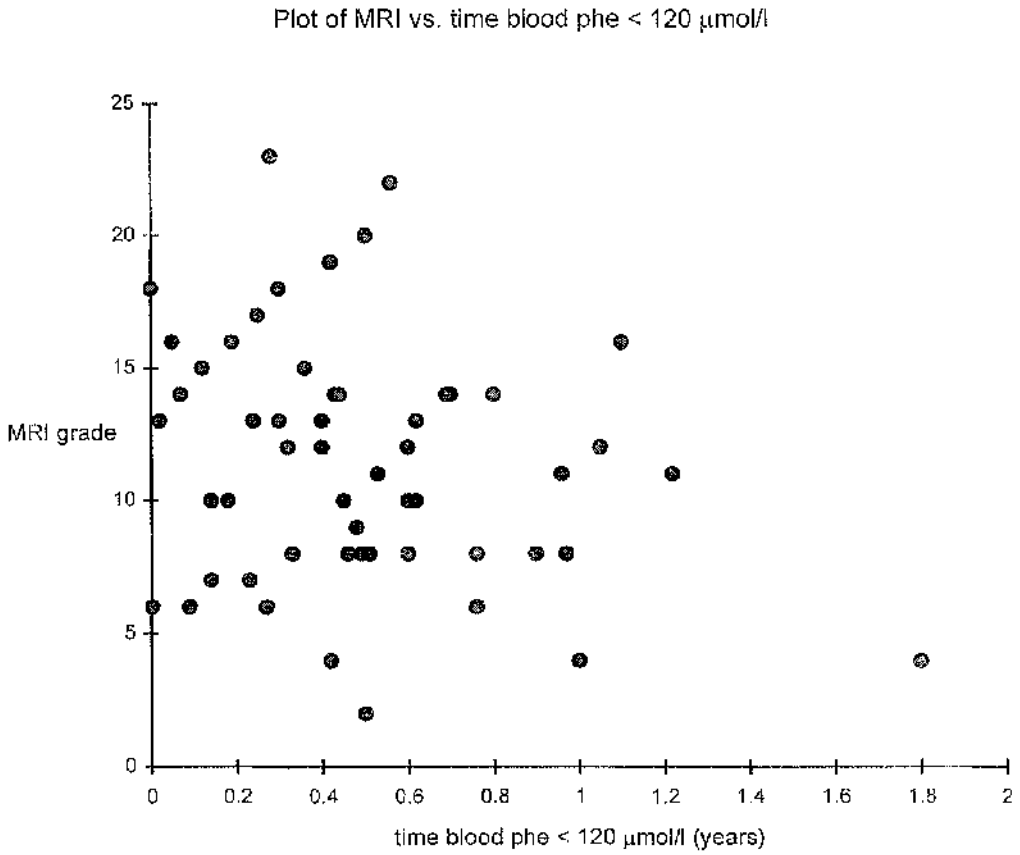
Appendix 6: Plot of MRI grade against lateral popliteal nerve conduction velocity



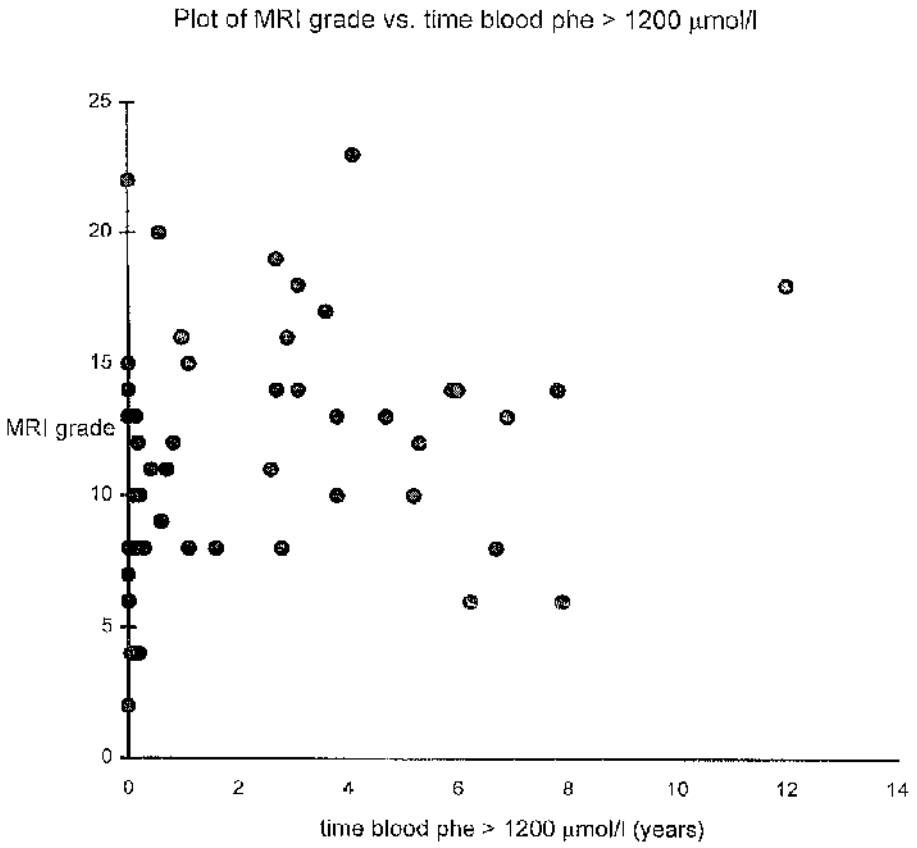
Appendix 7: Plot of MRI grade against sural nerve conduction velocity



