European Journal of Pediatrics (2019) 178:903–911
https://doi.org/10.1007/s00431-019-03365-0

ORIGINAL ARTICLE

Asymmetric dimethylarginine as a potential biomarker for management and follow-up of phenylketonuria

Fernando Andrade[1] · Olatz Villate[1] · María L. Couce[2] · María A. Bueno[3] · Carlos Alcalde[4] · Javier de las Heras[1,5,6] · Leticia Ceberio[1,7] · Sergio Núñez-Marcos[1] · Pablo Suárez Nambo[1] · Luis Aldámiz-Echevarría[1,5,6]

Received: 21 December 2018 / Revised: 7 March 2019 / Accepted: 13 March 2019 / Published online: 2 April 2019

Abstract

Phenylketonuria's (PKU) treatment based on low-protein diet may affect other metabolic pathways, such as that of asymmetric dimethylarginine (ADMA). The aim of this study was to evaluate the reliability of ADMA as a biomarker of adequate metabolic control and possible nutritional risk in a long-term PKU patient population. One hundred and six dietary-treated PKU patients from four hospitals in Spain were enrolled in this cross-sectional study. Their lipid profile, total homocysteine, ADMA, and symmetric dimethylarginine (SDMA) concentrations were analyzed and compared with a control group. Sensitivity, specificity, and likelihood ratios of the proposed biomarker were calculated. PKU patients had statistically significant lower plasmatic ADMA, SDMA, and arginine concentrations as compared with the control group ($p < 0.001$). Significant correlations were found between ADMA, phenylalanine, and total homocysteine levels. The ADMA/creatinine ratio correlated with phenylalanine levels as metabolic control and nutritional risk in PKU patients. Its reliability as a management biomarker was studied with positive results. The ADMA/creatinine ratio might serve as an independent biomarker in the management of PKU patients, different from blood phenylalanine levels. It could be of particular usefulness to detect those who are following an unbalanced diet that could have long-term negative effects.

Conclusion: In this study, we have evaluated the reliability of ADMA as a potential biomarker of adequate metabolic control and possible nutritional risk in a long-term PKU patient population.

What is Known:

• *Although PKU individuals have lower values of ADMA even with blood Phe levels in the recommended range, little attention is payed to other metabolic pathways.*

What is New:

• *ADMA could be used as new biomarker for PKU management and follow-up of the diet, after evaluating their reliability in a long-term PKU patient population.*

Keywords Asymmetric dimethylarginine · Biomarker · Phenylketonuria

Communicated by Peter de Winter

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00431-019-03365-0) contains supplementary material, which is available to authorized users.

✉ Luis Aldámiz-Echevarría
luisjose.aldamiz-echevarazuara@osakidetza.eus

1 Group of Metabolism, Biocruces Bizkaia Health Research Institute, CIBER de Enfermedades Raras (CIBERER), Plaza de Cruces 12, 48903 Barakaldo, Spain

2 Metabolic Disorders Unit, Santiago de Compostela University Hospital, IDIS, CIBERER, Travesía de Choupana s/n, 15706 Santiago de Compostela, Spain

3 Metabolic Disorders, Dietetics and Nutrition Unit, Virgen del Rocío University Hospital, Manuel Siurot Avenue, s/n, 41013 Sevilla, Spain

4 Paediatrics Unit, Río Hortega University Hospital, Calle Dulzaina 2, 47012 Valladolid, Spain

5 Division of Pediatric Metabolism, University Cruces Hospital, Barakaldo, Spain

6 University of the Basque Country (UPV/EHU), Barakaldo, Spain

7 Department of Internal Medicine, University Cruces Hospital, Barakaldo, Spain

Abbreviations

ADMA	Asymmetric dimethylarginine
Arg	Arginine
Crn	Creatinine
DDAH	Dimethylarginine dimethylamino hydrolase
HPA	Hyperphenylalaninaemia
LR	Likelihood ratios
NO	Nitric oxide
NOS	Nitric oxide synthase
Phe	Phenylalanine
PKU	Phenylketonuria
PV	Predictive values
SDMA	Symmetric dimethylarginine
tHcys	Total homocysteine

Introduction

The deficiency of the enzyme phenylalanine-4-hydroxylase (PAH), which catalyzes the conversion of the essential amino acid phenylalanine (Phe) to tyrosine [11], results in phenylketonuria (PKU, MIM #261600). This disease is the most common inborn error of amino acid metabolism. Since PKU is included in the newborn screening programs, dietary treatment, which consists of a low-Phe diet, is usually initiated soon after birth to avoid neurological damage and developmental delay. This diet consists of a marked reduction in natural protein and requires supplementation with Phe-free protein substitutes and specially manufactured low-protein foods [12, 24, 29] without specific medications.

