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Is basic bone profile affected in phenyloketonuria and hyperphenylalaninemia? The experience of the greek reference center

Maria Kalogerakou[1,2], George I. Lambrou[2,3], Artemis Doulgeraki[4], Ioannis K. Triantafyllopoulos[2,5], Kleopatra H. Schulpis[1]

[1]*Department of Inborn Errors of Metabolism, Institute of Child Health, "Aghia Sofia" Children's Hospital, Thivon 1, 11527, Athens, Greece*
[2] *Postgraduate Program "Metabolic Bones Diseases", National and Kapodistrian University of Athens, Medical School, Mikras Asias 75, 11527, Goudi, Athens, Greece*
[3]*First Department of Pediatrics, University of Athens, Choremeio Research Laboratory, National and Kapodistrian University of Athens, Greece*
[4]*Department of bone and mineral metabolism, Institute of Child Health, "Aghia Sofia" Children's Hospital, Thivon 1, 11527, Athens, Greece*
[5]*National and Kapodistrian University of Athens, Medical School, Laboratory for the Research of Musculoskeletal Disorders, Nikis 2, 14561, Kifissia, Athens, Greece*

ABSTRACT

Aim: To investigate the relationship between different basic bone profile biochemical parameters in Pheylketonuric patients.
Materials and Methods: Study participants included 100 phenylketonuria patients, males and females, with an average age 11,82 ±10,21 years, early diagnosed by Newborn Screening and dietary treated. Serum calcium, phosphate, magnesium, alkaline phosphatase, creatinine, albumin, triglycerides, cholesterol, HDL, LDL, vitamin D, hemoglobin and ferritin were determined for a routine biochemistry and total blood count. Z-BMI, Z-Wt and Z-Ht were estimated for the assessment of normal or delayed growth.
Results: no significant differences were observed to BMI, weight and height (Z-scores) in all subtypes of the disease (PKU-HPA), as well as in groups with good or poor compliance to diet. All the biochemical/ hematological measurements were within normal ranges. Significant differences ($p<0.05$) were presented with respect to phenylalanine levels (Phe) between the patients with good and poor compliance and to

CORRESPONDING AUTHOR, GUARANTOR

George I. Lambrou
First Department of Pediatrics, University of Athens,
Thivon & Levadeias 8, 11527, Athens, Greece.
Email: glamprou@med.uoa.gr

VOLUME 70 | ISSUE 3 | JULY - SEPTEMBER 2019

triglycerides, phosphorus and alkaline phosphatase between the subtypes of the disease. Interestingly, significant statistical differences were observed, between different age groups, such as prepubertal <11years and pubertal>11 years, in the follow biochemical factors: calcium, phosphorus, cholesterol, creatinine, phenylalanine levels and vitamin D. A negative correlation was found between vitamin D, phosphorus and ALP with Phe levels and a positive correlation between creatinine and Phe levels, in all patients. Finally, only in PKU patients, calcium was negatively correlated and Z-BMI was positively correlated to Phe levels. **Conclusion:** These findings suggest that a systematic and periodic biochemical monitoring of basic bone profile may be useful in order to detect and evaluate subtle changes in bone metabolism in Phenylketonuric patients.

KEY WORDS: Phenylketonuria; Phenylalanine; BMI; Vitamin D, Creatinine

Introduction

Phenylketonuria (PKU) (OMIM 261600) is one of the most common inborn errors of aminoacid metabolism, with an overall incidence 1:10.000-20.000 live births in Europe and in the U.S.A. (1, 2). PKU is caused by mutations of the phenylalanine hydroxylase enzyme (PAH-EC 1.14.16.1) gene, which is located on the long arm of chromosome 12 and is inherited in an autosomal recessive manner. The PAH enzyme, with its cofactor tetrahydrobiopterin (BH4), are responsible for catalyzing the conversion of the amino acid phenylalanine (Phe) to tyrosine (Tyr) in the liver. Due to deficiency or absent activity of the PAH enzyme, phenylalanine accumulates in blood and other tissues (brain), resulting in severe and irreversible mental retardation, progressive neurologic impairments and behavioral problems (3). PKU was the first disease of the inborn metabolism which was detected by Newborn Screening (NBS) in the 1960's. Later, the establishment of NBS programs in most of the developed countries resulted in the early diagnosis and treatment of the patients (4). In Greece, PKU screening started in 1973. The classification of PKU types is based on the pre-treatment phenylalanine levels and reflect the severity of the disease (5, 6). The most severe form is referred as "classical PKU" with phenylalanine levels above 20mg/dl. The other three subtypes are: Moderate PKU (15-20mg/dl), Mild PKU (10-15mg/dl), Mild hyperphenylalaninemia (HPA) (6-10mg/dl). The disease classification, its clinical presentation and the treatment are presented according to the protocol of Greek NBS program are illustrated in (**Table 1**).

The aim of the treatment is to reduce the Phenylalanine levels in the blood, in order to protect brain development. There is much debate on the Phe concentrations cut-offs above which treatment should be initiated (6). A life-long dietary therapy includes Phe-free formula diet (Amino Acid Medical Food-AA MF) and is strongly recommended, especially in the classic type of the disease (**Table 1**). When compliance is good, the treatment is very effective (7, 8). Measurements of Phe concentrations must be performed regularly. Published national guidelines for monitoring the frequency and the acceptable therapeutic ranges for phenylalanine levels differ between countries and occasionally between the screening centers of the same country (5, 6).

Despite the remarkable success of dietary therapy for PKU individuals, there is a variety of adverse outcomes, such as: neurocognitive, psychosocial, poor quality of life, growth, nutrition, bone pathology and pregnancy (9, 10). With regards to skeletal status, in particular, numerous studies have demonstrated that PKU has a negative impact (11, 12). In children and adolescents, PKU may cause disturbances in bone metabolism and this is evident by measuring its laboratory indexes, predicting a risk of bone disease later in life (13-20).

Moreover, imaging studies have revealed osteopenia, as a result of low bone mineral density in

VOLUME 70 | ISSUE 3 | JULY - SEPTEMBER 2019

TABLE 1. *PKU Types, clinical presentation and Treatment, according to Greek NBS program (adopted and modified from Burgard P et al (2009) (1)*

PKU Type	Clinical Presentation	PHE(mg/dL)	Treatment
	(INFANCY/ADULTHOOD)		
1. CLASSIC	Seizures, mental retardation, behavioral abnormalities, mousy odor	>20	Lifelong
2. Moderate-Mild PKU			
	If untreated, the same manifestations with the classical type	10-19	Until adulthood and lifelong for girls.
3. Non PKU-HPA/ mild HPA	Normal	<10	Until adulthood and lifelong for girls.
4. BH4-PAH	Depending on type	2-35 and BH4 response.	Depending on type

TABLE 2. *Descriptive statistics of study population*

	PKU	HPA
N	59	41
Age(years)	13.74±11.61	8.73±6.78
Males (n)	26	27
Females (n)	33	14
Prepubertal (n)	33	27
Pubertal (n)	26	14
Z-Wt	-0.1±0.8	-0.1±0.70
Z-Ht	0.0±0.9	0.2±0.66
Z-BMI	0.0±0.9	0.0±1.0
Mean PHE (mg/dl)	5.44±4.0	4.7±2.4

TABLE 3. *Normal ranges of blood Phe values by age according to Greek NBS program for all PKU types*

Age(years)	Target Phe (mg/dl)
0-3	Up to 3.0
3-5	Up to 5.0
6-12	Up to 7.0
13-18	<10
>18	<10

this group of patients has been frequently reported, with the evidence of low bone mineral density, in PKU patients (15, 18, 19) by some researchers but not universally (12, 21, 22).

The etiology of these findings is still a matter of continuous research, in order to identify the risk factors which negatively affect bone health in this disease. Is the genotype of the PKU disease itself or is the long standing dietary treatment the explanation of the suboptimal bone status (23, 24)? This study aims to investigate the correlation between PKU parameters and basic biochemistry bone profile.

Patients and methods

Patients

Fifty nine patients with PKU (Phe levels>10 mg/dl), aged 13.93±11.6 years and forty one patients with hyperphenylalaninemia (HPA, Phe levels6-10mg/dl)), with mean age 8.73±6.7, early diagnosed and treated, were included in the present study. They were all detected by the Greek National Newborn Screening program, in the Institute of Child Health ("*Aghia Sophia*" Children's Hospital, Athens, Greece) and follow a Phe-free formula diet (Amino Acid Medical Food, AA-MF), enriched in calcium, vitamins and minerals (Ca^{+2}:410-1410 mg/100g

VOLUME 70 | ISSUE 3 | JULY - SEPTEMBER 2019

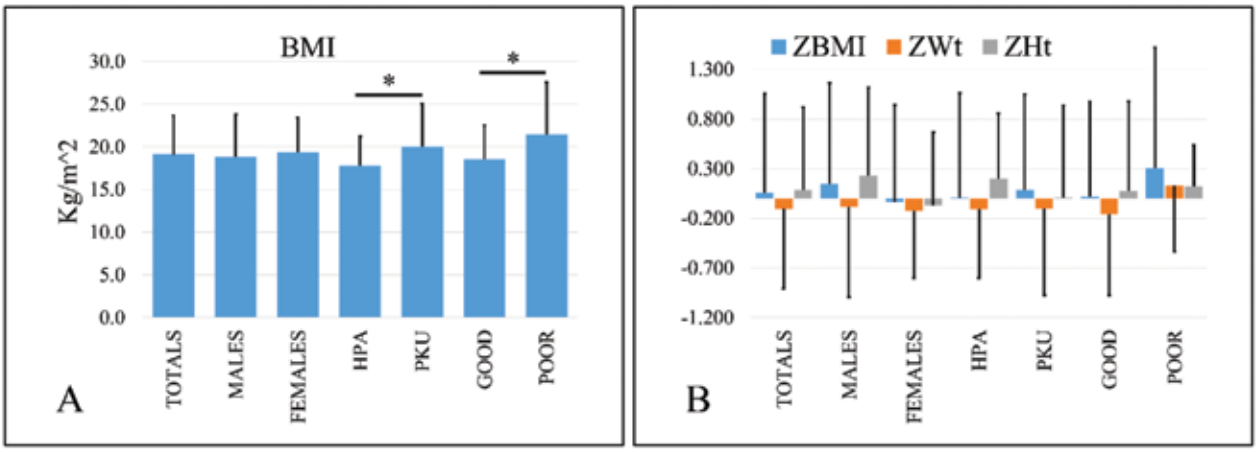

Figure 1. *Descriptive statistics of BMI measurements* (***A***) *as well as Z-BMI, Z-Wt and Z-Ht* (***B***). *Negative values for ZWt were obtained in all studied groups* (***B***), *while positive values were obtained for ZBMI and ZHt in all studied groups* (***B***). *Significant differences were observed between PKU and HPA with respect to BMI as well as between poor and good compliance to diet with respect to BMI* (***B***). *(* depicts a significance at the p<0.05 level. "TOTALS" indicate the mean of all samples irrespectively of gender, PKU subtype and compliance to diet. "MALES" and "FEMALES" indicate samples separated by gender and irrespectively of PKU subtype and compliance to diet. "PKU" and "HPA" indicate samples separated by PKU subtype and irrespectively of gender and compliance to diet. "GOOD" and "POOR" indicate samples separated by diet compliance and irrespectively of gender and PKU subtype. Legend:* ***BMI****: Body-Mass Index,* ***HPA****: Hyperphenylalaninemia,* ***PKU****: Classical PKU type,* ***GOOD****: subjects with good diet compliance, POOR: subjects with poor diet compliance).*

powder and vitamin D_3: 8.7-28μg/100gr powder). Therefore, the patients are supplemented with calcium and/or vitamin D only when indicated (e.g. proven deficiency). Finally, the yearly mean phenylalanine levels have been estimated for PKU and HPA patients.

