

**THE EFFECT OF LARGE NEUTRAL AMINO ACIDS ON MATERNAL  
PHENYLKETONURIA OFFSPRING**

A Thesis by

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I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirement for the degree of Master of Science with a major in Biological Sciences

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We have read this thesis and recommend its acceptance:

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**DEDICATION**

**To**

**MY PARENTS**

“Arise, Awake and Stop not till the goal is reached”  
**Swami Vivekananda**

## **ACKNOWLEDGEMENTS**

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## ABSTRACT

Women with untreated phenylketonuria (PKU), tend to give birth to infants with multiple congenital anomalies, as elevated maternal phenylalanine (Phe) level is teratogenic. The best outcomes occur when strict control of maternal Phe levels is achieved before conception and maintained throughout pregnancy. Such diets are not highly palatable and therefore there is often loss of dietary compliance. An alternative to low Phe diet would be a more normal diet that is altered so that the Phe content is less problematic. Hence, there is an interest in the use of large neutral amino acids (LNAA), which compete with Phe for membrane transport sites in the intestines and the placental barrier and thus alleviate the problems associated with PKU. In our research, we used a PKU mouse model to examine the effect of LNAA supplementation on the maternal and fetal blood Phe levels. 3 different doses of LNAA supplementation were given to different animal groups to observe its effect on the blood Phe levels. Results showed that 16.7% and 33.4% LNAA supplementation caused significant decrease in the blood Phe levels over a period of time, but was associated with adverse effects.

## TABLE OF CONTENTS

Chapter	Page
1) Introduction	
a) Phenylketonuria .....	1
b) Maternal PKU.....	6
c) Exploration of alternative methods for the treatment of MPKU .....	9
d) Animal Models.....	10
2) Hypotheses and Aims .....	11
3) Materials and Methods	
a) 5001 diet.....	13
b) LNAA.....	14
c) Reproductive Anatomy and Physiology of a Mouse.....	14
d) Tail Bleeding .....	16
e) Necropsy.....	17
f) Spectrofluorometry.....	18
g) Experimental design.....	20
4) Statistical Analysis.....	22
5) Results.....	24
6) Discussion.....	33
7) Conclusion.....	35
8) References .....	36

**LIST OF TABLES**

Table #	Caption	Page
1	Contents of the 5001 diet .....	13
2	Composition of the LNAA powder.....	14
3	Strategy for LNAA supplementation.....	21
4	Comparison of the maternal blood Phe levels between the control and the experimental group.....	24
5	Percentage reduction in the maternal blood Phe levels during second and third bleed as compared to the 1 <sup>st</sup> bleed.....	25
6	Pre and Post LNAA maternal blood Phe levels – Control group....	26
7	Pre and Post LNAA maternal blood Phe levels – 16.7% group.....	26
8	Pre and Post LNAA maternal blood Phe levels – 20% group.....	26
9	Pre and Post LNAA maternal blood Phe levels - 33.4%.....	26
10	Comparison of mean fetal blood Phe levels between the control and the experimental group.....	27
11	Fetal adverse effects.....	28
12	PreKUnil (LNAA) composition per tablet.....	34



## List of Figures

Figure #	Caption	Page
1	Metabolism of Phenylalanine .....	3
2	Bicornuate uterus of the mouse.....	15
3	Gravid uterus of the mouse.....	17
4	Experimental design.....	20

## LIST OF ABBREVIATIONS

PKU .....	Phenylketonuria
LNAA .....	Large Neutral Amino Acids
Phe .....	Phenylalanine
PAH.....	Phenylalanine hydroxylase
dpc.....	Day post coitum
MPKU.....	Maternal PKU
Avg.....	Average

## **Introduction**

### **Phenylketonuria (PKU)**

**History:** A Norwegian doctor named Asbjorn Folling was the first to describe Phenylketonuria, in the year 1934; he had named the disease ‘imbecillitas phenylpyruvica’ because of the severe mental retardation and phenylpyruvic acid in the urine. Dr Lionel Penrose, a geneticist from England coined the term “Phenylketonuria” for this disease because of the characteristic appearance of a phenylketone (phenylpyruvic acid) in the urine (1). H. Bickel was the first to suggest and implement a Phe restricted diet as a treatment for PKU (2). Woo in 1983 first described the PAH gene. Most often, deficiency of the enzyme PAH is due to a mutation in the gene coding the enzyme (3).

**Incidence:** The incidence of PKU is one in 10,000 live births in USA, with a carrier frequency of approximately 1 to 2 %, commonly seen in Caucasians (4). Internationally, Turkey has the highest incidence in the world with approximately one in 2600 births (4). High incidence is also reported in the Yemenite Jewish population, as well as in regions of northern and eastern Europe, Italy and China (4).

**Etiology:** PKU is a metabolic disorder included in a group of disorders called ‘the inborn errors of metabolism’ by Sir Archibald Garrod (5). It was the first of the inborn errors in which Garrod’s triad of criteria- gene, enzyme and clinical abnormality was convincingly demonstrated (5). Homozygote with this autosomal recessive disorder classically have a severe lack of phenylalanine hydroxylase, leading to hyperphenylalaninemia (presence of high phenylalanine {Phe} levels in the blood) and PKU (4).

**Pathogenesis:** Phe is an essential large neutral amino acid, present in all foods containing protein (21). Normally, less than 50% of the dietary phenylalanine is utilized for protein synthesis and the rest is converted to tyrosine by phenylalanine hydroxylase (PAH) (20, 21). As the enzyme that catalyses the rate limiting step in Phe catabolism, the phenylalanine hydroxylating system is an important determinant in phenylalanine homeostasis in humans (20, 21). Its action initiates a series of events that ultimately results in the complete oxidation of phenylalanine to CO<sub>2</sub> and water as diagrammed in Figure-1 (6). The system consists of at least three essential components, two enzymes (PAH and dihydropterin reductase) and a coenzyme tetrahydrobiopterin (7, 8). The tyrosine that is formed is essential for the synthesis of important neurotransmitters like epinephrine, norepinephrine, dopamine and a pigment called melanin (21).