Arginine (Arg), as essential amino acid, follows a specific methylation pathway because Arg residues within proteins can be methylated by means of protein-arginine-methyltransferases (PRMTs). This methylation results in three different derivates: N-monomethyl-L-arginine (NMMA), asymmetric dimethylarginine (ADMA), and symmetric dimethylarginine (SDMA). ADMA levels depend on dimethylarginine dimethyaminohydrolase (DDAH) activity which metabolizes ADMA. Arg can be oxidated to citrulline (Citr) and nitric oxide (NO) by nitric oxide synthase (NOS) [17, 28]. ADMA is an inhibitor of NOS, and healthy, normally functioning endothelial cells depend on the bioactivity of NO as an important physiological mediator of vascular tone and vascular structure [3, 4]. Altered ADMA levels, and subsequently disturbed NO synthesis, are the result of reduced DDAH activity, which is expressed in the liver and kidneys [18] and degrades ADMA, but not SDMA, to Citr and dimethylamine (Fig. 1). This metabolic pathway is related to methylation capacity and the necessity of some molecules to be methylated for being active, such as guanidinoacetate to produce creatine, or DNA, neurotransmitters, hormones, and immune cells, competing with homocysteine remethylation to methionine (Met) which also consumes methyl groups. So, the methylation capacity has a key influence in DNA/RNA synthesis, brain chemical production of dopamine and serotonin, hormonal breakdown, creation of immune cells (NK cells and T cells), myelin formation, or detoxification.

Early diagnosis by newborn screening and Phe restriction has been successful in preventing disturbances in the majority of patients. However, several current studies described that PKU individuals have lower ADMA values and a higher prevalence of oxidative stress status due to restricted diets and accumulation of toxic metabolites even with blood Phe levels in the recommended range [15, 16, 19]. Oxidative stress parameters are measured by nitrates/nitrites, cytokines [3], thiobarbituric acid-reactive species (TBA-RS), total antioxidant reactivity (TAR), antioxidant enzymes catalase (CAT), superoxide dismutase (SOD), or glutathione peroxidase (GSH-Px) [25]. Nevertheless, the management of PKU has been solely based on blood Phe levels with little attention to long-term markers related to other metabolic pathways. Until now, there was no valuable test to optimize dietary treatment based on ADMA pathway, taking into account the fact that ADMA is a methylated and stable molecule which does not change during a period of time without any modification of the diet. These methyl groups come from Met that is another essential amino acid coming from diet. Low-protein diet of PKU patients could reduce the amount of methyl groups available to complete this methylation cycle [13]. Once Met loses its methyl group, it is converted to homocysteine by transmethylation [27]. Due to this methylation process, ADMA itself gives us a non-punctual value for PKU management in contrast to Phe in blood, because the methylation reactions produce a more stable compound which remains constant during a short period of time, although the diet followed by the patient could slightly change.

We hypothesize that levels of cardiovascular markers, such as dimethylated arginines, could be used as new biomarker for PKU management and follow-up of the diet. The aim of this study was to evaluate the reliability of ADMA as a biomarker in a long-term PKU patient population. For this purpose, sensitivity, specificity, and likelihood ratios (LR, both positive and negative) of the proposed biomarker were calculated.

Materials and methods

Patients

In this cross-sectional study, 106 patients with PKU from Cruces University Hospital, Santiago de Compostela Clinic University Hospital, Virgen del Rocío University Hospital, and Río Hortega University Hospital were enrolled. The patients enrolled in the study including 69 treated exclusively with Phe-restricted diet, and 37 patients receiving (6R)-L-erythro-5,6,7,8-tetrahydrobiopterin (6R-BH_4, sapropterin