Based on the same follow up strategy, growth assessment is performed during each visit. Therefore weight (kg), Length/Height (cm, for ages <2y or ≥ 2y, respectively) and Body Mass Index (BMI) (kg/m²) are recorded monthly during the first three years of life and then annually until adulthood. Patient biometric data are summarized in **Table 2**. Due to the wide age range of the participants the absolute values have been converted to Z-scores using, the Greek growth charts Z-BMI, Z-Wt, Z-Ht respectively (25, 26).

Phenylalanine Screening Assay

A fluorescent ninydrin method (Neonatal phenylalanine kit/NP4000, Perkin Elmer) is used for the quantitative determination of phenylalanine concentration in the presence of other amino acids (excitation wave length of 390nm and an emission wave length of 486 nm) (27).The assay is performed in dried blood spot samples, collected in filter papers (*Guthrie Card*-Whatman 902) by heel puncture from the newborns at the age 3-5 days. The normal Phe ranges by age are laboratory depended and for Greek NBS are summarized in **Table 3**.

Biochemical-hematological investigations

Fasting serum samples for routine biochemistry and complete blood count are obtained from all patients, every 3 months until 2 years, every 6 months from 2 to 6 years and every year for children older than 6 years, in order to examine: a) nutritional status and b) the degree of dietary compliance. The determined parameters which are included in the study were calcium (mg/dl), phosphorus (mg/dl), magnesium (mg/dl), albumin (g/dl), alkaline phosphatase (IU/L), triglycerides (mg/dl), cholesterol (mg/dl), HDL (mg/dl), LDL (mg/dl), creatinine (mg/dl), 25(OH)D (ng/ml), hemoglobin(g/dl)

VOLUME 70 | ISSUE 3 | JULY - SEPTEMBER 2019

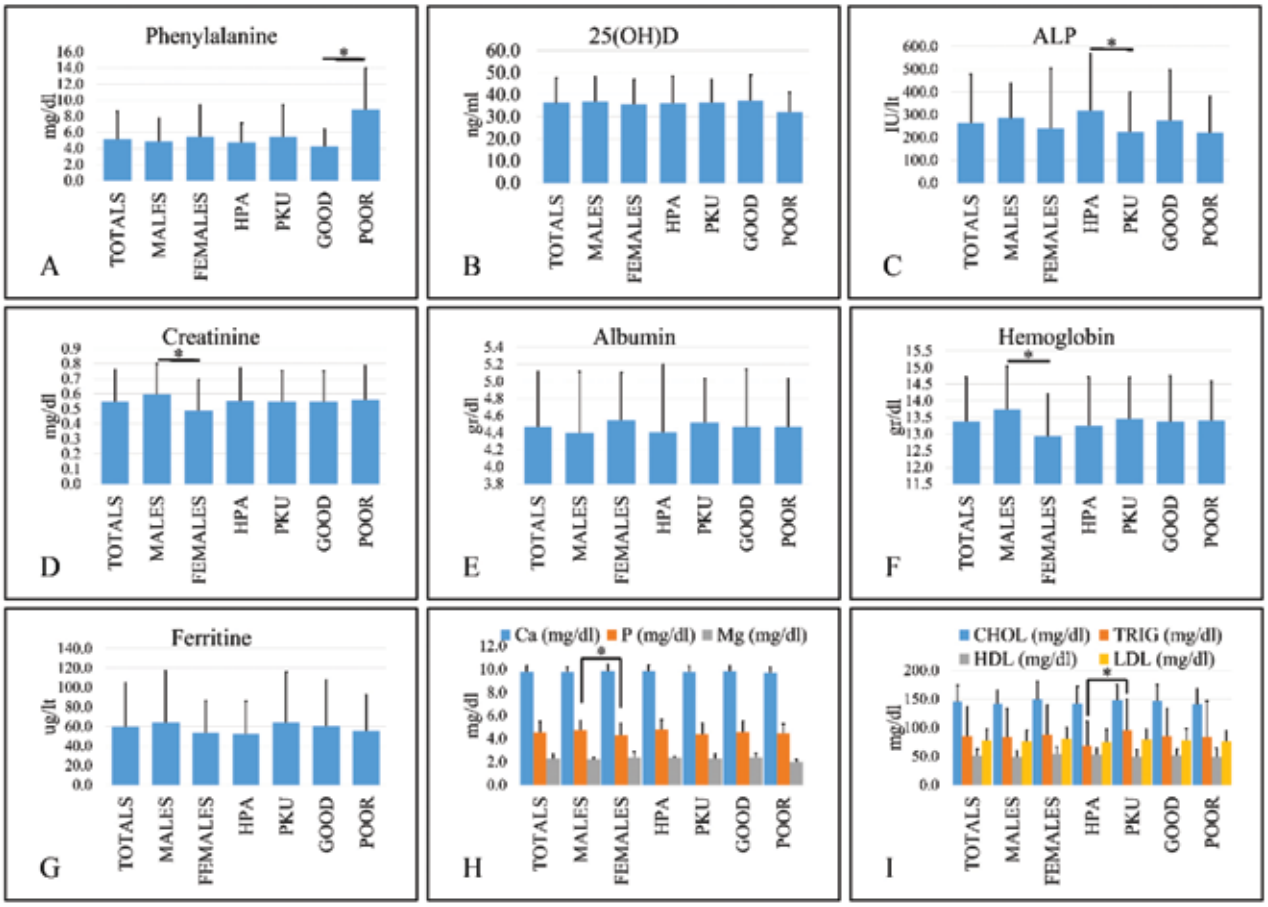

***Figure 2**. Descriptive statistics of biochemical factors in patients with PKU. In particular, measurements included phenylalanine (**A**), 25-OH-Vitamin D (**B**), Alkaline Phosphatase (ALP) (**C**), Creatinine (**D**), Albumin (**E**), Hemoglobin (**F**), Ferritine (**G**), Ca^{+2}, P^{+4} and Mg^{+2} (**H**) and lipidemic profile, which included cholesterol (CHOL), triglycerides (TRIG), high-density lipoprotein (HDL) and low-density lipoprotein (LDL) (**I**). Significant differences were observed between patients with good and poor compliance to diet with respect to phenylalanine (**A**), between patients with PKU and HPA with respect to ALP (**C**), between males and females with respect to creatinine (**D**), between males and females with respect to hemoglobin (**F**), between males and females with respect to P^{+4} (**H**) and between patients with PKU and HPA with respect to triglycerides (**I**) (* depicts a significance at the $p<0.05$ level. "TOTALS" indicate the mean of all samples irrespectively of gender, PKU subtype and compliance to diet. "MALES" and "FEMALES" indicate samples separated by gender and irrespectively of PKU subtype and compliance to diet. "PKU" and "HPA" indicate samples separated by PKU subtype and irrespectively of gender and compliance to diet. "GOOD" and "POOR" indicate samples separated by diet compliance and irrespectively of gender and PKU subtype. Legend: **BMI**: Body-Mass Index, **HPA**: Hyperphenylalaninemia, **PKU**: Classical PKU type, **GOOD**: subjects with good diet compliance, **POOR**: subjects with poor diet compliance, **25(OH)D**: 25-OH-Vitamin D, **ALP**: Alkaline Phosphatase, **CHOL**: Cholesterol, **TRIG**: triglycerides, **HDL**: High-Density Lipoprotein, **LDL**: Low-Density Lipoprotein).*

and ferritin (μg/lt). The routine Biochemistry was performed in an automatic biochemical analyzer (Advia 1800 Siemens). Hematological parameters were determined using an automatic hematological analyzer (Advia 2120i, Siemens).

Data Analysis

The multi-parameter analyses were performed with the MATLAB® simulation environment (The Mathworks, Inc., Natick, MA) and SPSS version 16.0.The one- and two-way ANOVA tests were used to test the

VOLUME 70 | ISSUE 3 | JULY - SEPTEMBER 2019

TABLE 4. *Biochemical/hematological descriptive statistics in classic PKU patients*

	Mean	Std. Deviation	Paediatric Normal ranges
			(adult range in parentheses)
PHE(mg/dl)	5.449	4.03	Table 4
VitD (ng/ml)	36.514	10.85	<12 deficiency;12-20 insufficiency;>20 sufficiency
Ca(mg/dl)	9.754	0.52	<12 years:8.7-10.8
P(mg/dl)	4.433	0.92	4.0-6.0 (2.4-5.1)
ALP(U/lt)	228.86	176.51	60-240 (45-129)
ALB(gr/dl)	4.512	0.53	3.4-4.8
Cr(mg/dl)	0.5360	0.20	0.20-1.0 (0.7-1.3)
CHOL(mg/dl)	147.34	27.43	120-200 (<200)
TG(mg/dl)	96.02	54.82	30-130(<150)
HDL(mg/dl)	49.56	12.53	35-84(>40)
LDL(mg/dl)	79.14	18.65	<150(<130)
Mg(mg/dl)	2.2830	0.43	1.5-2.3
HB(g/dl)	13.452	1.28	11.5-14.5
FERRITIN(μg/L)	64.22	52.09	10-150 22-322

mean differences between groups. Continuous variables are expressed as median±standard deviation unless indicated differently. Correlations between variables were calculated using *Pearson's* Correlation coefficient. Linear regressions were performed using the $y=ax+b$, $y=ax^2+bx+c$ form and curves were estimated using a least-chi-square approach. K-means, clustered scatter plots as well as Hierarchical Clustering algorithms were implemented with the MATLAB ® simulation environment (The Mathworks, Inc., Natick, MA).