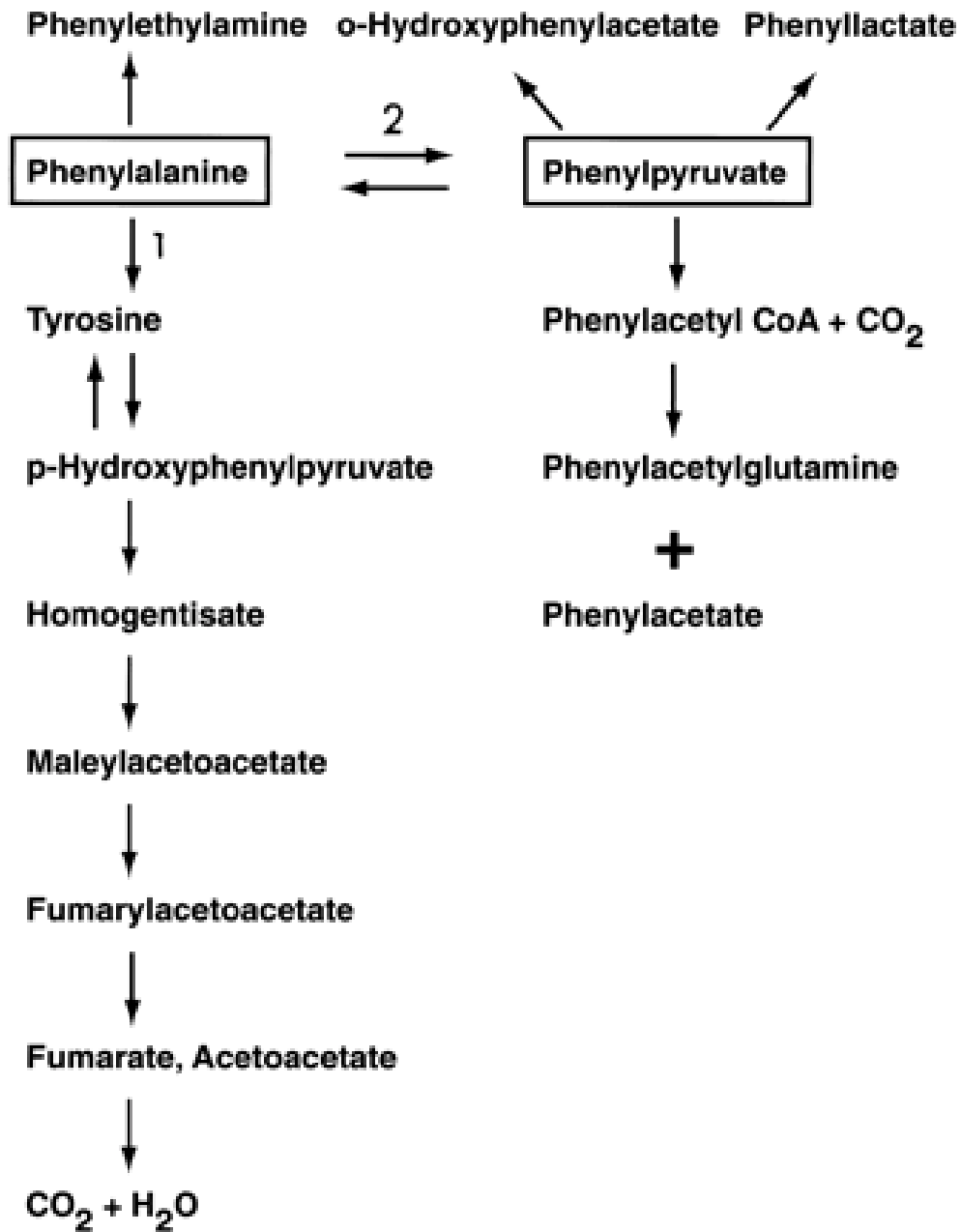


Figure 1: Metabolism of Phenylalanine (21)

**Clinical Manifestations:** The affected individual is normal at birth, but gradually develops mental retardation which may not be evident during the initial few months (9). If left untreated, the mental retardation is usually severe and may require institutional care (10). Untreated older children become hyperactive with purposeless movements, rhythmic rocking and athetosis (10). On physical examination, the affected infants have blonde hair, abnormally fair skin and blue eyes as result of hypopigmentation due to hypotyrosinemia (10). Some of them may suffer from seborrheic or eczematoid skin rash, which is usually mild and may disappear as the child grows older(11-13). They also have an unpleasant odor of phenylacetic acid, which is usually described as musty or a mousy odor (1). Microcephaly, prominent maxilla with wide spaced teeth, enamel hypoplasia and growth retardation are other common findings in untreated children(14, 15). Seizures occur in about one fourth of patients, predominantly in those who are severely retarded (10). The clinical manifestations of classical PKU are not common in those countries in which neonatal screening programs for detection of PKU are in effect and the patient has been initiated on treatment as soon as the disease has been detected (10,16). Clinical manifestations may also be seen in patients who are treated early in life but have discontinued therapy subsequently. Behavioral problems, including restlessness, aggression and sleep disturbances are common in such patients (17).

**Diagnosis:** PKU should be diagnosed at birth. But infants with PKU clinically appear normal at birth and tests of their urine for phenylpyruvic acid are negative for the first few days of life; therefore, diagnosis depends on measuring the blood levels of phenylalanine (10). Until recently, the bacterial inhibition assay, a method developed by

Guthrie (18), was widely used. But now it has been replaced by a spectrofluorometric assay, which is a modified version of McCaman and Robin's method (19).

The criteria for diagnosis of classical PKU are (10):

- 1) A plasma Phe level above 20 mg/dl(1.2mM) (20)
- 2) Increased urinary levels of metabolites of Phe (phenylpyruvic and o-hydroxyphenylacetic acids) and
- 3) A normal concentration of the cofactor tetrahydrobiopterin.

### **Treatment**

The primary goal of therapy is to reduce the blood and brain Phe levels in order to prevent or minimize brain damage (10). This can be achieved by instituting a diet low in Phe; formulas low in this amino acid is now available commercially (10). All clinical manifestations of classic PKU can be completely prevented by restriction of dietary intake of Phe (10). The treatment should be instituted as soon as the disease has been diagnosed (10).

The duration of the therapy is controversial (10). Though the patient need not follow the rigid dietary restriction after the first six years of life, it is advantageous if the patient is on some form of Phe restriction indefinitely (10, 21). The rationale for this long term therapy is because cerebral white matter hypomyelination or demyelination has been demonstrated in patients who were either inadequately treated or in whom the treatment was terminated later in life (10). Patient compliance with this treatment is the most difficult part because of the emotional problems resulting from the restricted diet (10, 21).

## **Maternal PKU**

**History:** Dent was the first to suggest, based on an observation, that the toxicity of a PKU mother's high Phe levels might damage the fetal brain in utero (22). Mabry confirmed the observations and coined the term 'Maternal PKU Syndrome' (23).

Nielsen and Wamberg first reported that the low Phe diet introduced before conception resulted in a successful outcome of pregnancy in a PKU women (24).

**Incidence:** 1/15000 live births (25)

**Etiopathogenesis:** Maternal PKU syndrome is an embryopathy and fetopathy as a result of a maternal genetic defect (25). The term "Maternal PKU" refers to the cause of the damage rather than to the features of the disease (25). The high Phe levels in the maternal blood are teratogenic to the developing fetus (26). The pathogenesis of the maternal PKU is not very well understood, but it is suspected that the hyperphenylalaninemia during embryogenesis has deleterious effects on neural crest migration, thereby causing heart defects (25). It is also suspected that during fetogenesis / organogenesis, Phe affects the multiplication of neurons and myelin synthesis, which persists even after birth (25,27-29).