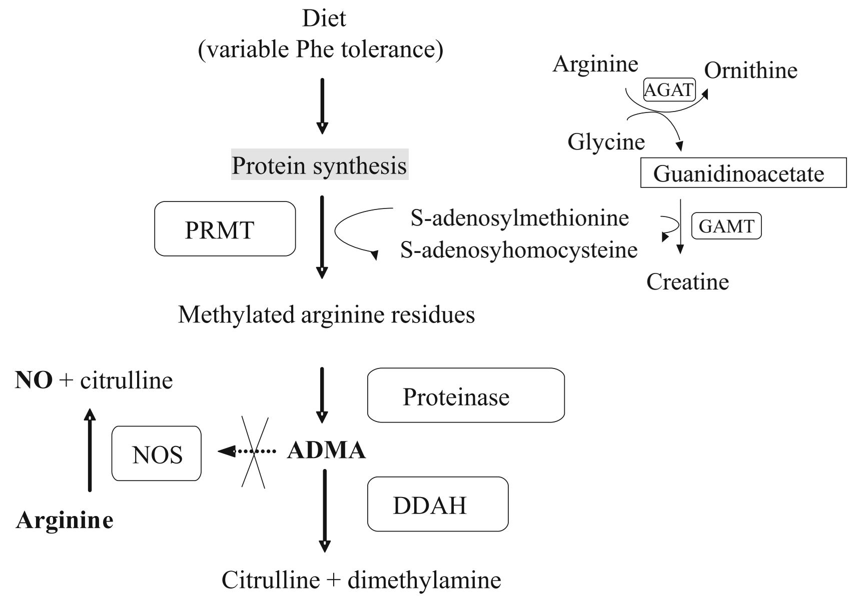

Fig. 1 Arginine (Arg) residues within proteins can be methylated by means of protein-arginine-methyltransferases (PRMT) to give asymmetric dimethylarginine (ADMA). ADMA levels depend on dimethylarginine dimethyaminohydrolase (DDAH) activity which metabolizes ADMA to citrulline (Citr) and dimethylamine. Arg can be oxidated to Citr and nitric oxide (NO) by nitric oxide synthase (NOS), but ADMA is a competitive inhibitor of NOS

dihydrochloride [KUVAN®, Merck, Madrid, Spain]) with a less restrictive diet, were diagnosed through newborn screening programs. A definitive diagnosis was obtained by mutation analysis of the *PAH* gene. Taking into account the European guidelines and the classification [30] depending on plasma Phe levels at the time of diagnosis and the Phe tolerance and median blood Phe levels in the previous year, patients were classified into one of three phenotypic categories [6]: mild hyperphenylalaninaemia (mild HPA) (360–600 μM), mild–moderate PKU (600–1200 μM), and classic PKU (> 1200 μM). The inclusion criteria for the study were (a) early diagnosis of PKU; (b) early and continuous treatment with Phe-restricted diet, supplemented with Phe-free substitutes and specially manufactured low-protein foods; (c) absence of any other diseases known to affect physical development; and (d) regular attendance to their scheduled clinical checkups. Adherence to treatment in patients with PKU was established according to their metabolic control by annual median blood Phe levels and the pre-established "safe" thresholds for each age [29].

Collected data for each patient were age, gender, phenotype, protein tolerance, Phe tolerance, lipid profile, total homocysteine (tHcys), ADMA, SDMA, and Arg. Control values for amino acids, ADMA, SDMA, and tHcy levels in plasma were determined in 40 healthy volunteers matched by age and gender. For the other variables, the reference ranges used in Biochemistry Laboratory at Cruces University Hospital (Spain) were taken.

The study protocol was approved by Clinical Research Ethics Committees at the hospitals involved and performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. Written informed consent was obtained from patients and parents or legal guardians of all the children included as patients or controls.

Analytical methods

Biological samples were taken in the morning, after an overnight fasting, at one time during regular follow-up hospital visits. Blood samples using EDTA as anticoagulant were immediately centrifuged at 1000×g for 5 min at 4 °C. The platelet-poor plasmas were aliquoted and stored at − 80 °C until the assay was performed, usually within a few days. Cholesterol, triglycerides, tHcys, and creatinine (Crn) were measured using standard laboratory techniques in Biochemistry Laboratories at four hospitals. Protein intake and Phe tolerance were calculated according to the 3-day food records completed by adult patients or parents/legal guardians.

Arg levels and its derivatives (ADMA and SDMA) were determined by a 1100 high-performance liquid chromatography (HPLC) system coupled to a 6410 triple quadrupole mass spectrometer (both from Agilent Technologies, Madrid, Spain), as previously described [2]. Briefly, sample preparation plasma proteins were removed by ultrafiltration using centrifugal filters, avoiding derivatization, solid-phase extraction, and organic solvents or acids. Reverse phase chromatography was performed for ADMA-SDMA separation in order to use the most sensitive mass transition (m/z 203.2–70.1). Positive electrospray ionization was performed, and analytes were detected by multiple reaction monitoring.

Statistical analysis

Statistical analysis was performed using SPSS® 23.0 for Windows (IBM, Chicago, IL). Descriptive statistics are presented as median and 3rd–97th percentile range. Initially, all data were analyzed using the Kolmogorov-Smirnov test to check the normal distribution of the data. Differences between groups were assessed using the Student's *t* test. With the purpose of determining correlations, logarithmic function was applied to non-normal variables to convert them into normal variables. Pearson correlation coefficients (r) were used to assess bivariate relationships. Statistical significance was set at the $p < 0.05$ level.