Data Availability

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics Statement

All investigations were conducted in compliance with the international biomedical studies stipula-

TABLE 5. *Biochemical/haematological descriptive statistics in HPA patients*

	Mean	Std. Deviation
	Statistic	Statistic
PHE(mg/dl)	4.727	2.45
VITD(ng/ml)	36.128	12.40
Ca(mg/dl)	9.877	0.48
P(mg/dl)	4.801	0.83
ALP(U/lt)	317.93	255.17
ALB(gr/dl)	4.406	0.80
Cr(mg/dl)	0.5533	0.22
CHOL(mg/dl)	142.40	29.86
TRIG(mg/dl)	68.93	42.10
HDL(mg/dl)	52.71	12.17
LDL(mg/dl)	75.33	22.01
Mg(mg/dl)	2.3250	0.10
HB(g/dl)	13.248	1.49
FERRITINE(μg/L)	52.45	33.95

VOLUME 70 | ISSUE 3 | JULY - SEPTEMBER 2019

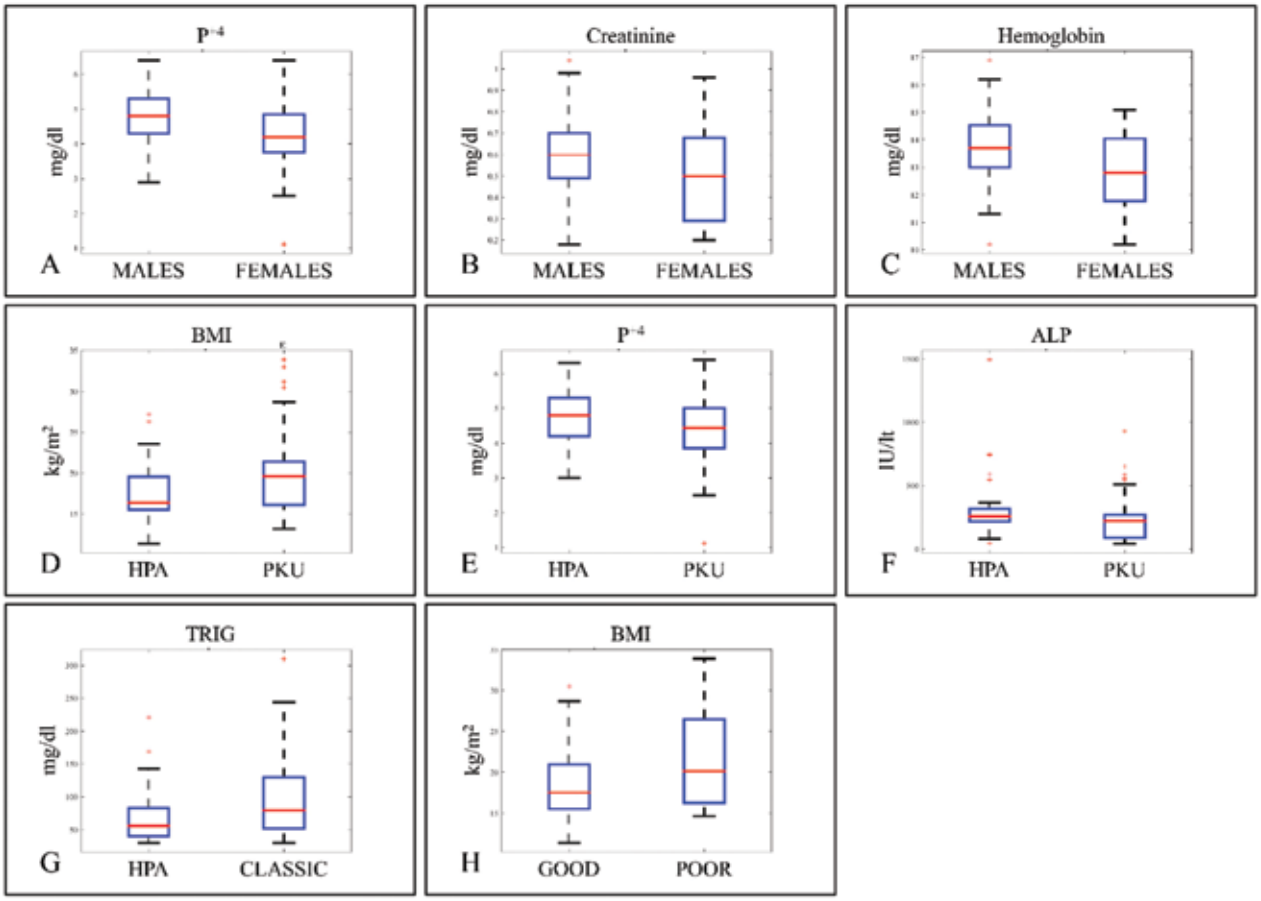

Figure 3*. ANOVA analysis of biochemical factors in patients with PKU. In particular, significant differences were manifested between males and females with respect to P^{+4} (**A**), Creatinine (**B**) and hemoglobin (**C**). Further on, significant differences were observed between PKU and HPA with respect to BMI (**D**), P^{+4} (**E**), Alkaline Phosphatase (ALP) (**F**) and Triglycerides (TRIG) (**G**). Finally,significant difference was manifested between patients with good and poor compliance to diet with respect to BMI (**H**) (all differences presented are at a significance level of $p<0.05$. "TOTALS" indicate the mean of all samples irrespectively of gender, PKU subtype and compliance to diet. "MALES" and "FEMALES" indicate samples separated by gender and irrespectively of PKU subtype and compliance to diet. "PKU" and "HPA" indicate samples separated by PKU subtype and irrespectively of gender and compliance to diet. "GOOD" and "POOR" indicate samples separated by diet compliance and irrespectively of gender and PKU subtype. Legend: **BMI**: Body-Mass Index, **HPA**: Hyperphenylalaninemia, **PKU**: Classical PKU type, **GOOD**: subjects with good diet compliance, **POOR**: subjects with poor diet compliance, **ALP**: Alkaline Phosphatase, **CHOL**: Cholesterol, **TRIG**: triglycerides).*

tions, with reference to the Declaration of Helsinki of the World Medical Association. There's no reference to patients' personal data.

Results

Descriptive Statistics of Phenylketonuria (PKU) and Hyperphenylalaninamia (HPA) Patients

Descriptive statistics of BMI measurements (**Figure 1A**) manifested significant differences between patients with HPA and PKU (p=0.016) (**Figure 1A**) as well as between patients with good diet compliance and poor diet compliance (p=0.014) (**Figure 1A**). Further on, as far as Z-BMI, Z-Wt and Z-Ht were concerned (**Figure 1B**), negative values of ZWt were obtained in all studied groups, while positive values were obtained for ZBMI and ZHt in all studied groups.

VOLUME 70 | ISSUE 3 | JULY - SEPTEMBER 2019

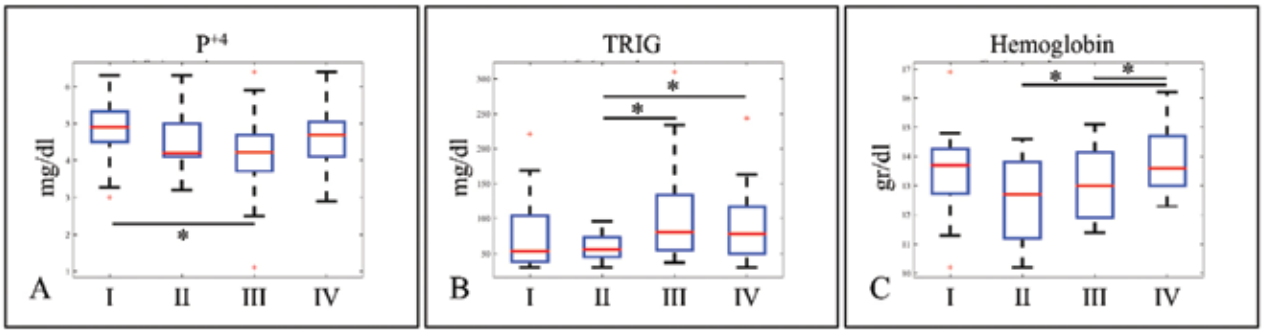

***Figure 4**. ANOVA analysis of biochemical factors in multiple groups of patients with respect to gender and PKU subtype irrespectively of diet compliance. In particular, significant differences were manifested with respect to P^{+4} (**A**), Triglycerides (TRIG) (**B**) and hemoglobin (**C**). In particular, significant differences were observed between Males with HPA (I) and Females with PKU (III) (p=0.0101) (**A**). Further on, significant differences were observed between Females with HPA (II) and Females with PKU (III) (p=0.012) (**B**) and between Females with HPA (II) and Females with PKU (III) (p=0.02) (**B**). Finally, hemoglobin also manifested significant differences and in particular, between Females with HPA (II) and Males with PKU (IV) (p=0.014) (**C**) as well as between Females with PKU (III) and Males with PKU (IV) (p=0.0108) (**C**) (Legend: **HPA**: Hyperphenylalaninemia, **TRIG**: triglycerides, **I**: Males with HPA, **II**: Females with HPA, **III**: Females with PKU, **IV**: Males with PKU).*

With respect to biochemical factors in patients with PKU, measurements included phenylalanine (p=2.8×10^{-8}) (**Figure 2A**), 25-OH-Vitamin D (**Figure 2B**), Alkaline Phosphatase (ALP) (p=0.037) (**Figure 2C**), Creatinine (p=0.041) (**Figure 2D**), Albumin (**Figure 2E**), Hemoglobin (p=0.006) (**Figure 2F**), Ferritine (**Figure 2G**), Ca^{+2}, P^{+4} (p=0.02) and Mg^{+2} (**Figure 2H**) and lipidemic profile, which included cholesterol (CHOL), triglycerides (TRIG) (p=0.009), high-density lipoprotein (HDL) and low-density lipoprotein (LDL) (**Figure 2I**). Significant differences were observed between patients with good and poor compliance to diet with respect to phenylalanine (**Figure 2A**), where patients with poor diet compliance manifested higher levels of phenylalanine, between patients with PKU and HPA with respect to ALP, where patients with HPA manifested higher levels of ALP (**Figure 2C**), between males and females with respect to creatinine, where males presented higher levels of creatinine (**Figure 2D**), between males and females with respect to hemoglobin (**Figure 2F**), between males and females with respect to P^{+4}, where males manifested higher levels of Phosphorus as compared to females (**Figure 2H**) and between patients with PKU and HPA with respect to triglycerides, where patients with HPA manifested higher levels of triglycerides as compared to PKU patients (**Figure 2I**).