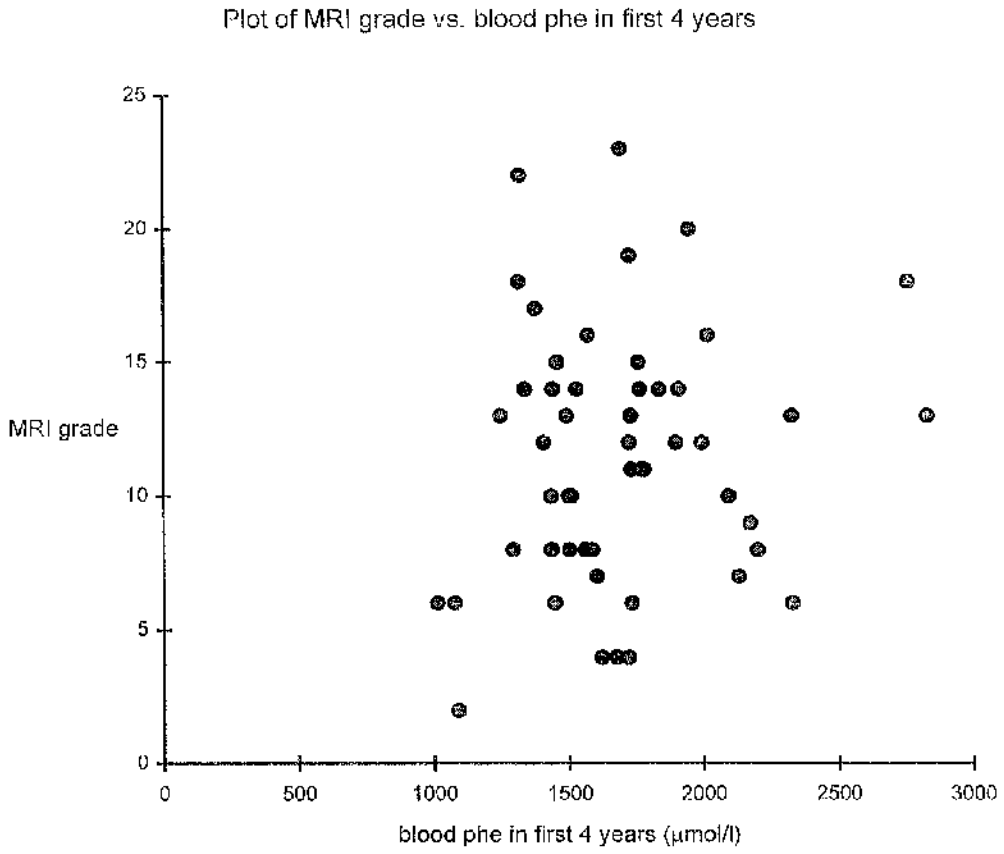
Appendix 8: Plot of MRI grade against time blood phe was less than 120 μmol/l