Results

Our study included 106 dietary-treated PKU patients (52 males, 54 females): 59 classic PKU, 34 moderate PKU, and 13 mild HPA. Median age was 10.7 years; P_3 and P_{97} were 1.2 and 42.1 years, respectively. Supplementary Table 1 shows patient classification by genetic mutations and BH_4 sensitivity, showing missense mutations in 79.7% of the cases, splicing in 9.0%, nonsense in 3.3%, and frameshift in 2.8%. Two patients below 12 years of age had Phe values above 360 μM which is the maximum value for this age. Eight patients above 12 years of age displayed Phe values above 600 μM, which is the cutoff value for this age. Supplementary Table 2 shows the nutritional and lipid profile for PKU population.

Patients with PKU had statistically significant lower plasmatic ADMA and SDMA ($p < 0.001$) levels (Supplementary Table 3), as well as Arg levels ($p < 0.001$) than the control group (Fig. 2).

We performed statistical analyses between males and females for ADMA (0.2–0.6 μM for males, 0.2–0.6 μM for females) and SDMA (0.3–0.6 μM for males, 0.3–0.6 μM for females) among PKU patients, but there was not statistically significance (p not significant) between ranges for both gender groups. There was significant difference between males and females for Crn (0.16–1.02 μM for males, 0.20–0.77 μM for females, $p = 0.034$).

Analyzing the correlations among variables, we found that there was a statistically significant inverse correlation between ADMA levels and tHcys levels ($r = -0.279$, $p = 0.020$). Positive correlations were observed between the median Phe plasma levels obtained from the measurements performed during 1 year and total cholesterol ($r = 0.204$, $p = 0.044$) and triglycerides ($r = 0.210$, $p = 0.037$). Median Phe plasma concentrations significantly correlated with age ($r = 0.562$, $p < 0.001$) and Crn ($r = 0.575$, $p < 0.001$), but inversely with ADMA ($r = -0.310$, $p = 0.002$) (Fig. 3) and Arg ($r = -0.261$, $p = 0.009$). As it is reported, ADMA levels are higher for children and adolescents [1]. In this study, ADMA levels have an inverse correlation with age ($r = -0.547$, $p < 0.001$). Crn plasmatic concentrations significantly correlated with tHcys ($r = 0.490$, $p < 0.001$), but inversely with ADMA ($r = -0.451$, $p < 0.001$), and there was no correlation with SDMA for our population ($r = 0.033$, $p = 0.747$). SDMA was only positively correlated with ADMA ($r = 0.543$, $p < 0.001$) as expected, but not with any other variable. ADMA/SDMA ratio is shown in Table 2.

Taking into account the correlation of ADMA with median Phe levels and Crn, the values of ADMA were normalized by Crn. This ratio ADMA/Crn has a correlation with median Phe recorded during the previous year ($r = -0.463$, $p < 0.001$), so this variable was used in a linear regression representation (Fig. 4).

This ratio was tested as a new cutoff biomarker for PKU management, because when this ratio increases, the Phe level decreases. So, it could be used to predict the good adherence to the diet and the quality of the follow-up. Taking into account several cutoffs for this ratio, the best results for sensitivity and specificity were obtained with 0.7 (Table 1).

Therefore, the reliability of the ADMA/Crn as a new biomarker for PKU management and follow-up was analyzed through its specificity, sensitivity, predictive values (PVs), and LR (positive and negative) (Table 1). Trying to define the ambiguous categories for these calculations, ADMA/Crn ratio < 0.7 was considered a positive result because in most cases, the patients have the risk of a poor metabolic control (Phe > 360 μM). Its counterpart, ADMA/Crn ratio > 0.7 was a negative result as Phe levels are supposed to be lower than 360 μM or suitable control for the patient (Table 1). It has been suggested that a new biomarker is clinically useful when its lowest LR and highest PV reach an equilibrium giving sense to our casuistic and empirical results [5].

Considering PKU population as two separate groups depending on being under BH_4 treatment or not, levels of ADMA, SDMA, Arg, ADMA/Arg, and ADMA/Crn ratios did not show any statistical difference between both groups. However, ADMA/Crn ratio has a negative correlation with median Phe values ($r = -0.502$, $p < 0.001$ for exclusively protein-restricted diet group, and $r = -0.432$, $p = 0.011$ for BH_4 group) in the same way as the whole PKU group. Moreover, if the exclusively protein-restricted diet group not under BH_4 is taken, the statistical results for ADMA/Crn ratio as biomarker are improved (Table 2), increasing the specificity, positive PV, and positive LR.

If the PKU population is divided into two groups depending on age, above or below 12 years, there are no significant results for ADMA levels and ADMA/Crn ratio.