**Clinical Manifestations:** Microcephaly, low birth weight, congenital heart defects, and mental retardation are some of the most common clinical features seen in this syndrome (25). Others manifestations include facial dysmorphism, corpus callosum agenesis and tracheoesophageal malformations (25, 30).



The clinical manifestation of maternal PKU syndrome is dependent on the maternal plasma concentration of Phe (25, 26). With a plasma concentration of more than 20 mg/dl (normal values are 0.5 – 2.0 mg/dl), the frequency of each feature was the following (26):

Mental Retardation – 92%,

Microcephaly – 72%,

Congenital Heart Disease – 12%,

Birth Weight < 2500g – 40%.

The international maternal PKU collaborative study was started in 1984 to determine fetal outcome with improved control of maternal Phe levels during pregnancy (31). The results of the study showed that the best outcomes occurred when strict control of Phe levels was implemented before conception and maintained throughout pregnancy within the normal range (31). There was not only a decrease in the incidence of microcephaly, but no congenital heart defects were observed in women whose blood Phe levels were within the recommended treatment range of 120 – 360  $\mu$  mol / l before 8 weeks of gestation (31). However, congenital heart defects were observed in patients who did not have blood Phe levels under control during the first trimester of pregnancy (31).

Spontaneous abortion rates were comparable to those observed in normal pregnancies (31). The pregnancy outcomes of well treated PKU women had more offspring with microcephaly and mental retardation than normal pregnancies (31).

**Diagnosis:** It is usually based on the mother's medical history, that is, whether she is heterozygous / homozygous mutant for the disease (25). But if the maternal history is not known, it can be detected during the prenatal period by ultra sonography, which shows a

fetus with growth retardation, microcephaly, and congenital heart disease (25). The diagnosis can be confirmed by measuring the maternal blood Phe levels (25).

**Treatment:** The best treatment is a Phe restricted diet started before conception (26, 44 - 47, 51). But the main drawback is poor patient compliance, because not only are commercially available formulations expensive, they are also unpalatable (42, 43, 51). In addition, these women need constant monitoring to ensure proper dietary intake. The fact that PKU women are cognitively and hence economically and socially backward makes it difficult to adhere to the commercially available modified diet (42, 43, 51). The only way one can achieve good patient compliance and hence a reduction in the MPKU is by formulating a drug that can be consumed with normal food, such that the Phe content is less problematic (48 – 51). Our study was designed to test the hypothesis that LNAA can reduce the blood Phe levels significantly when consumed in a normal diet

## **Exploration of alternative methods for the treatment of MPKU**

### **Large Neutral Amino Acids (LNAA)**

Pardridge conducted an experiment based on Oldendorf's work (32), which stated that there are transporters for LNAA at the blood brain barrier and that the LNAA compete with each other to get across this barrier. Pardridge's study showed that there is competitive inhibition of Phe when the dose of other LNAA is increased and that Phe has the lowest Michaelis Menton constant ( $K_m$ ) (33). Studies conducted by Christenson (et al 1990), showed similar amino acid transporters at the basolateral membrane of the intestinal epithelial cells and placental barrier (34). It is also known that the concentration gradient of the blood Phe levels across the placental barrier is more than twice the concentration in the PKU mother's blood. There have been many clinical trials conducted with partial LNAAs, but without success. The supplementation that we used in this study consisted of all large neutral amino acids and other amino acids like histidine, arginine, and threonine (Table 2). The LNAA supplementation was given in three dosages, 16.7% LNAA, 20% LNAA and 33.4% LNAA (Table 3). The dosages were formulated based on two factors

- The study conducted by Hidalgo et al , (1990) on Caco cells, showed that  $10\mu\text{M}$  of Phe in buffer applied to cells in the presence of  $1\text{mM}$  of LNAA inhibited the Phe transport across the intestinal barrier completely (35).
- The concentration of Phe in the rodent diet.

## Animal Models

The animal model used in this experiment was the BTBR *Pah* ENU2 mouse strain. This is a useful genetic animal model that has a mutation for the PAH enzyme and exhibits some important clinical manifestations very similar to that seen in human PKU patients. The mutation was induced using the chemical mutagen N-ethyl-N-Nitrosourea (ENU) in the mouse germline (36). The treatment with the ENU mutagen resulted in the development of two different mutant alleles, *Pah enu1* and *Pah enu2*. The *Pah enu1* mutation is associated with a marked reduction in PAH protein but still retains residual activity sufficient to ameliorate the PKU phenotype. The *Pah enu2* mutant exhibited more pronounced PKU phenotypes than the original, similar to human classical PKU. These animals displayed phenylalanine dependent hypopigmentation, reduction in head circumferences and showed behavioral abnormalities, which includes relatively uncoordinated behavior and decreased vigilance compared to their heterozygous siblings. In addition, the PAH *enu2* mutant females exhibited severe maternal effects in which the fetuses had features that indicate damage during prenatal development. All these characteristics were very similar to those seen in the human PKU. This mutation has been localized to the exon 7 of chromosome 10, a region that encodes the active site for PAH enzyme, and this is also the most frequent site for PKU mutations in humans(37).

## Hypotheses and Aims

### Hypotheses:

Our two hypotheses are that:

- a) Supplementation of LNAA in the diet will competitively inhibit the dietary Phe uptake at the intestinal epithelium, resulting in decreased maternal blood Phe levels, and
- b) The LNAA will also inhibit the Phe transport from the maternal to fetal blood, thereby decreasing the fetal blood Phe levels.

### Null Hypotheses ( $H_0$ )

- Maternal blood Phe levels prior to LNAA exposure in the experimental group will not be different from maternal blood Phe levels after exposure to LNAA supplementation in the experimental group
- Blood Phe levels in the animals supplemented with LNAA will not be different from blood Phe levels of animals in control group.
- Blood Phe levels in the fetuses born to the animals supplemented with the LNAA will not be different from blood Phe levels in the fetuses born to control group.

### Alternative Hypotheses

- Maternal blood Phe levels after exposure to LNAA supplemented diet in the experimental group will be less than maternal blood Phe levels prior to LNAA supplemented in the experimental group.
- Blood Phe levels in the LNAA supplemented animals will be less than blood Phe levels in the control animals.

- Blood Phe levels in the fetuses born to females on LNAA supplemented diet will be less than blood Phe levels in the fetuses born to females in the control group.

### **Aims**

Given the above hypotheses, our aims are:

- 1) To determine the maternal pre-LNAA blood Phe levels and post-LNAA blood Phe levels of the animals in the experimental group.
- 2) To determine the blood Phe levels in the fetuses born to the LNAA supplemented animals and fetuses born to the control animals and compare the results of both groups.
- 3) To determine the blood Phe levels in the LNAA supplemented animals and control animals and compare the results of both animal groups.

## Materials and Methods

**5001 diet:** This is the standard rodent diet for biomedical research. The 5001 diet has a constant nutrition formula recommended for laboratory mice and is designed to minimize the nutritional variations in long term studies. It is available in pelleted form and an adult mouse will consume approximately 5 to 6 grams of the pellets per day. The pellets were ground to powdered form before feeding the animals. The contents in the pellet are as shown in the Table 1 below.