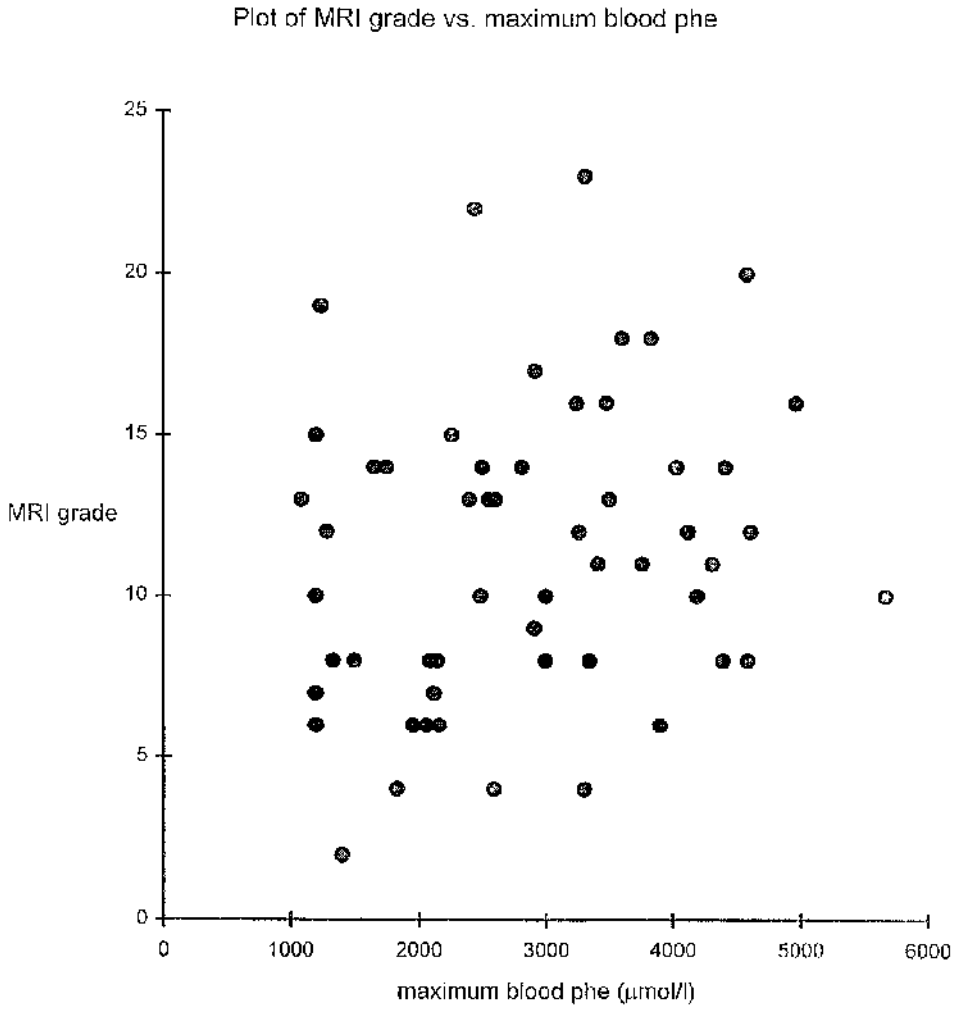
Appendix 9: Plot of MRI grade against time that blood phe greater than 1200 μmol/l