Discussion

Novel aspects of NO metabolism related to the pathway of ADMA as an inhibitor of NOS and higher cardiovascular risk as arterial stiffness are investigated in PKU patients with low-protein diets. This work aimed to analyze the reliability of ADMA as a new cardiovascular biomarker for management

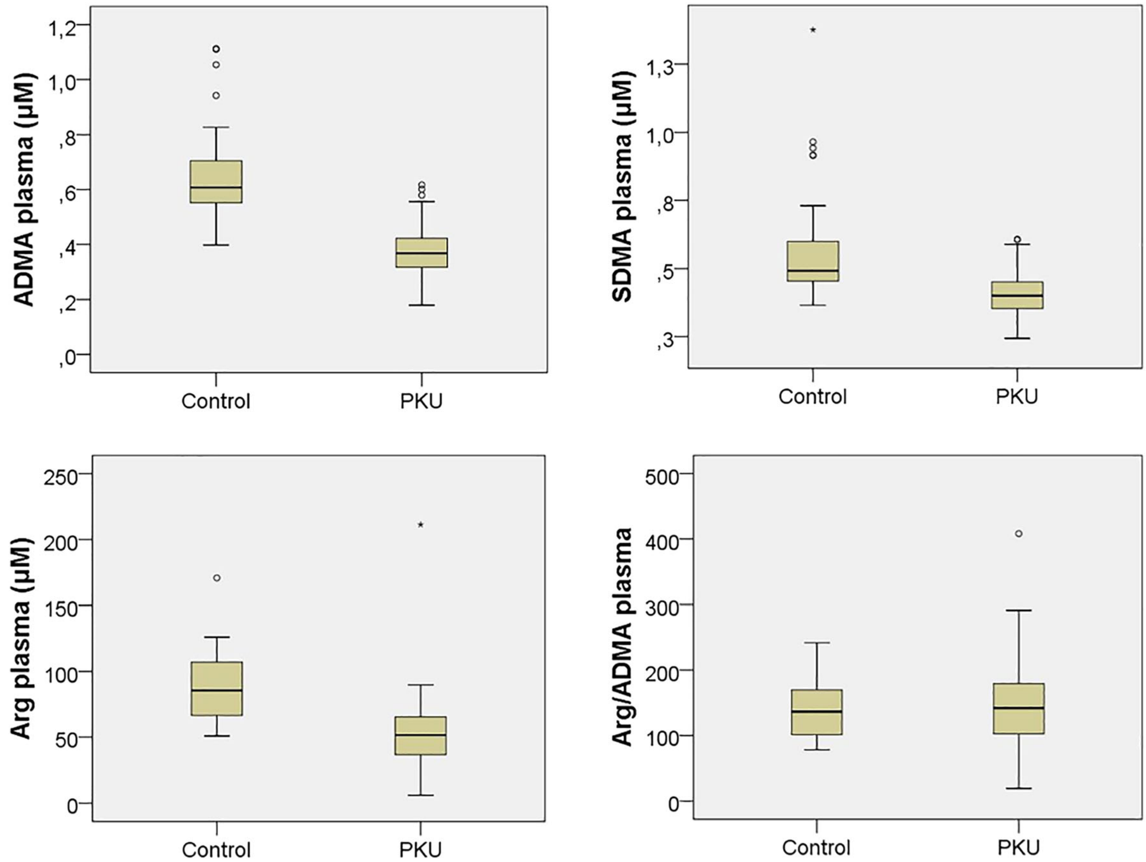

Fig. 2 Plasma levels of asymmetric dimethylarginine (ADMA), symmetric dimethylarginine (SDMA), arginine (Arg), and Arg/ADMA ratio in patients with PKU in comparison with the control group

and follow-up in PKU patients in order to establish a relationship between cardiovascular risk and nutritional status.

Nowadays, the Phe measured at one point in time is used as a biomarker for patients' follow-up. Due to the variability of this determination and the lack of information about adherence, the median of blood Phe levels is commonly used to give information about the status for a period of time, usually for 1 year [7].

It should be pointed out that hypocholesterolemia has been related to patients with PKU who had good adherence to the diet [9, 22]. In our study, there was significant correlation between Phe levels and triglycerides and total cholesterol.