**Table – 1: Contents of 5001 Diet**

<b>Protein</b>	<b>23.4%</b>
Arginine	1.38%
Cystine	0.32%
Glycine	1.2%
Histidine	0.55%
Isoleucine	1.18%
Leucine	1.7%
Lysine	1.42%
Methionine	0.43%
Phenylalanine	1.03%
Tyrosine	0.68%
Threonine	0.91%
Tryptophan	0.29%
Valine	1.21%
Serine	1.21%
Aspartic acid	2.83%
Glutamic Acid	4.54%
Alanine	1.44%
Proline	1.55%
Taurine	0.02%
<b>Fat</b>	<b>10%</b>
<b>Carbohydrates</b>	<b>55.2%</b>
<b>Others (Vitamins, Minerals etc)</b>	<b>11.4%</b>

**LNAA Powder:** The LNAA powder was obtained from the lab of Dr Reuben Matalon, UTMB, Galveston, Texas. The contents of the powder are shown in the Table 2.

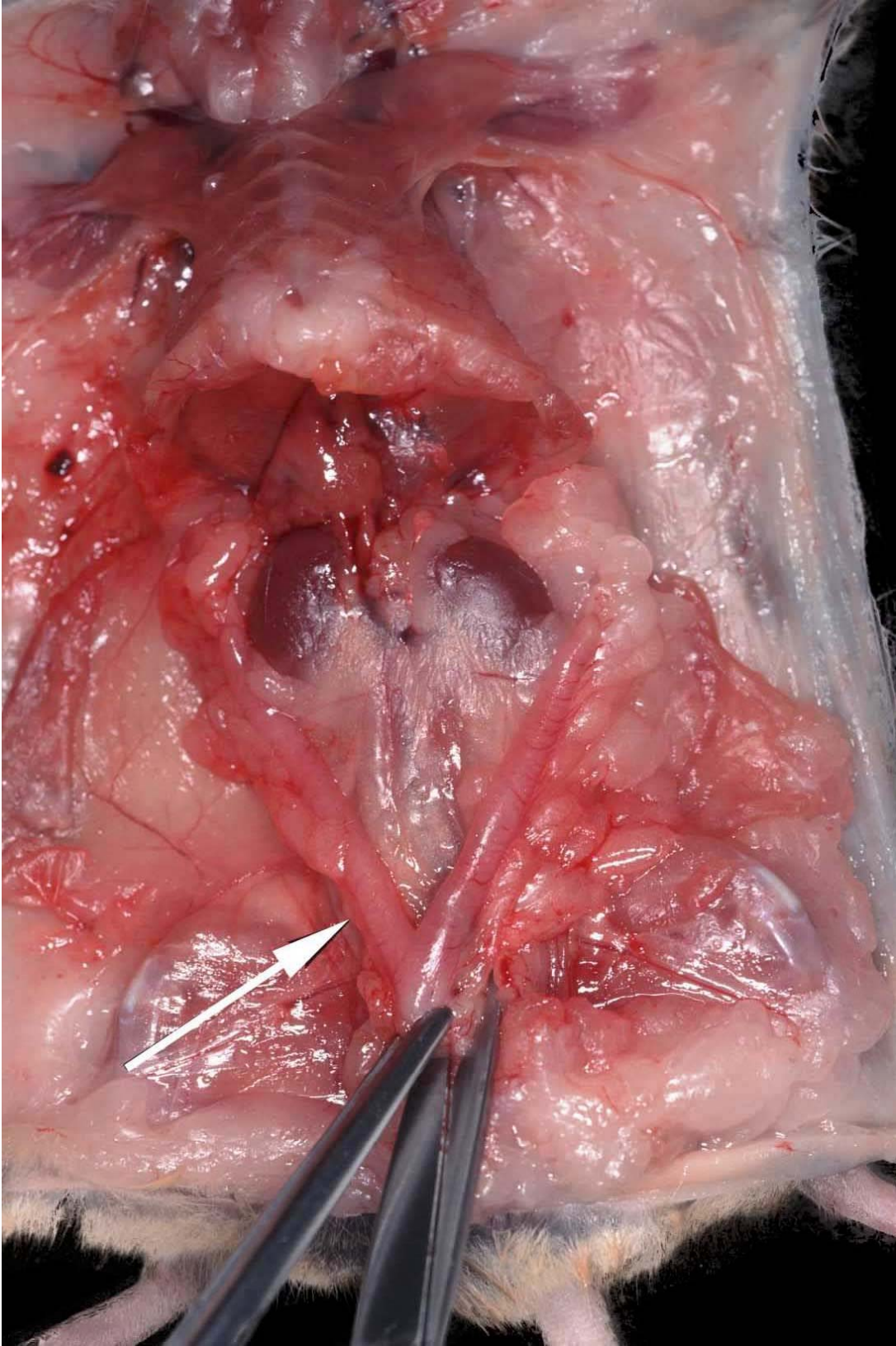
**Table 2:** Composition of the LNAA powder

Amino Acid	Mg
L -Tyrosine	162.4
L - Tryptophan	42.46
L - Methionine	26.64
L - Isoleucine	24.19
L - Leucine	108.25
L - Threonine	26.64
L - Histidine	25.01
L - Lysine	25.01
L - Arginine	25.01
L - Valine	29.19

**Schleicher and Schuell BioScience 903 Specimen Collection Paper:** was used to smear blood and stored for later use. It has been the standard for neonatal screening sample collection for over 40 years. It is also widely used for specimen collection for a range of non-neonatal applications, including long-term sample archiving, epidemiological studies, forensic analysis, HLA typing, at-home patient monitoring and paternity testing.