All the descriptive statistics of growth Z-scores and annualized mean values of Phe levels for each group of patients (PKU/HPA) are presented in **Table 2**. The results of Z-BMI, Z-Wt and Z-Ht, did not reveal any statistically significant difference (**Figure 1B**), in all studied groups (PKU/HPA, Good /Poor dietary compliance, Males/Females). 81% of the study population is characterized as good adherent to diet, whereas the other 19% as poor adherent, according to the expected Phe levels indicated for their age (**Table 3**). Additionally, a percentage of 84.3% of 19% with poor dietary compliance refers to PKU patients (Phe levels>10mg/dl). No significant differences were observed between Z-BMI and vitamin D for all subtypes of the disease (PKU/HPA). All laboratory parameters were within reference range (**Tables 4** and **Table 5**).

Statistical Analysis of Patient Groups: PKU vs HPA and Good vs Poor dietary compliance

Statistical Analysis of Patient Groups: Gender

ANOVA analysis of biochemical factors in patients with PKU manifested significant differences in a number of metabolic factors. In particular, signif-

VOLUME 70 | ISSUE 3 | JULY - SEPTEMBER 2019

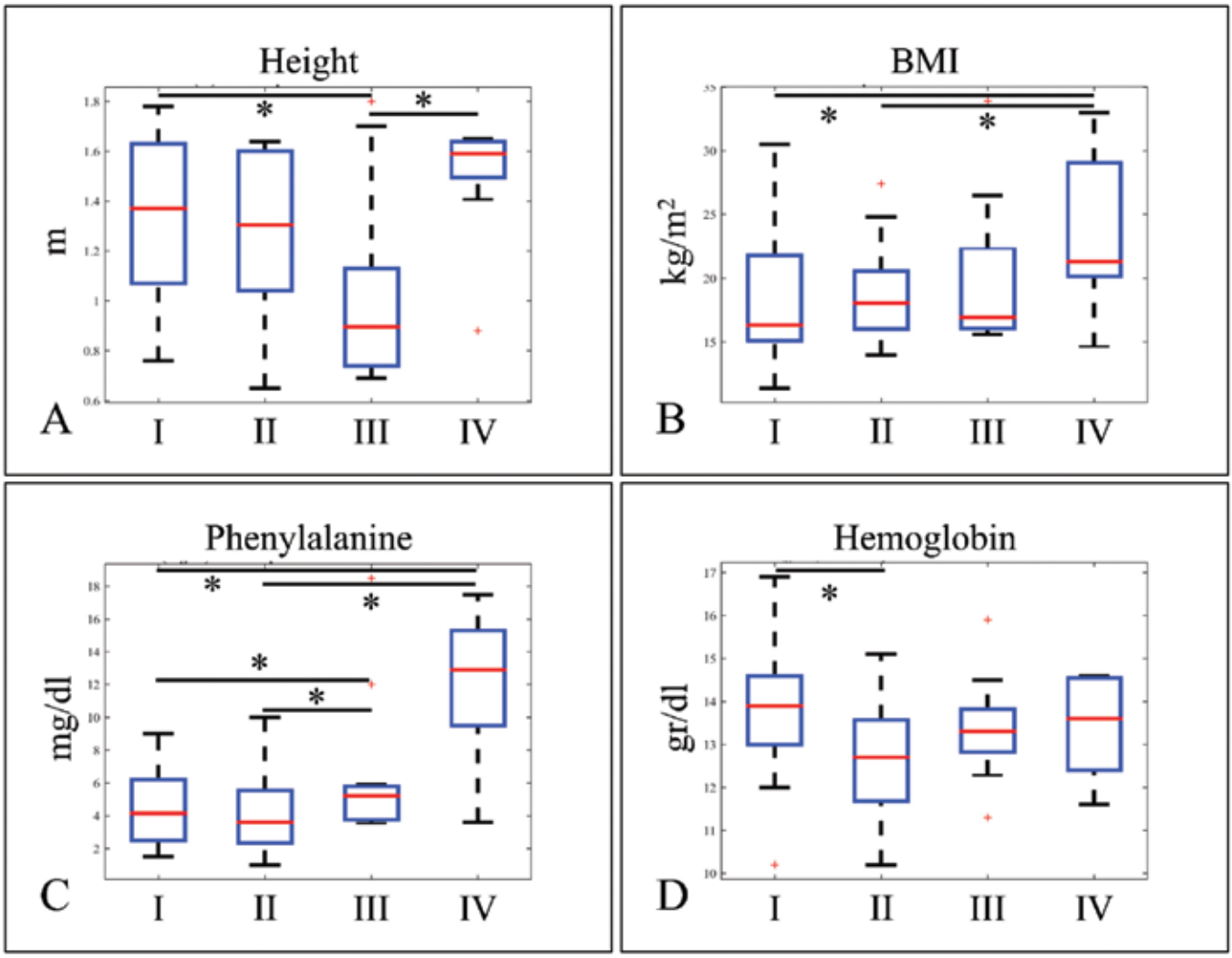

Figure 5. *ANOVA analysis of biochemical and anthropometric factors in multiple groups of patients with respect to gender and diet compliance and irrespectively of PKU subtype. In particular, significant differences were manifested with respect to height (**A**), BMI (**B**), Phenylalanine (**C**) and Hemoglobin (**D**). In particular, significant differences were observed between Males with Good Compliance to Diet (I) and Males with Poor Compliance to Diet (III) ($p=0.017$) (**A**) and between Males with Poor Compliance to Diet (III) and Females with Poor Compliance to Diet (IV) ($p=0.012$) (**A**). Further on, significant differences were observed between Males with Good Compliance to Diet (I) and Females with Poor Compliance to Diet (IV) ($p=0.014$) and between Females with Good Compliance to Diet (II) and Females with Poor Compliance to Diet (IV) ($p=0.0009$) (**B**). In addition, significant differences were observed between Males with Good Compliance to Diet (I) and Males with Poor Compliance to Diet (III) ($p=0.03$) (**C**), between Males with Good Compliance to Diet (I) and Females with Poor Compliance to Diet (IV) ($p=9.4 \cdot 10^{-4}$) (**C**), between Females with Good Compliance to Diet (II) and Males with Poor Compliance to Diet (III) ($p=0.018$) (**C**) and between Females with Good Compliance to Diet (II) and Females with Poor Compliance to Diet (IV) ($p=1.33 \cdot 10^{-9}$) (**C**). Finally, hemoglobin also manifested significant differences and in particular, between Males with Good Compliance to Diet (I) and Females with Good Compliance to Diet (II) ($p=0.002$) (**D**) (Legend: **BMI**: Body-Mass Index, **I**: Males with Good Compliance to Diet (irrespectively of PKU subtype), **II**: Females with Good Compliance to Diet (irrespectively of PKU subtype), **III**: Males with Poor Compliance to Diet (irrespectively of PKU subtype), **IV**: Females with Poor Compliance to Diet (irrespectively of PKU subtype)).*

VOLUME 70 | ISSUE 3 | JULY - SEPTEMBER 2019

icant differences were manifested with respect to gender, between males and females, irrespectively of PKU subtype or diet compliance with respect to P^{+4} (p=0.02) (**Figure 3A**), Creatinine (p=0.041) (**Figure 3B**) and hemoglobin (p=0.006) (**Figure 3C**).

Statistical Analysis of Patient Groups: PKU and HPA

Further on, significant differences were observed between classical PKU and HPA with respect to BMI (p=0.01) (**Figure 3D**), P^{+4} (p=0.043) (**Figure 3E**), Alkaline Phosphatase (ALP) (p=0.037) (**Figure 3F**) and Triglycerides (TRIG) (p=0.009) (**Figure 3G**).

Statistical Analysis of Patient Groups: Diet Compliance

Finally, significant difference was manifested between patients with good and poor compliance to diet with respect to BMI (p=0.014) (**Figure 3H**).

Statistical Analysis of Multiple Groups with Respect to Gender and PKU Subtype

ANOVA analysis of biochemical factors in multiple groups of patients with respect to gender and PKU subtype irrespectively of diet compliance manifested, significant differences with respect to P^{+4} (**Figure 4A**), Triglycerides (TRIG) (**Figure 4B**) and hemoglobin (**Figure 4C**). In particular, significant differences were observed between Males with HPA-PKU (**Figure 4A***I*) and Females with Classical PKU (**Figure 4A***III*) (p=0.0101). Further on, significant differences were observed between Females with HPA-PKU (**Figure 4B***II*) and Females with Classical PKU (**Figure 4B***III*) (p=0.012) and between Females with HPA-PKU (**Figure 4B***II*) and Females with Classical PKU (**Figure 4B***III*) (p=0.02). Finally, hemoglobin also manifested significant differences and in particular, between Females with HPA-PKU (**Figure 4C***II*) and Males with Classical PKU (**Figure 4C***IV*) (p=0.014) as well as between Females with Classical PKU (**Figure 4C***III*) and Males with Classical PKU (*IV*) (p=0.0108).

Statistical Analysis of Multiple Groups with Respect to Gender and Diet Compliance

Further on, we performed ANOVA analysis of biochemical and anthropometric factors in multiple groups of patients with respect to gender and diet compliance and irrespectively of PKU subtype. In particular, significant differences were manifested with respect to height (**Figure 5A**), BMI (**Figure 5B**), Phenylalanine (**Figure 5C**) and Hemoglobin (**Figure 5D**). Specifically, significant differences were observed between Males with Good Compliance to Diet (**Figure 5A***I*) and Males with Poor Compliance to Diet (**Figure 5A***III*) (p=0.017) and between Males with Poor Compliance to Diet (**Figure 5A***III*) and Females with Poor Compliance to Diet (**Figure 5A***IV*) (p=0.012). Further on, significant differences were observed between Males with Good Compliance to Diet (**Figure 5B***I*) and Females with Poor Compliance to Diet (**Figure 5B***IV*) (p=0.014) and between Females with Good Compliance to Diet (**Figure 5B***II*) and Females with Poor Compliance to Diet (**Figure 5B***IV*) (p=0.0009). In addition, significant differences were observed between Males with Good Compliance to Diet (**Figure 5C***I*) and Males with Poor Compliance to Diet (**Figure 5C***III*) (p=0.03), between Males with Good Compliance to Diet (**Figure 5C***I*) and Females with Poor Compliance to Diet (**Figure 5C***IV*) (p=9.4 $\cdot 10^{-4}$), between Females with Good Compliance to Diet (**Figure 5C***II*) and Males with Poor Compliance to Diet (**Figure 5C***III*) (p=0.018) and between Females with Good Compliance to Diet (**Figure 5C***II*) and Females with Poor Compliance to Diet (**Figure 5C***IV*) (p=1.33 $\cdot 10^{-9}$). Finally, hemoglobin also manifested significant differences and in particular, between Males with Good Compliance to Diet (**Figure 5D***I*) and Females with Good Compliance to Diet (**Figure 5D***II*) (p=0.002).