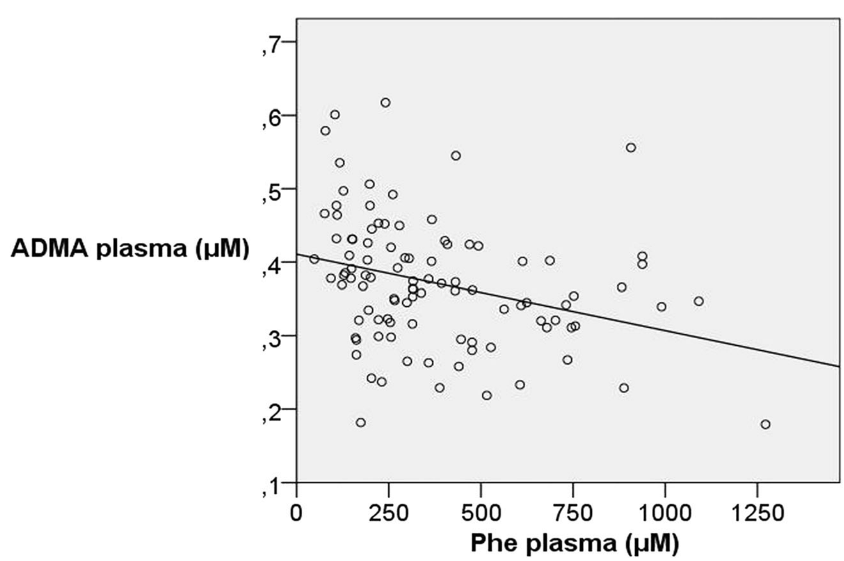

Fig. 3 Correlation between Phe (µM) and ADMA (µM) by linear regression ($r = -0.310$, $p = 0.002$)

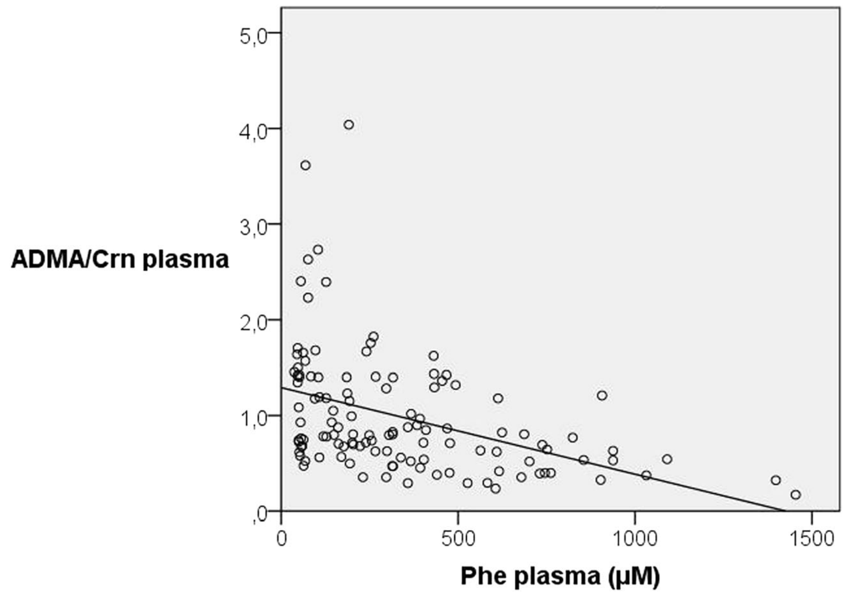

Fig. 4 Correlation between Phe (μM) and ADMA/Crn ratio by linear regression ($r = -0.463$, $p < 0.001$)

This result suggests that low cholesterol intake due to low-protein diet may be the cause of hypocholesterolemia, rather than a role of Phe in the inhibition of cholesterol biosynthesis [8]. However, cholesterol fractions should not be used as follow-up biomarkers because of low sensitivity.

The prealbumin was also said to be a biomarker of protein nutritional status [20]. However, the prealbumin could suffer from a turnover of 24–48 h and does not represent an effective evaluation of diet follow-up for a long period of time. In this sense, if the patients take care of their diets before the sample extraction and clinical control, the prealbumin levels could be affected and non-representative. Other biomarkers should be used as a long-term biomarker for diet management, not affected by the turnover.

In our study, there were no differences for ADMA or SDMA between males and females, probably due to low-protein diet altering competition for methyl groups between ADMA, creatine, or homocyesteine remethylation. So, low-protein diet affects equally the creatine levels for males and females. However, there is significant difference for Crn which is higher for male patients because this metabolite is more gender-related than age-related.

It is widely accepted that dietary management and guidance of PKU individuals should be lifelong; otherwise, the PKU patients non-compliant with diet and consequently with high Phe levels could suffer from neurocognitive dysfunction. For PKU children compliant with diet, normal neurocognitive development is the main goal; however, higher cardiovascular risk than the healthy population is a possible effect of low-protein diet [21, 31] and higher Phe levels [14]. Among the cardiovascular problems, the PKU patients could suffer from increased aortic stiffness, lower blood pressure, higher prevalence of obesity, lower total cholesterol, and LDL cholesterol [23]. High blood Phe is known to inhibit protein and peptide synthesis, and consequently, compounds as ADMA that comes from residues within proteins. This may explain why PKU infants and children are more susceptible to intellectual disability and microcephaly, and PKU adults tend to develop neuropsychological disturbances, such as anxiety or depressive disorders, which are probably due to secondary chronic neurotransmitter deficiencies [10]. PKU individuals who are under poor dietary control are reported to have ADMA deficiencies, as evidenced by Özcan et al. [19]. The results of this study also show low levels of tHcys and ADMA in PKU patients indicating the possible influence of ADMA/Crn ratio