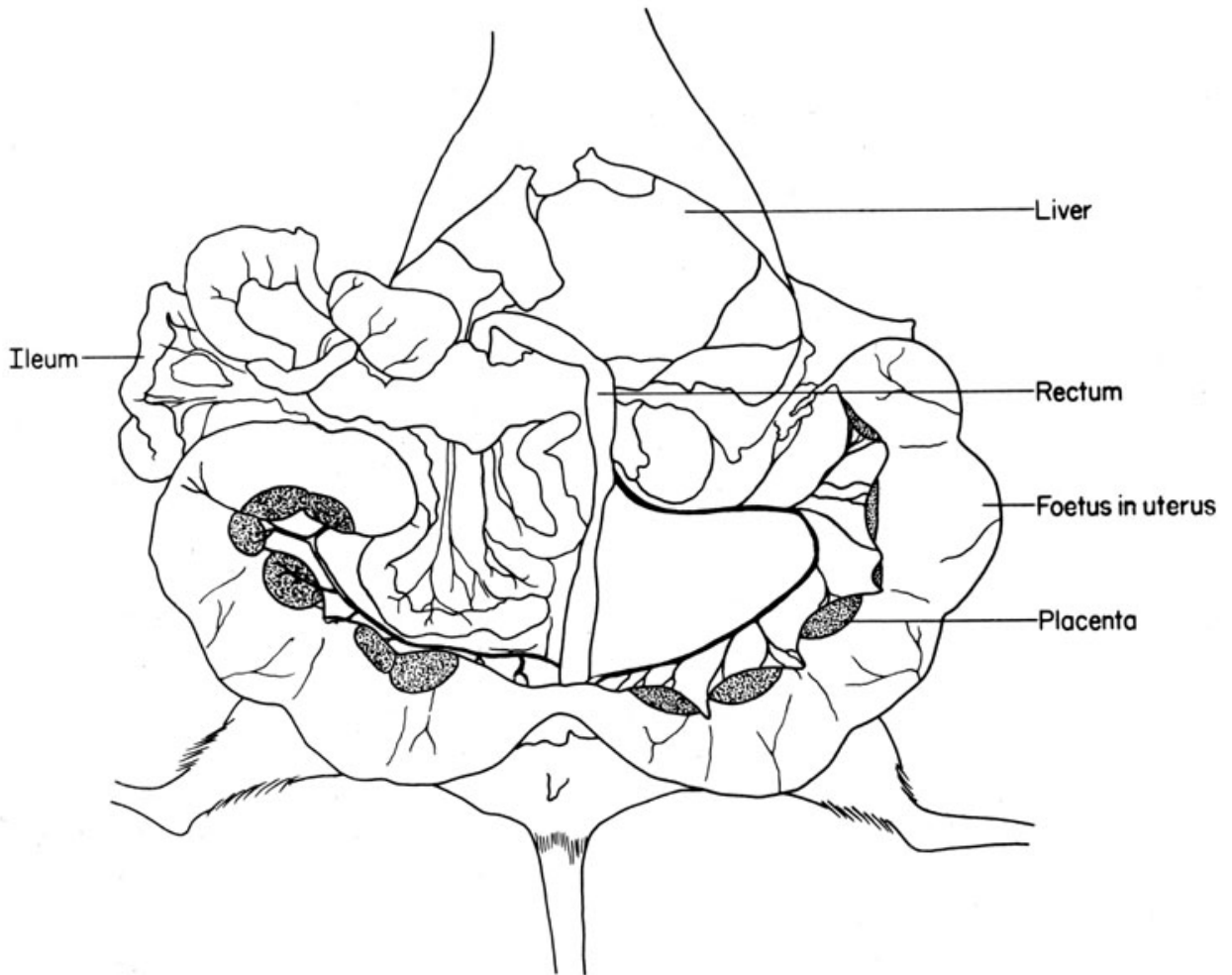
**Anatomy and Physiology of the mouse:** The scientific name of the mouse strain used in our study is *Mus Musculus*. It has a bicornuate uterus, with left and right horns (Figure1). Each horn has two ends, the ovarian end and the cervical end. The horns join at their cervical end to form the cervix. They have an estrus cycle every 4-5 days. Gestation period is about 19 – 21 days. On the day of mating the male mouse leaves behind a waxy substance in the female vagina which is usually referred to as the “post - coital plug” or “plug”. The day of plug detection is considered as 0.5 day post coitum (dpc).





**Figure 2:** Bicornuate uterus of the mouse

**Tail Bleeding:** All animal manipulations were performed following approval by the Wichita State University IACUC. The blood Phe levels of both the control and experimental animals were assessed by collecting blood by tail bleeding. Tail bleeding was performed under aseptic precautions. The mouse was removed from the cage by gently holding the tail at the tip and was placed on the wire bar cage lid to permit the animal to grip the bars. Care was taken not to excite the animal very much. About 2mm of the tail tip was snipped off with disinfected scissors. Starting at the base of the tail, blood was gently milked with fingers. About two to three drops of blood were absorbed onto the filter paper. To stop the bleeding we applied styptic powder to the tail and provided direct pressure to the bleeding part for about 30 seconds and / or until the bleeding stopped.



**Figure 3:** Gravid bicornuate uterus of the mouse.

**Necropsy** was performed on 18.5 dpc (day post coitum) or a day prior to normal parturition. The pregnant female was euthanized by carbon-dioxide asphyxiation. The animal was exposed to carbon-dioxide for about five minutes and checked for the presence of life by observing for any body movements; if absent the animal was prepared for dissection. However, if the animal showed any movements then it was exposed to carbon-dioxide for another five minutes. This procedure was continued until the animal showed no signs of life. On the dissection table, the animal was laid on its dorsal surface exposing the abdominal or ventral surface. The abdominal surface was disinfected by scrubbing with ethanol. The abdominal skin was picked up with toothed (V- Groove)

forceps and a small nick made with scissors at the junction of the upper two thirds and lower one third of the abdomen. The nick was extended horizontally on both sides of the abdomen. The skin was then pulled away to expose the peritoneum. The peritoneum was treated in a similar way as the skin and the abdominal contents were exposed. The intestines were moved to one side to expose the uterine horns on both sides of the abdomen. Extraction of the fetus was done from the left horn and always began at the ovarian end. An incision was made in the uterine wall and the incision extended to extract the fetuses. The umbilical cord was cut to free the fetus. Fetal blood was collected in a heparinised hematocrit tube by making an incision in the neck of the fetus and cutting through the carotid artery. The blood was smeared onto the filter paper, which was then air-dried and stored at 4°C one to two weeks prior to spectrofluorometric determination of Phe content.

**Spectrofluorometry:** The procedure used here is a modified version of the fluorometric method described by McCaman and Robins in 1962 (19). The assay is based on the enhancement of the fluorescence of a phenylalanine –ninhydrin reaction product by dipeptide, L-leucyl-L-alanine. A succinate buffer is used to optimize the fluorescence and increase the specificity. The copper reagent was used to further enhance the reaction and reduce background. This method measures phenylalanine quantitatively in the presence of other amino acids.

## Assay procedure

### 1) Preparation of reagents

-PKU reagent: 6.5ml of PKU reconstitution buffer added to one vial of PKU reagent, mix until completely dissolved.

-Extraction solution: Add two thirds of the total of Zinc Sulfate reagent to one third of the total of denatured alcohol.

2) Placed one 3mm disk from a dried blood spot into each labeled microtube. Only one disk was added per well.

3) 15  $\mu$ l of extraction solution was added to each well containing a filter paper disk. Gently tapped the tube to ensure that all the disks are well immersed in the extraction solution.

4) The samples with extraction solution were incubated for 30 minutes at room temperature.

5) 40  $\mu$ l of distilled water was added to each tube containing the blood spot and the contents of the tube pipetted up and down to ensure mixing of the solutions.

6) 25  $\mu$ l of the contents of each tube was transferred to corresponding labeled 1.5ml microtube.