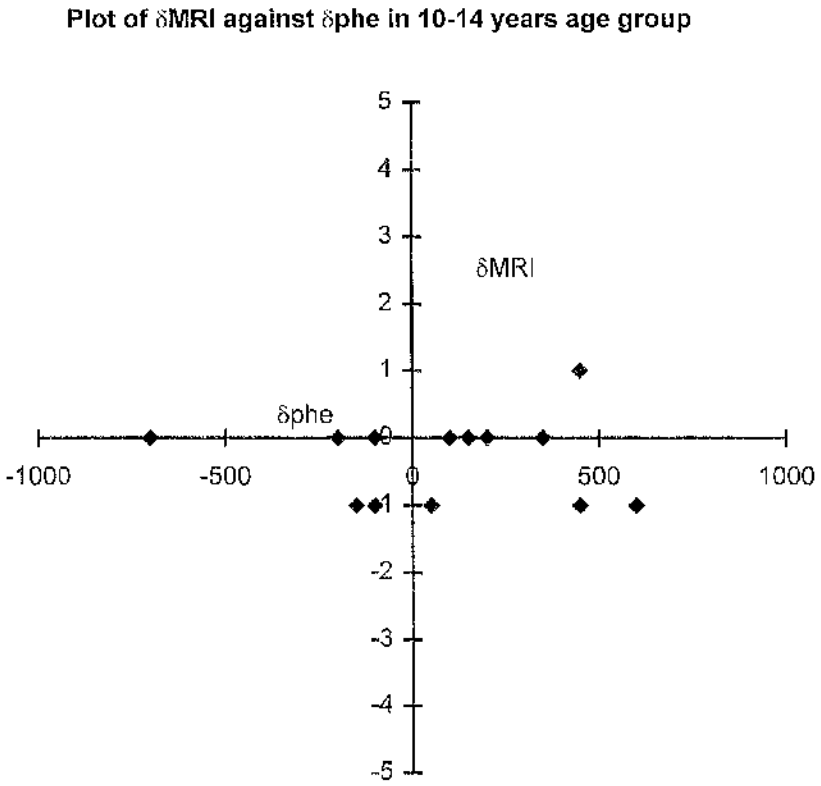
Appendix 10: Plot of MRI grade against average blood phe in the first four years of life



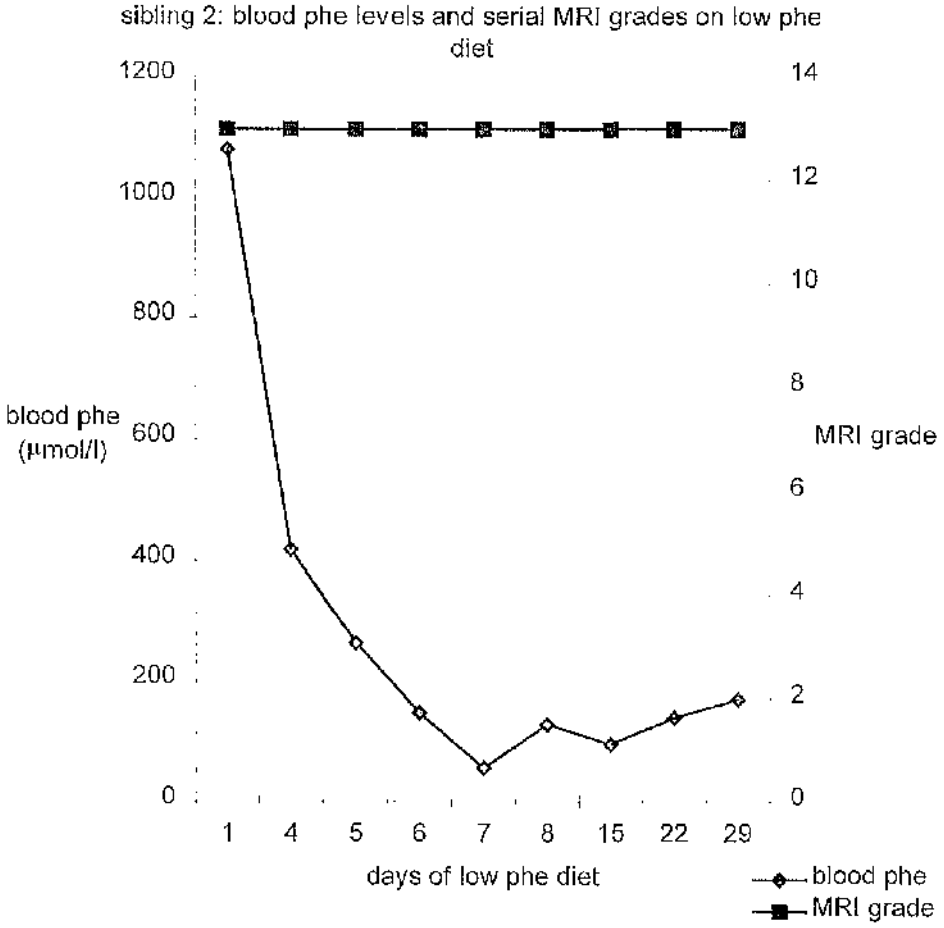
Appendix 11: Plot of MRI grade against maximum blood phe level recorded



Appendix 12: Plot of δMRI against δphe in younger group (aged 10-14 years) of PKU patients



Appendix 13: Plot of serial MRI grades and blood phe levels in sibling 2 on returning to strict low phe diet



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