Statistical Analysis of Multiple Groups with Respect to PKU Subtype and Diet Compliance

Next step towards our analysis was the ANOVA analysis of biochemical factors in multiple groups of patients with PKU with respect to PKU subtype and compliance to diet irrespectively to gender. Significant differences were manifested with respect to BMI (**Figure 6A**) and Phenylalanine (**Figure 6B**). In particular, significant differences were observed between HPA patients with Good Diet Compliance (**Figure 6A***I*) and patients with PKU with Poor Diet Compliance (**Figure 6A***III*)

VOLUME 70 | ISSUE 3 | JULY - SEPTEMBER 2019

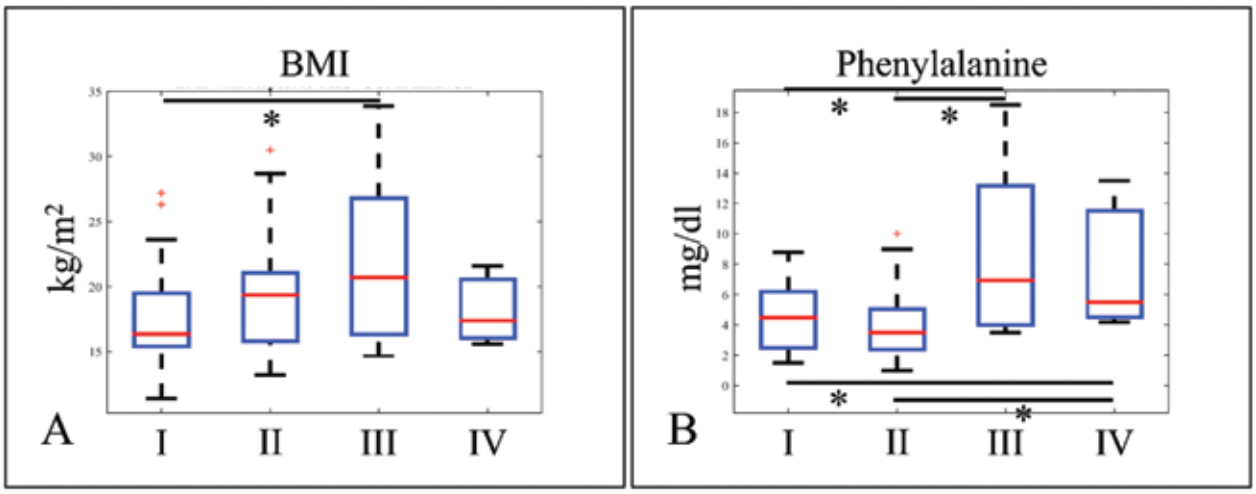

***Figure 6**. ANOVA analysis of biochemical factors in multiple groups of patients with PKU with respect to PKU subtype and compliance to diet irrespectively to gender. In particular, significant differences were manifested with respect to BMI (**A**) and Phenylalanine (**B**). In particular, significant differences were observed between HPA patients with Good Diet Compliance (I) and patients with PKU with Poor Diet Compliance (III) (p=0.002) (**A**). Finally, significant differences were observed between patients with HPA and Good Diet Compliance (I) and patients with PKU and Poor Diet Compliance (III) ($p=4 \cdot 10^{-5}$) (**B**), between patients with HPA and Good Diet Compliance (I) and patients with HPA and Poor Diet Compliance (IV) (p=0.025) (**B**), between patients with PKU with Good Diet Compliance (II) and patients with PKU with Poor Diet Compliance (III) ($p=4.8 \cdot 10^{-6}$) (**B**), as well as between patients with PKU and Good Diet Compliance (II) and patients with HPA and Poor Diet Compliance ($p=4 \cdot 10^{-5}$) (**B**) (Legend: **BMI**: Body-Mass Index, **I**: HPA with Good Diet Compliance, **II**: PKU with Good Diet Compliance, **III**: PKU with Poor Diet Compliance and **IV**: HPA with Poor Diet Compliance).*

(p=0.002). Further on, significant differences were observed between patients with HPA and Good Diet Compliance (**Figure 6B***I*) and patients with PKU and Poor Diet Compliance (**Figure 6B***III*) ($p=4 \cdot 10^{-5}$), between patients with HPA and Good Diet Compliance (**Figure 6B***I*) and patients with HPA and Poor Diet Compliance (**Figure 6B***IV*) (p=0.025), between patients with PKU with Good Diet Compliance (**Figure 6B***II*) and patients with PKU with Poor Diet Compliance (**Figure 6B***III*) ($p=4.8 \cdot 10^{-6}$), as well as between patients with PKU and Good Diet Compliance (**Figure 6B***II*) and patients with HPA and Poor Diet Compliance ($p=4 \cdot 10^{-5}$) (**Figure 6B***IV*).

Statistical Analysis of Multiple Groups with Respect to PKU Subtype, Diet Compliance and Gender

As a final step towards our analysis, we have performed ANOVA analysis of biochemical factors in multiple groups of patients with PKU with respect to PKU subtype, compliance to diet and gender. In particular, significant differences were manifested with respect to phenylalanine. Specifically, significant differences were observed between Females with Classical PKU and Good Diet Compliance (**Figure 7***C*) and Females with Classical PKU and Poor Diet Compliance (**Figure 7***F*) ($p=3.2 \cdot 10^{-7}$), between Females with Classical PKU and Good Diet Compliance (**Figure 7***C*) and Males with Classical PKU and Poor Diet Compliance (**Figure 7***E*) (p=0.02), between Females with HPA-PKU and Good Diet Compliance (**Figure 7***B*) and Females with Classical PKU and Poor Diet Compliance (**Figure 7***F*) (p=0.0002), between Males with Classical PKU and Good Diet Compliance (**Figure 7***D*) Females with Classical PKU and Poor Diet Compliance (**Figure 7***F*) and ($p=3.06 \cdot 10^{-5}$) and finally between Males with HPA-PKU and Good Diet Compliance (**Figure 7***A*) and Females with Classical PKU and Poor Diet Compliance (**Figure 7***F*) ($p=9.5 \cdot 10^{-7}$).

VOLUME 70 | ISSUE 3 | JULY - SEPTEMBER 2019

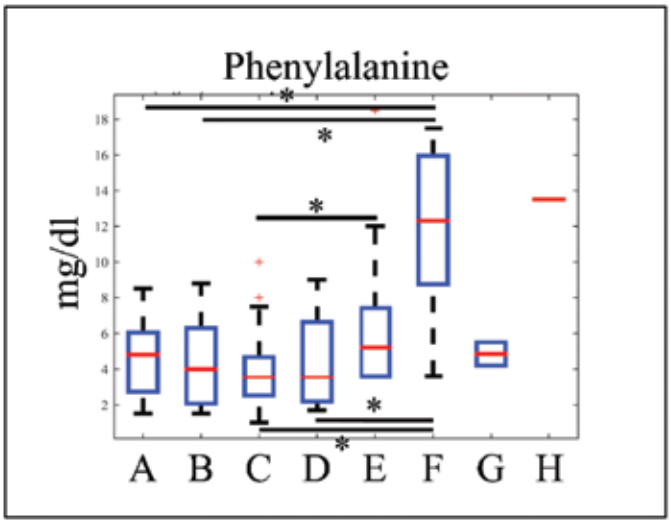

Figure 7. *ANOVA analysis of biochemical factors in multiple groups of patients with PKU with respect to PKU subtype, compliance to diet and gender. In particular, significant differences were manifested with respect to phenylalanine. Specifically, significant differences were observed between Females with Classical PKU and Good Diet Compliance (C) and Females with Classical PKU and Poor Diet Compliance (F) ($p=3.2 \cdot 10^{-7}$), between Females with Classical PKU and Good Diet Compliance (C) and Males with Classical PKU and Poor Diet Compliance (E) (p=0.02), between Females with HPA-PKU and Good Diet Compliance (B) and Females with Classical PKU and Poor Diet Compliance (F) (p=0.0002), between Males with Classical PKU and Good Diet Compliance (D) Females with Classical PKU and Poor Diet Compliance (F) and ($p=3.06 \cdot 10^{-5}$) and finally between Males with HPA-PKU and Good Diet Compliance (A) and Females with Classical PKU and Poor Diet Compliance (F) ($p=9.5 \cdot 10^{-7}$) (Legend:* ***BMI****: Body-Mass Index,* ***A****: Males with HPA-PKU and Good Diet Compliance,* ***B****: Females with HPA-PKU and Good Diet Compliance,* ***C****: Females with Classical PKU and Good Diet Compliance,* ***D****: Males with Classical PKU and Good Diet Compliance,* ***E****: Males with Classical PKU and Poor Diet Compliance,* ***F****: Females with Classical PKU and Poor Diet Compliance,* ***G****: Males with HPA-PKU and Poor Diet Compliance and* ***H****: Females with HPA-PKU and Poor Diet Compliance).*

TABLE 6. *T-test between prepubertal and pubertal patients with PKU and HPA.*

	AGE	Mean	Std. deviation	Sig. (2-tailed)
VITD	>= 11	29.592	9.48	0.00
	< 11	41.034	10.30	0.00
Ca	>= 11	9.640	0.51	0.00
	< 11	9.922	0.46	0.00
P	>= 11	4.050	0.87	0.00
	< 11	4.923	0.78	0.00
ALP	>= 11	141.51	102.17	0.00
	< 11	356.80	232.49	0.00
ALB	>= 11	4.595	0.76	0.21
	< 11	4.404	0.57	0.25
CHOL	>= 11	153.26	34.05	0.01
	< 11	139.54	21.45	0.02
Cr	>= 11	0.6903	0.17	0.00
	< 11	0.4103	0.14	0.00
TRIG	>= 11	85.33	50.9	0.96
	< 11	84.81	52.0	0.96
HDL	>= 11	52.31	14.36	0.29
	< 11	49.69	10.61	0.31
LDL	>= 11	83.71	20.73	0.01
	< 11	73.26	18.47	0.01
Mg	>= 11	2.2060	0.29	0.56
	< 11	2.3500	0.46	0.52
HB	>= 11	14.021	1.32	0.00
	< 11	12.791	1.09	0.00
FERRITIN	>= 11	77.01	58.95	0.00
	< 11	42.90	16.26	0.00
PHE	>= 11	7.095	4.22	0.00
		3.633	1.54	0.00

Statistical Analysis of Pubertal Status

T-Test statistical analysis was subsequently performed to test for differences between patients, based on their pubertal status, thus two subgroups were studied: prepubertal<11years (Tanner stages