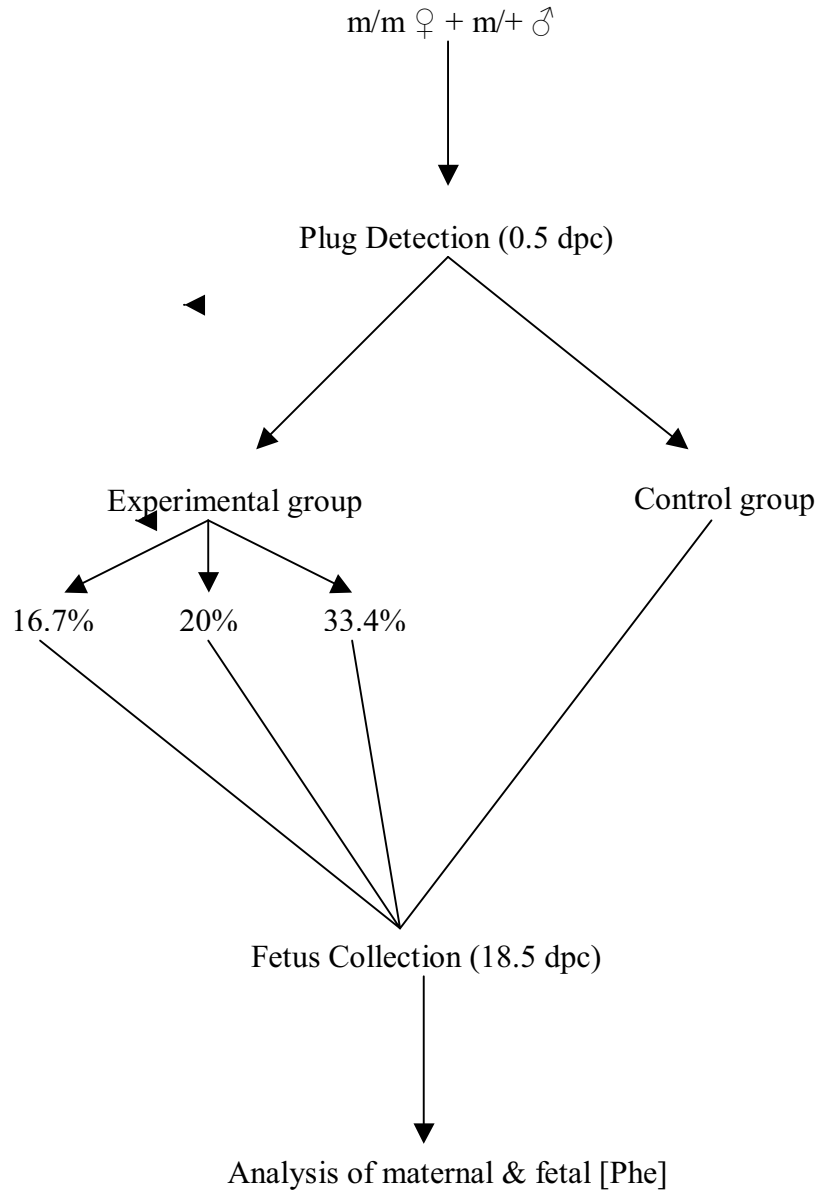
7) 50  $\mu$ l of the reconstituted PKU reagent was added to each tube and the contents mixed by pipetting it up and down three times.

8) The tubes were incubated in a prewarmed incubator at 60° C for 30 minutes.

9) 1.5 ml of diluted copper reagent (0.2ml Copper solution + 1.3ml distilled water) was added to each tube and the tubes incubated again for 45 minutes at room temperature.

10) Quantitative analysis of the unknown blood samples was done by creating a standard curve with controls (blood samples with known [Phe]) provided with the neonatal Phe assay kit.

### Experimental design



**Flowchart 4: Experimental design for the LNAA supplementation study**  
**m/m = homozygous mutant**  
**m/+ = heterozygous mutant**

Production crosses were set up by pairing homozygous mutant females with heterozygous mutant males. Animals were checked everyday for a post-coital plug. On the day of plug detection or mating, the pairs were randomly assigned to the control group and experimental groups. The animals in the control group were fed on the powdered 5001 diet, 6g/mouse/day. The animals in the experimental group were further assigned to different supplemental doses. The following dosage levels (Table 3) were tried based on the theory explained above.

**Table 3: Strategy for LNAA supplementation**

Animal group	LNAA (g)	5001 diet (g)	Total food intake
0 % (Control)	0	6g	6g
16.7 %	1g	5g	6g
20 %	1.2g	4.8g	6g
33.4%	2g	4g	6g

For blood Phe analysis, maternal blood was collected from the animal by tail bleeding (except the last bleed) on three days:

- 1) On the day of plug detection (0.5 dpc).
- 2) On the 10.5<sup>th</sup> day of pregnancy.
- 3) On the 18.5<sup>th</sup> day of pregnancy from the descending (abdominal aorta).

The fetal blood was collected after termination of pregnancy from the carotid arteries.

The collected blood was smeared onto a filter paper and stored for later use.

The blood Phe analysis was done by spectrofluorometry as described above.

## **Statistical Analysis**

ANCOVA (Analysis of covariance) and ANOVA (Analysis of variance) of statistical analysis were used to analyze the data collected. Three levels of analysis were Necessary, including:

- 1) Maternal blood Phe analysis among different supplemental groups.
- 2) Maternal blood Phe analysis within the supplemental (16.7%, 20% and 33.4%) groups.
- 3) Fetal blood Phe analysis.

In order to analyze maternal blood Phe levels between different supplemental groups, we used ANCOVA (Analysis of Covariance). Many factors (inherent as well as external), like the age of the animal, the varying blood Phe levels in each animal, and assay-to-assay variation contributed to variability in the study. These could mask the effect of LNAA on blood Phe levels. To minimize the effect of such variability, we used the ANCOVA method of statistical analysis.

The analysis of covariance is useful in a pretest – posttest randomized experimental design. The pretest score / measure, also known as the covariate, is the measure of a variable tested before program intervention and the posttest score is the measure of the same variable after program intervention. ANCOVA design is one of the “noise reduction” experimental designs, in which information about pretest scores are used to remove the noise / variability in the study. In other words, it is used to control for initial differences in the pretest scores. This is done by ‘co-varying’ the pretest scores (hence the name covariate) with posttest scores to adjust the mean of the pretest scores in such a way that it is equivalent/constant in all groups. In a study like ours where animal –



animal variability is quite common, we always wonder if the pretest scores held constant, and would there be a significant difference in the posttest scores? This concern was addressed by using ANCOVA. It removes 'selection biases or 'covariate biases.

ANOVA (analysis of variance) was used to compare

- 1) the pre and post LNAA effect within an experimental group and
- 2) fetal blood Phe levels in the control group with the fetal blood Phe levels in the experimental groups

An ANOVA sometimes called an F test is closely related to t – test, but differs by the fact that t-test can measure the difference between only two groups, ANOVA can test the differences between the means of two or more groups. The advantage of using ANOVA instead of multiple t – tests is that it reduces probability of a type – 1 error. Multiple comparisons can increase the likelihood of finding something by chance, which is nothing but a type 1 error. A disadvantage with ANOVA is that the F test can tell us whether there is a significant difference between groups, but not which groups are significantly different from each other. In order to measure which groups are particularly different from each other, I used a post-hoc comparison of means. Post hoc comparison compares the highest mean with the lowest mean, specifically taking into account the fact that there are more than two samples. Commonly used post-hoc tests are Scheffe's and Tukey's.