Table 1 Reliability of ADMA/Crn as PKU management biomarker

	Median Phe < 360 μM	Median Phe > 360 μM
ADMA/Crn < 0.7	15% (FP)	29% (TP)
ADMA/Crn > 0.7	43% (TN)	13% (FN)

Sensitivity = TP / (TP + FN) = 69%. Specificity = TN / (FP + TN) = 74%. Positive predictive value = TP / (TP + FP) = 66%. Negative predictive value = TN / (TN + FN) = 77%. Positive likelihood ratio = sensitivity / (1 − specificity) = 2.7. Negative likelihood ratio = (1 − sensitivity) / specificity = 0.4

Table 2 Reliability of ADMA/Crn as PKU management biomarker for protein-restricted diet group not under BH_4

	Median Phe < 360 μM	Median Phe > 360 μM
ADMA/Crn < 0.7	14% (FP)	33% (TP)
ADMA/Crn > 0.7	45% (TN)	8% (FN)

Sensitivity = 57%. Specificity = 85%. Positive predictive value = 80%. Negative predictive value = 65%. Positive likelihood ratio = 3.7. Negative likelihood ratio = 0.5

in PKU management, and being useful evaluating the adherence and the alteration of secondary metabolic pathways.

As far as the metabolic pathways are concerned, it should be noted that tHcys synthesis is partially ensured due to polyvitamin status provided in the amino acid supplements, which are enriched in vitamin B_{12} and folic acid, cofactors of remethylation and transsulfuration. To the best of our knowledge, there is no evidence of an impaired methylation cycle for PKU patients. However, as it has been previously described [13, 15, 16, 19], we have observed decreased ADMA levels and tHcys levels at the lower limit of the normal range in our PKU population with amino acid supplementation. This fact could alter the NO formation by higher NOS activity and the methylation cycle. However, previous study measuring nitrate and nitrite levels stated that NO metabolism was almost unaltered in PKU patients [16]. In spite of this, neuronal NOS should be studied in future studies on PKU relating neurocognitive function and cardiovascular risk.

It is known that increased levels of tHcys may cause accumulation of ADMA due to the homocysteine effect on the enzymatic activity of DDAH, which degrades ADMA to citrulline and dimethylamine. In particular, the union to homocysteine modifies DDAH spatial configuration and inhibits its activity [26]. Nevertheless, we found that tHcys levels were at the lower limit of normality (Table 2) and inversely correlated with ADMA levels in this PKU population. This result shows that there is a strong competition for methyl group between Arg methylation to produce ADMA and homocysteine remethylation because several compounds as Arg, guanidinoacetate, or hormones demand methyl groups to be methylated in ADMA, SDMA, creatine, or methylated hormones, respectively, as stable metabolites. In fact, low-protein diet for PKU diets means a low intake of Met, so this fact could restrict the source of methyl groups to methylate the described demethylated compounds. This theory could be an issue to be studied in further research. Our study also shows direct evidence that ADMA metabolism is disturbed in PKU population under low-protein diet with significant effect and relation with blood Phe levels. The ADMA levels did not reach control levels; thus, it may be necessary to analyze the status of this pathway by checking ADMA/Crn ratio to achieve further improvement by means of supplementation.

Several therapeutic possibilities could be suggested in order to improve PKU diet and treatment. Nevertheless, we firstly consider the ADMA/Crn ratio as a long-term marker for PKU due to its direct relation with Phe levels. The reliability of the ADMA/Crn ratio as a new biomarker for follow-up of PKU patients was not previously tested. The sensitivity and specificity of the ADMA/Crn ratio were 69% and 74%, respectively, when this ratio was below 0.7, predicting Phe levels above 360 μM which means a poor analytical control. In our study, the ADMA/Crn analysis can produce false-negative results in PKU patients with very low ADMA levels, which is more likely to happen in poor-controlled patients with higher Phe levels. As far as the LR are concerned (PLR and NLR), the values provided by the ADMA/Crn ratio (PLR = 2.7; NLR = 0.4) are near to those which have been proposed as clinically useful. The ADMA/Crn ratio is clearly able to distinguish between patients with poor diet management and appropriate control by means of even PV. Concerning LR, from our point of view, analyzing more PKU patients, higher PLR and lower NLR would be obtained. For this reason, it has been used in several centers with PKU population of some collaborating hospitals in Spain to detect poor-controlled PKU patients with nutritional issues. So, we tested the reliability of the ADMA/Crn ratio as progress evaluation test, because the patients were under long-term low-protein diet. Therefore, this ratio could be used as a method to follow up the evolution of patients, in addition to its possible use as a tool for poor methylation capacity.