VOLUME 70 | ISSUE 3 | JULY - SEPTEMBER 2019

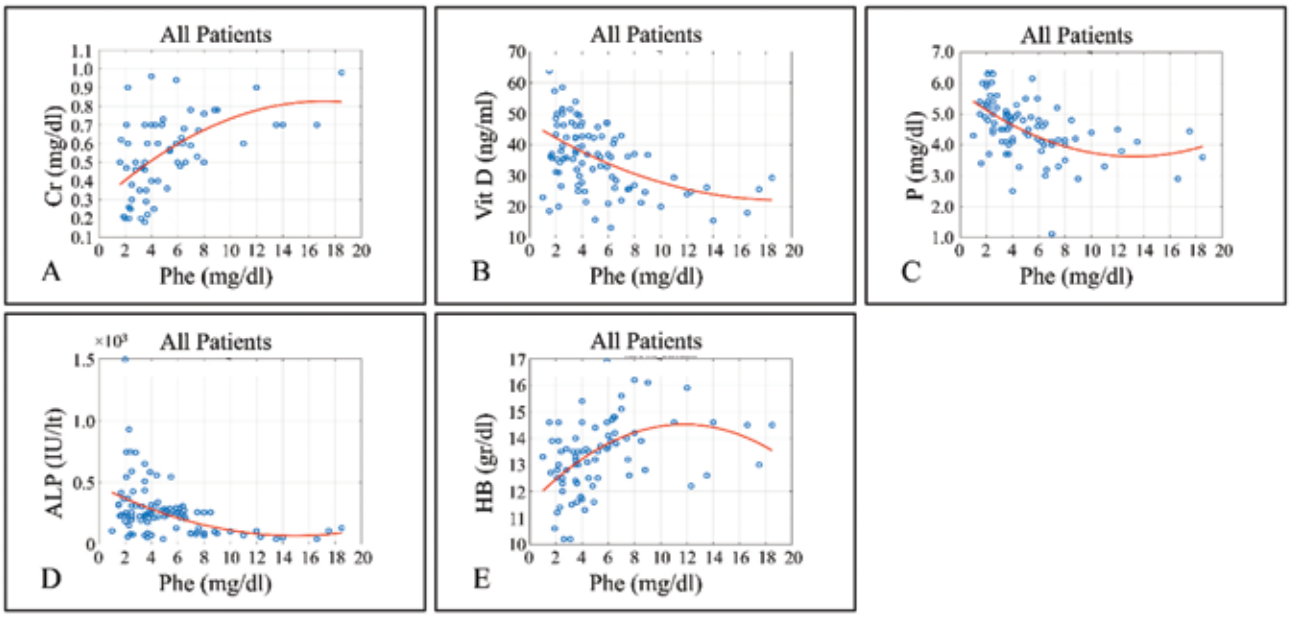

***Figure 8**. Binomial regressions of Phe vs. biochemical factors in all patients, independently of gender, PKU type and diet compliance. In particular, significant regressions were observed between Phe and Cr ($p<<0.01$) ($R^2=0.3$) (**A**), Vitamin D ($p<<0.01$) ($R^2=0.21$) (**B**), Phosphorus ($p<<0.01$) ($R^2=0.25$) (**C**), Alkaline Phosphatase ($p<<0.01$) ($R^2=0.16$) (**D**) and Hemoglobin ($p<<0.01$) ($R^2=0.22$) (**E**) (Legend: **Cr**: Creatinine, **Vit D**: Vitamin D, **P**: Phosphorus, **ALP**: Alkaline Phosphatase, **HB**: Hemoglobin).*

1,2) and pubertal>11 years (Tanner stages 3,4,5) (**Table 6**).The biochemical parameters with a statistically significant difference ($p<0.05$) were as follows: Vitamin D ($p<0.01$), Ca ($p<0.01$), P ($p<0.01$), Chol ($p<0.05$),Cr ($p<0.01$),mean Phe ($p<0.01$).Specifically, for Vitamin D, Ca and P the values in prepubertal group were higher than in pubertal group, whereas for Cholesterol, Creatinine and mean Phe, the values were higher in pubertal group than in prepubertal. No significant difference was observed with respect to Z-BMI in these two age groups.

Regression Analysis

Regression Analysis of Phe in the Total Population

Regression analysis was performed between the study biomarkers and Phe levels, in which significant correlations have been found. In particular, Phe manifested significant correlation, using binomial regressions, svs. biochemical factors in all patients, independently of gender, PKU type and diet compliance. In particular, significant regressions were observed between Phe and Creatinine (Cr) ($R^2=0.3$, $p<<0.01$) (**Figure 8A**), Vit D (Vitamin D) ($R^2=0.21$, $p<<0.01$) (**Figure 8B**), Phosphorus (P) ($R^2=0.25$, $p<<0.01$) (**Figure 8C**), Alkaline Phosphatase (ALP) ($R^2=0.16$, $p<<0.01$) (**Figure 8D**) and Hemoglobin (HB) ($R^2=0.22$, $p<<0.01$) (**Figure 8E**).

Regression Analysis of Phe in the PKU Population

Further on, Phe manifested significant correlation, using binomial regressions, vs. biochemical factors in PKU patients, independently of gender and diet compliance. In particular, significant regressions were observed between Phe and Creatinine (Cr) ($R^2=0.3$, $p<<0.01$) (**Figure 9A**), Vit D (Vitamin D) ($R^2=0.21$, $p=0.019$) (**Figure 9B**), Phosphorus (P) ($R^2=0.25$, $p=0.015$) (**Figure 9C**), Alkaline Phosphatase (ALP) ($R^2=0.16$, $p<<0.01$) (**Figure 9D**), Hemoglobin (HB) ($R^2=0.22$, $p<<0.01$) (**Figure 9E**) and Ferritin ($R^2=0.14$, $p=0.04$) (**Figure 9F**). Ferritin was the factor that differed the PKU patients from the total population.

Regression Analysis of Phe in the HPA Population

Finally, Phe manifested significant correlation, us-

VOLUME 70 | ISSUE 3 | JULY - SEPTEMBER 2019

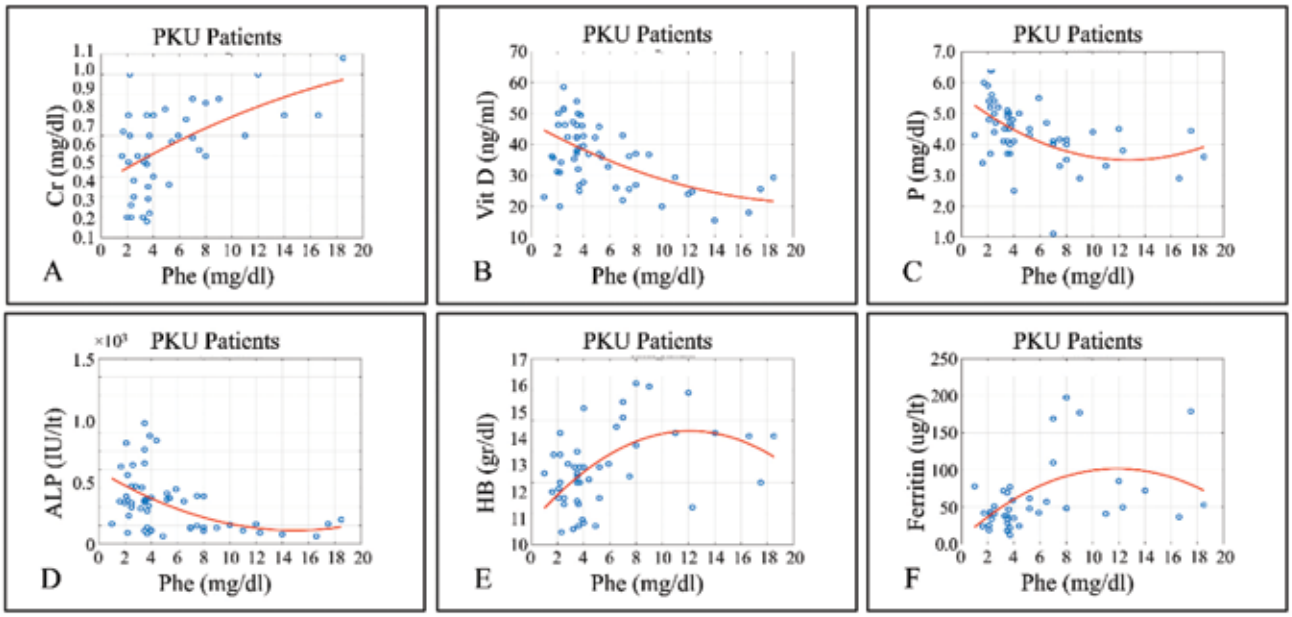

***Figure 9**. Binomial regressions of Phe vs. biochemical factors in PKU patients, independently of gender and diet compliance. In particular, significant regressions were observed between Phe and Cr (R^2=0.24) (**A**), Vitamin D (R^2=0.27) (**B**), Phosphorus (R^2=0.24) (**C**), Alkaline Phosphatase (R^2=0.17) (**D**), Hemoglobin (R^2=0.26) (**E**) and Ferritin (R^2=0.14) (**F**) (Legend: **Cr**: Creatinine, **Vit D**: Vitamin D, **P**: Phosphorus, **ALP**: Alkaline Phosphatase, **HB**: Hemoglobin).*