## RESULTS

### A) Maternal Blood Phe Levels

#### 1) Control group vs. Experimental group

**Table – 4: Comparison of the maternal blood Phe levels between the control and experimental groups**

Dependent Variable	Control			16.7%			20%			33.4%		
	M	SE	N	M	SE	N	M	SE	N	M	SE	N
Phe T2 <sup>b</sup>	32.6 <sup>a</sup>	3.0	4	26.9 <sup>a</sup>	3.0	4	29.4 <sup>a</sup>	1.5	15	29 <sup>a</sup>	2.1	8
Phe T3 <sup>c</sup>	29.4 <sup>a</sup>	3.2	4	19.2 <sup>a</sup>	3.3	4	23.2 <sup>a</sup>	1.7	15	14.1 <sup>a</sup>	2.1	8

a. Covariates appearing in the model were evaluated at the following values: Phe level of mother at 1<sup>st</sup> (T1) bleed = 35.83mg/dl

b and c : Phe level at second and third bleed respectively.

d = measure of dependent variable = mg/dl

M = Mean, SE = Standard Error, N = number of animals

Comparison between the maternal blood Phe levels of control with that of experimental group was done using ANCOVA method of statistical analysis. As mentioned earlier, the animals had been bled at three different times,

- 1) 1<sup>st</sup> bleed taken at 0.5 dpc was designated as Phe T1
- 2) 2<sup>nd</sup> bleed taken at 10.5 dpc was designated as Phe T2
- 3) 3<sup>rd</sup> bleed taken at 18.5 dpc was designated as Phe T3

Phe T1 values from all groups (control as well as experimental groups) were taken as the pretest score or covariate, and the covariate mean was adjusted by covarying it with the posttest scores of different groups. The Phe T1 value calculated was 35.83mg/dl.

Statistical analysis showed a significant decrease with 16.7% and 33.4%

supplementation. The decrease was seen at the time of 3<sup>rd</sup> bleed.

**Table – 5: Percentage reduction in the maternal blood Phe levels during the 2<sup>nd</sup> and 3<sup>rd</sup> bleed as compared to the 1<sup>st</sup> bleed (pretest score = 35.83mg/dl).**

Dependent variable	Control	16.7%	20%	33.4%
Phe T2 <sup>b</sup>	9%	15%	18%	19%
Phe T3 <sup>c</sup>	18%	40%	35%	61%

b and c : Phe level at second and third bleed respectively.

The percentage reduction in maternal blood Phe after LNAA supplementation seems to have a direct relationship with the dosage, in other words, we see greater percentage reduction at higher LNAA supplementation exception being 20% LNAA supplementation.

## 2) Pre LNAA vs. Post LNAA maternal blood Phe levels

**Table – 6: Control group**

Timeline	M (mg/dl)	SE	N	p value* when compared with Phe level at 1 <sup>st</sup> bleed
0.5 dpc / 1 <sup>st</sup> bleed	28.4	2.7	8	
10.5 dpc / 2 <sup>nd</sup> bleed	28.9	4.3	4	p > 0.05
18.5 dpc / 3 <sup>rd</sup> bleed	25.5	3.3	7	p > 0.05

**Table – 7: 16.7% LNAA group**

Timeline	M (mg/dl)	SE	N	p value* when compared with Phe level at 1 <sup>st</sup> bleed
0.5 dpc / 1 <sup>st</sup> bleed	26.2	1.0	7	
10.5 dpc / 2 <sup>nd</sup> bleed	22.2	2.2	4	p > 0.05
18.5 dpc / 3 <sup>rd</sup> bleed	16.9	1.9	7	p < 0.05

**Table – 8: 20% LNAA group**

Timeline	M (mg/dl)	SE	N	p value* when compared with Phe level at 1 <sup>st</sup> bleed
0.5 dpc / 1 <sup>st</sup> bleed	37.4	3.9	15	
10.5 dpc / 2 <sup>nd</sup> bleed	30.2	2.8	15	p > 0.05
18.5 dpc / 3 <sup>rd</sup> bleed	24.0	2.9	15	p < 0.05

**Table – 9: 33.4% LNAA group**

Timeline	M (mg/dl)	SE	N	p value* when compared with Phe level at 1 <sup>st</sup> bleed
0.5 dpc / 1 <sup>st</sup> bleed	40.7	5.6	8	
10.5 dpc / 2 <sup>nd</sup> bleed	31.7	2.8	8	p > 0.05
18.5 dpc / 3 <sup>rd</sup> bleed	15.9	1.7	9	p < 0.05

\*p value < 0.05 is statistically significant

ANOVA method of statistical analyses was used to compare Pre and Post LNAA exposure blood Phe levels. All the animals in the experimental group (16.7%, 20% and 33.4%) show a significant difference at the time of third bleed when compared to blood Phe levels prior to exposure (i.e. 1<sup>st</sup> bleed).

## B) Fetal blood Phe Levels

**Table 10: Comparison of mean fetal blood Phe levels between the control and the experimental groups.**

LNAA supplementation Level	Fetal blood Phe levels		
	M (mg/dl)	SE	N
0% (Control group)	49.0	1.7	76
16.7%	34.3	1.0	55
20%	40.4	1.6	106
33.4%	25.3	1.2	73

The blood Phe levels collected from fetuses at the termination of pregnancy were compared using ANOVA method of statistical analysis followed by Post hoc comparisons using Scheffe's and Tukey's. Fetal blood Phe levels in the experimental groups i.e. 16.7%, 20% and 33.4% showed a significant decrease ( $p < 0.05$ ) when compared to the control group.

**C) Adverse effects observed in the control and LNAA supplementation groups**

In addition to seeing some beneficial effects with LNAA supplementation, we also saw few adverse effects.

**Table – 11: Fetal adverse effects**

LNAA supplementation level	Adverse Effect Frequency
0%	# ED = 1
	# FD = 3
	# Preg/# Fetuses = 11/80
16.7%	# ED = 1
	# FD = 3
	# Preg/# Fetuses = 8/60
20%	# ED = 3
	# FD = 3
	# Preg/# Fetuses = 15/120
33.4%	# ED = 8
	# FD = 1
	# Preg/# Fetuses = 9/83*

ED, Embryonic death

FD, Fetal death

\*2 terms fetuses noted with anencephaly

All the groups exhibited atleast one adverse effect either in the form of embryonic deaths or in the form of fetal death or both. Embryonic deaths were more commonly seen in the experimental groups than in the control group. Fetal deaths were more or less similar in both the experimental as well as the control group. However, severe birth defect in the form anencephaly was observed in the 33.4% LNAA group, which is an important finding.

The results suggest that LNAA may play an important role in reducing the amount of Phe transported across the intestinal epithelium and perhaps more effectively across the placental barrier. To summarize the results:

### **16.7% LNAA Supplementation**

With this dose, the following was observed

- Decrease in maternal blood Phe levels was seen when compared with control at the time of the third bleed (Table 4).
- There was a statistically significant reduction when the blood Phe levels prior to a supplementation were compared with blood Phe levels at the time of the third bleed (Table 7).
- The decrease in blood Phe levels was also seen in fetal blood when compared with the control group (Table 10).
- The average number of fetuses per pregnancy per animal was eight.
- There was only one incidence of fetal death but three embryonic deaths (Table 11).

### **20% LNAA supplementation**

With the 20 % LNAA it was observed:

- That the decrease in maternal blood Phe levels was not significant when compared with the control group (Table 4).
- That there was a statistically significant decrease when the maternal blood Phe levels after LNAA supplementation was compared with maternal blood Phe before LNAA supplementation (Table 8).

- That the decrease seen in fetal blood Phe when compared to the control group was statistically significant (Table 10).
- The average number of fetuses per pregnancy per animal was eight.
- There was one animal with visible vaginal bleeding.
- Three embryonic deaths and three fetal deaths were also observed (Table 11).

There was an animal which had difficulty to conceive in the control group, but when the animal was supplemented with 20% LNAA, the animal conceived and sustained the pregnancy until term.

### **33.4% LNAA supplementation**

The following observations were noted,

- Significant reduction in the maternal blood Phe levels when compared with the control group at time of the third bleed. (Table 4)
- Decrease in maternal blood Phe levels after supplementation was statistically significant when compared to blood Phe levels before LNAA supplementation (Table 9).
- The fetal blood Phe levels also showed a significant reduction when compared to the control group (Table 10).
- The average number of fetuses per pregnancy per animal was nine.
- Embryonic deaths were more common in females on this dosage, which was eight and there was one fetal death. The most important finding in this group was two fetuses with anencephaly; one each in two consecutive litters.
- Visible vaginal bleeding was seen more often in this group than the others.



The most astonishing find was the anencephalic fetuses, but at this point it is very difficult to pinpoint the etiology for anencephaly; it could be due to the function of LNAA or due to maternal factor or a combination of both. Such a birth is extremely rare in this mPKU mouse model. But since this was not observed in the control group, the possible role of LNAA for this adverse effect is very likely.

One animal was started on 50% LNAA on the day of mating, but the plug did not result in pregnancy nor did she plug again and so she was started on 33.4% LNAA when she plugged and conceived.

### **Control group**

- Three animals had visible vaginal bleeding and one animal had miscarriages in two consecutive pregnancies.
- There was one embryonic death and three fetal deaths.

Based on all these observations, we can conclude that though increasing the dosage of LNAA seemed to decrease the blood Phe levels, it was not without adverse effects. We see less of fetal and embryonic deaths in the control group than the experimental groups. This could mean one thing that in the experimental group the fetus was affected not only by the maternal disease but also by the presence of large amounts of LNAA in the maternal blood, which then crosses the placental barrier to supply the fetus. The same trend emerged in previous studies conducted on pregnant women in New York. These patients were supplemented with a high protein diet during their pregnancy and it was observed that there was a high incidence of low birth weight offspring and / or birth defects in neonates born to these patients [38].

It was also observed, that a significant decrease in the blood Phe levels was seen in the experimental only at the time of the third bleed. This has been observed when the control group was compared with the experimental group (Table – 4) as well as when the comparison was made between the Pre and Post LNAA exposures (Tables 6 – 9). This indicates that the effect of LNAA supplementation on blood Phe levels can be seen after a certain exposure period.

From the above observations, the beneficial and adverse effects of LNAA are listed as follows:

- **Therapeutics Effects of LNAA** observed in this study:
  - Decrease in maternal blood Phe levels.
  - Decrease in fetal blood Phe.
  - Increase in the average number of fetuses born per animal per pregnancy
- **Adverse Effects of LNAA** observed in this study:
  - Anencephaly (33.4% LNAA).
  - Embryonic deaths were more common.

## Discussion

The LNAA formulation used in our study is commercially available as “NeoPhe” tablets (500 mg each). Ours is the first study conducted so far using this formulation, and also the first to be conducted to determine the LNAA effect on maternal and fetal blood Phe levels; hence it is difficult to compare these results with other studies. The first LNAA supplementation study conducted by Dotremont et al (39) on four patients used 0.8g / kg of LNAA and a low protein diet 0.6 g / kg. Their results showed that there was no significant change in the serum Phe levels. In the most recent times, another commercially available LNAA formula, PreKUnil, has been used in many studies on both humans as well as on mice. The composition of PreKUnil is shown in Table 12. One such study conducted by Koch (40) reported the lowering of blood Phe levels when the individuals were treated with PreKUnil at 0.4g/kg body weight. The decrease in blood Phe levels was 5% after 48 hrs and 9% after six months of therapy. But when the PKU mice (PAH ENU 2) (41) were treated with PreKUnil at 0.5g / kg and 1.0 g / kg, it was observed that there was a 15% and 50% decrease in maternal blood Phe levels. The LNAA treated mice also had a sustained decrease in blood Phe level over the six weeks of study. In our study we treated the animals with LNAA at 0.05mg/kg (16.7%), 0.06 mg / kg (20%) and 0.1 mg / kg (33.4%). We observed 40%, 35% and 61% (Table 5) decreases in maternal blood Phe levels at 18.5 dpc and 30%, 18% and 49% (respectively) decreases in fetal blood Phe levels. From these comparisons we can conclude that lower doses of NeoPhe, when administered over a prolonged period of time decreases blood Phe levels at a greater percentage than PreKUnil.

**Table – 12: PreKUnil (LNAA) composition per tablet**

Amino Acid	Mg
Tyrosine	256
Tryptophan	256
Arginine	35
Leucine	35
Isoleucine	35
Valine	35
Methionine	35
Threonine	35

## **Summary and Conclusion**

The main goal of this study was to develop a treatment formula that will enable the patient to consume normal food and maintain treatment compliance. The commercially available Phe restricted diet is not only expensive but also unpalatable, hence resulting in poor patient compliance. From the results of our experiment, though 16.7% LNAA supplementation seemed to be an ideal dosage, it did not decrease the Phe levels to the desired levels (clinically the levels are still high). A dosage of 33.4% dosage decreased the blood Phe levels significantly but was associated with unwanted side effects. Hence from these observations we can conclude that 16.7% LNAA supplementation can be used only as an adjunct therapy. The more important aspect of this therapy will be the effect of these LNAA supplementation on the brain Phe levels and on the incidence of congenital heart defects. Another interesting study would be to determine the effect of LNAA supplementation, which has been modified in such a way that there is an increase in the dietary LNAA without increasing the total dietary amino acid level.

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