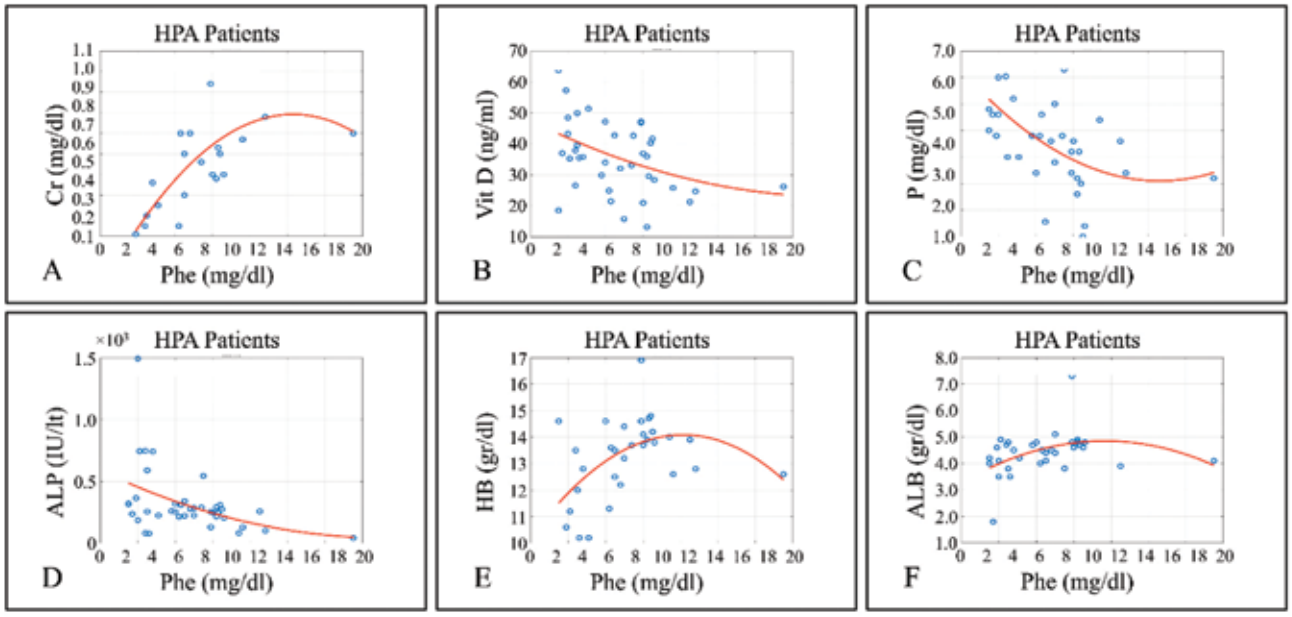

***Figure 10**. Binomial regressions of Phe vs. biochemical factors in HPA patients, independently of gender and diet compliance. In particular, significant regressions were observed between Phe and Cr (R^2=0.5) (**A**), Vitamin D (R^2=0.11) (**B**), Phosphorus (R^2=0.28) (**C**), Alkaline Phosphatase (R^2=0.16) (**D**), Hemoglobin (R^2=0.22) (**E**) and ALB (R^2=0.14) (**F**) (Legend: **Cr**: Creatinine, **Vit D**: Vitamin D, **P**: Phosphorus, **ALP**: Alkaline Phosphatase, **HB**: Hemoglobin, **ALB**: Albumin).*

VOLUME 70 | ISSUE 3 | JULY - SEPTEMBER 2019

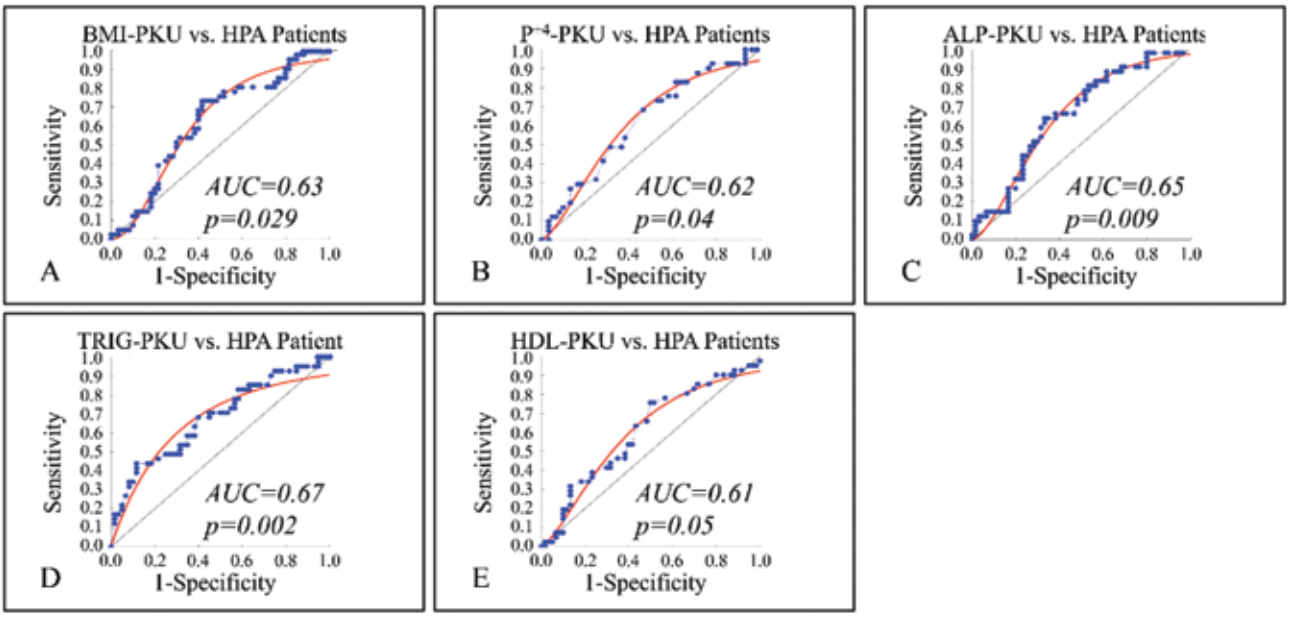

***Figure 11**. ROC analysis of clinical and biochemical factors with respect to PKU type (PKU and HPA). In particular, it appeared that significant Area Under the Curve (AUC) values were manifested by BMI (AUC=0.63, p=0.029) (**A**), Phosphorus (P^{+4}) (AUC=0.62, p=0.04) (**B**), Alkaline Phosphatase (ALP) (AUC=0.65, p=0.009) (**C**), Triglycerides (TRIG) (AUC=0.67, p=0.002) (**D**) and High Density Lipoprotein (HDL) (AUC=0.61, p=0.05) (**E**) (Legend: **AUC**: Area Under the Curve, **BMI**: Bone-Mass Index, **P^{+4}**: Phosphorus, **ALP**: Alkaline Phosphatase, **TRIG**: Triglycerides, **HDL**: High Density Lipoprotein).*

ing binomial regressions, vs. biochemical factors in HPA patients, independently of gender and diet compliance. In particular, significant regressions were observed between Phe and Creatinine (Cr) (R^2=0.5, p<<0.01) (**Figure 10A**), Vit D (Vitamin D) (R^2=0.11, p=0.019) (**Figure 10B**), Phosphorus (P) (R^2=0.28, p=0.015) (**Figure 10C**), Alkaline Phosphatase (ALP) (R^2=0.16, p<<0.01) (**Figure 10D**), Hemoglobin (HB) (R^2=0.22, p<<0.01) (**Figure 10E**) and Albumin (ALB) (R^2=0.14, p=0.04) (**Figure 10F**). ALB was the factor that differed the HPA patients from the PKU and the total population.

Receiver Operating Characteristic (ROC) Analysis

ROC analysis was performed in order to identify, which clinical and biochemical values differentiate between PKU type (i.e HPA and PKU) irrespectively of all other patient characteristics as well as compliance to diet in the patient group irrespectively of all other characteristics. In particular, it appeared that significant Area Under the Curve (AUC) values were manifested by BMI (AUC=0.63, p=0.029) (**Figure 11A**), Phosphorus (P^{+4}) (AUC=0.62, p=0.04) (**Figure 11B**), Alkaline Phosphatase (ALP) (AUC=0.65, p=0.009) (**Figure 11C**), Triglycerides (TRIG) (AUC=0.67, p=0.002) (**Figure 11D**) and High Density Lipoprotein (HDL) (AUC=0.61, p=0.05) (**Figure 11E**). Interestingly, ALP and Phosphorus, two bone-metabolic factors, as well as BMI and Triglycerides, two metabolism-related factors appeared to differentiate PKU types. HDL another metabolism-related factor manifested a marginal significance (**Figure 11E**).

Further on, a similar approach was attempted with respect to compliance to diet. In particular, it appeared that significant Area Under the Curve (AUC) values were manifested by Phenylalanine (Phe) (AUC=0.74, p=0.0005) (**Figure 12B**) alone. This was an interesting result, which showed that Phe levels could be a significant differentiating factor for patient's diet compliance, which is also in agreement with our previous observations as far as Phe is concerned. On the contrary, two factors that appeared in the comparison between PKU

VOLUME 70 | ISSUE 3 | JULY - SEPTEMBER 2019

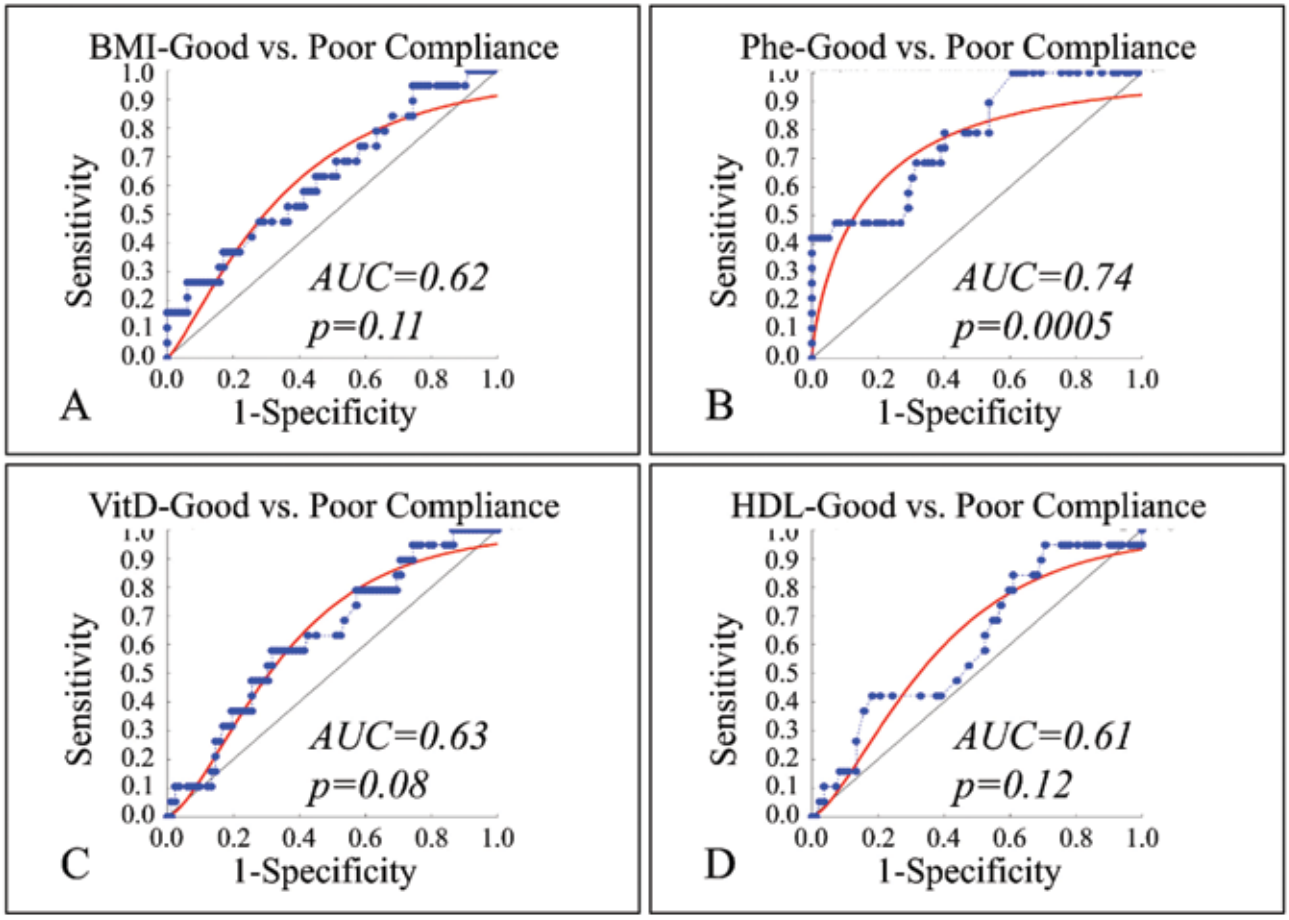

***Figure 12**. ROC analysis of clinical and biochemical factors with respect to diet compliance. In particular, it appeared that significant Area Under the Curve (AUC) values were manifested by Phenylalanine (Phe) (AUC=0.74, p=0.0005) (**B**). On the contrary, two factors that appeared in the comparison between PKU and HPA did now manifest significant AUC when compared with respect to diet compliance. In particular, BMI (AUC=0.62, p=0.11) (**A**) and HDL (AUC=0.61, p=0.12) (**D**) where those variables that could not differentiate between patients with good or poor compliance. Finally, another important factor that manifested marginally significant AUC was Vitamin D (VitD) (AUC=0.63, p=0.08) (**C**) (Legend: **AUC**: Area Under the Curve, **BMI**: Bone-Mass Index, **Phe**: Phenylalanine, **HDL**: High Density Lipoprotein).*

and HPA did now manifest significant AUC when compared with respect to diet compliance. In particular, BMI (AUC=0.62, *p*=0.11) (**Figure 12A**) and HDL (AUC=0.61, *p*=0.12) where those variables that could not differentiate between patients with good or poor compliance. Finally, another important factor that manifested marginally significant AUC was Vitamin D (VitD) (AUC=0.63, *p*=0.08) (**Figure 12D**).

Discussion

Numerous studies have reported low Bone Mineral Density (BMD) or an alteration on bone metabolism in PKU patients, as a well-known long term complication of the disease (16, 28, 29). The pathophysiology is still unclear, yet several factors are implicated: dietary deficiencies (low natural protein intake, calcium, vitamin D, essential fatty acids), PAH gene mutations which are responsible for enzyme loss activity and may probably cause a tendency of osteoporosis appearance in adulthood, possible defects in bone turnover related to the disease itself or possible negative effects of high PHE levels on bone status (17, 23).

VOLUME 70 | ISSUE 3 | JULY - SEPTEMBER 2019

BMI

In the present study population, statistical analysis of BMI demonstrated a significant difference, in the group of PKU patients, with respect to diet compliance, suggesting that poor compliance to diet may lead to overweight or obesity. Previous studies report that PKU patients manifest behavioral issues with regards to food intake (30, 31). During puberty, this problem is enlarged. In the current study, the finding of higher PHE levels in the pubertal group (>10y) vs pre-pubertal (<10y), support this claim and is in line with previous studies (32). It is probable that in adolescence, patients intent to avoid the nutritional supervision from their parents and loose the recommended strict diet, resulting in consuming excess calories and fat from "unhealthy" food (biscuits, chocolate, junk-food etc.) (33). *Doulgeraki* et al (2014) have reported that fat mass was significantly increased in teenagers with PKU, especially in those with poor dietary compliance, suggesting that excessive PHE levels may have negative effects on bone health through the cross-talk between adipocytes and bone cells (21). The high PHE levels may also negatively influence patient's mood (33), affecting some organizational functions (e.g. preparing healthy meals) or decreasing the physical activity (34-37).

Phenylalanine

On the other hand, the encouragement of low protein foods (rice, pasta or bread) consumption, could contribute to their excessive weight. Additionally, Vitamin D levels presented a negative correlation to PHE concentration in all subtypes of the disease (PKU/HPA). This observation has important clinical implication because it implies that patients with increased PHE levels should be targeted towards regular assessment of the vitamin D status and prompt treatment. In agreement with previous studies, life style, physical activity, sun exposure and nutritional habits, are some of the factors which may play an important role to this finding (17, 23, 38). Generally, a trend towards overweight or obesity has been reported in PKU patients but recent studies do not confirm this (39). Better understanding of the food consumption patterns, energy and nutrient intakes as well as detailed assessment of the level of physical activity of PKU patients are necessary in everyday clinical practice, in order to prevent comorbidities due to excessive weight gain.

Low PHE diet has been defined as non-atherogenic, with low serum cholesterol levels to be reported in previous studies, in well treated PKU individuals (40). The reduced intake of natural protein, which is the main feature of this specific diet, could be the explanation. In this study, cholesterol levels are higher in pubertal group as compared to the pre-pubertal group, showing that teenagers are trying to liberalize from the strict dietary compliance. In addition, no significant correlation has been found between cholesterol and phenylalanine levels, in all patients. Nevertheless, serum cholesterol levels are equally depended on diet intake and its biosynthesis (cholesterogenesis).

Previous studies have reported that increasing PHE levels may play a negative role in cholesterol biosynthesis, yet other reports don't support this relationship (41). Thus, the interpretation of low cholesterol concentration, in PKU patients, is more complicated. Longitudinal follow up of the cholesterol intake of the patients would allow safer conclusions. In addition, triglycerides present higher levels in PKU group than HPA, as a result of the low phenylalanine diet which is enriched in carbohydrates (42).

Phosphorus

Phosphorus is an essential nutrient for bone which with calcium forms hydroxyapatite. It's also a component of most foods in large amounts. Its adequate intake is necessary for optimal bone accretion and normal bone development among others (e.g. protein). Especially, in recent years, the amino acid PHE-free mixtures are enriched with Vitamins and Minerals (VM). In the current study, a negative linear regression is reported between phosphorus levels and PHE concentration, both in PKU as well as HPA patients, although phosphorus values were within the reference ranges. This result was in agreement with previous studies, yet with no

VOLUME 70 | ISSUE 3 | JULY - SEPTEMBER 2019

further identification of clinical importance (43). Considering that elevated PHE levels are reflecting poor dietary compliance, this tendency may manifest some nutritional deficiencies and/or an implication of the disease itself. Further and longitudinal investigations are needed in order to draw safe conclusions.

Alkaline Phosphatase (ALP)

A negative correlation was observed between ALP and PHE levels, as well as a positive correlation was found between creatinine and PHE values in all studied groups (PKU and HPA). ALP, which is a crucial factor of bone formation, may be adversely affected by high PHE levels (44, 45).

Calcium

In the PKU group, a negative correlation has been observed between calcium serum levels and PHE concentrations. This result is in line with previous studies which reported nutritional deficiencies in this population (Vitamin D, B12, Ca), due to the strict diet, leading to long-term complications like low bone mineral density (46, 47), as well as increased 24-h urinary excretion of calcium consequently of low PHE diet in combination with the diet "formula" (Amino Acid-Medical Food (AA-MF)) (19). Given that lifelong intake of this formula leads to high dietary acid load, high concentration of H^+ and decreasing pH results in increased bone resorption and higher urinary calcium excretion which is associated with a lower BMD and increased fracture risk (48, 49). *Hennerman* et al (2012), also refers in her study that 23% of the study population, all patients with PKU in lifelong diet, demonstrate hypecalciuria (50).

Creatinine

Finally, creatinine is a rough indicator of renal function, which manifested an increasing trend, positively correlated to PHE level, in all patients. This finding is in line with previous reports, which support the idea that lifelong intake of AA-MF may result in an impairment of renal function, contributing to the etiology of skeletal fragility in PKU (51). The inclusion of routine urinary tests in the follow up of those patients could be a safe strategy to ensure good renal function, as some experts suggest (48, 50, 51).

ROC Analysis

In addition, to our previous investigations we have used ROC analysis in order to identify biochemical or clinical factors that could differentiate between patient groups. In agreement to previous studies our results showed that ALP, Phosphorus and Phe are the most significant factors that discriminate between patient groups. Especially, the finding that Phenylalanine did not manifested significant results with respect to PKU type but only with respect to diet compliance, is very interesting since it indicates that Phe levels are affected in both PKU deficiencies in a similar manner, yet Phe appears to be significantly affected by the diet. It is possible that Phe levels could be used as a diagnostic tool for diet compliance identification, since BMI did not differentiate with respect to diet compliance.

Study Limitations

This is a cross-over study, where data capture the specific period of the patient's lifespan and do not reflect the lifelong interventions. Serial laboratory monitoring could be more informative and helpful for safer conclusions. Moreover, the assessment of their lifestyle (dietary habits and physical activity) was not recorded in detail and the study group was large, with a wide age range and on various therapy options. Finally, 25(OH) D measurement was included in the follow-up protocol only recently.

Conclusions

Of note, the investigation of PKU patients' basic bone profile showed some interesting correlations with phenylalanine levels. Good dietary compliance still remains the cornerstone of good health in these individuals. Additionally, a healthy lifestyle, which includes adequate intake of calcium and vitamin D and exercise, as well as diet and treatment improvements (e. g medical formula enriched in natural protein) would be beneficial for bone health. In conclusion, the degree of dietary compliance is key to the optimal biochemical

VOLUME 70 | ISSUE 3 | JULY - SEPTEMBER 2019

bone profile of the patients with PKU and HPA. Those individuals with high PHE levels and excessive weight should be the targets of counseling and regular monitoring of bone health.

Conflict of interest:

The authors declared no conflicts of interest.

Declarations

Ethics approval and consent to participate: Please refer to the "Materials and Methods" section.
Consent for publication: Not applicable
Availability of data and material: The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request. Please also refer to the "Materials and Methods" section.
Competing interests: Nothing to declare.
Funding: No funding received.
Authors' contributions: MK: Collected data, performed clinical evaluation), drafted the manuscript. KHS: Provided data. AD: Provided data and critically reviewed the manuscript. GIL: performed data analysis, drafted the manuscript, proof-edited the manuscript and gave final permission for publication.

Aknowledgements

We wish to thank the patients and their families.

LIST OF ABBREVIATIONS

Abbreviation	Explanation
ALP	Alkaline Phosphatase
BH4	Tetrahydrobiopterin
BMI	Body Mass Index
CHOL	Cholesterol
Cr	creatinine
HDL	High Density Lipoprotein
HPA	Hyperphenylalaninemia
LDL	Low Density Lipoprotein
NBS	Newborn Screening
PAH	Phenylalanine hydroxylase enzyme
PHE (or Phe)	Phenylalanine
PKU	Phenylketonuria
TG	Triglycerides
Tyr	Tyrosine

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VOLUME 70 | ISSUE 3 | JULY - SEPTEMBER 2019

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