Moreover, considering that BH_4 as a treatment could have a pleiotropic effect on ADMA metabolism, we have taken into account the exclusively protein-restricted diet group not receiving BH_4 as a group, improving specificity, PPV, and PLR for ADMA/Crn ratio (Table 2).

The present study has demonstrated the potential usefulness of the ADMA compound and its ADMA/Crn ratio as a tool for detecting PKU patients with poor long-term dietary control suffering from PKU. We have therefore developed a simple and mass spectrometric test based on the ADMA levels which has shown very good results, as far as sensitivity and specificity values are concerned. The test was designed for the evaluation of PKU management, given that ADMA/Crn ratio might produce false-negative results, due to their low ADMA levels for properly controlled patients. Further PKU samples would be necessary to ensure the tendency of statistical parameters.

In conclusion, our study suggests that ADMA analysis would be useful for an analysis of problematic follow-up among PKU patients under suspicion of suffering from adherence problems, as well as poor long-term nutritional management. The test would be able to make a follow-up evaluation and reduce the number of individuals with nutritional risk. ADMA and Crn may serve as independent long-term biomarkers, different from blood Phe, reflecting NO metabolism in the patients. Monitoring these biomarkers would optimize dietary therapy with medical food products which may prevent long-term cardiovascular disturbances, assuring normal neurocognitive development.

Acknowledgements We thank all patients for kindly participating in the study. This work was supported in part by NUTRICIA S.L.R. (Madrid, Spain). Research in this paper was partially financed by Biocruces Bizkaia Health Research Institute and Carlos III Health Research Institute.

Authors' contributions LAE, MLC, MAB, CA, JH, and LC designed the study, conducted the research, and corrected the final version of the manuscript. FA, OV, SNM, and PSN analyzed samples and data and wrote the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethics approval The study was approved by Clinical Research Ethics Committees at the hospitals involved and performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.

Informed consent Informed consent was obtained from all individual participants included in the study.

References

1. Aldámiz-Echevarría L, Andrade F (2012) Asymmetric dimethylarginine, endothelial dysfunction and renal disease. Int J Mol Sci 13:11288–11311
2. Andrade F, Llarena M, Lage S, Aldámiz-Echevarría L (2015) Quantification of arginine and its methylated derivatives in healthy children by liquid chromatography-tandem mass spectrometry. J Chromatogr Sci 53(5):787–792
3. Antoniades C, Tousoulis D, Marinou K, Vasiliadou C, Tentolouris C, Bouras G, Pitsavos C, Stefanadis C (2006) Asymmetrical dimethylarginine regulates endothelial function in methionine-induced but not in chronic homocystinemia in humans: effect of oxidative stress and proinflammatory cytokines. Am J Clin Nutr 84(4):781–788
4. Böger RH, Bode-Böger SM, Szuba A, Tsao PS, Chan JR, Tangphao O, Blaschke TF, Cooke JP (1998) Asymmetric dimethylarginine (ADMA): a novel risk factor for endothelial dysfunction. Circulation 98:1842–1847
5. Bossuyt PM (2010) Clinical validity: defining biomarker performance. Scand J Clin Lab Invest Suppl 242:46–52
6. Camp KM, Parisi MA, Acosta PB, Berry GT, Bilder DA, Blau N, Bodamer OA, Brosco JP, Brown CS, Burlina AB, Burton BK, Chang CS, Coates PM, Cunningham AC, Dobrowolski SF, Ferguson JH, Franklin TD, Frazier DM, Grange DK, Greene CL, Groft SC, Harding CO, Howell RR, Huntington KL, Hyatt-Knorr HD, Jevaji IP, Levy HL, Lichter-Konecki U, Lindegren ML, Lloyd-Puryear MA, Matalon K, MacDonald A, McPheeters ML, Mitchell JJ, Mofidi S, Moseley KD, Mueller CM, Mulberg AE, Nerurkar LS, Ogata BN, Pariser AR, Prasad S, Pridjian G, Rasmussen SA, Reddy UM, Rohr FJ, Singh RH, Sirrs SM, Stremer SE, Tagle DA, Thompson SM, Urv TK, Utz JR, van Spronsen F, Vockley J, Waisbren SE, Weglicki LS, White DA, Whitley CB, Wilfond BS, Yannicelli S, Young JM (2014) Phenylketonuria Scientific Review Conference: state of the science and future research needs. Mol Genet Metab 112:87–122
7. Cleary M, Trefz F, Muntau AC, Feillet F, van Spronsen FJ, Burlina A, Bélanger-Quintana A, Giżewska M, Gasteyger C, Bettiol E, Blau N, MacDonald A (2013) Fluctuations in phenylalanine concentrations in phenylketonuria: a review of possible relationships with outcomes. Mol Genet Metab 110(4):418–423
8. Colomé C, Artuch R, Lambruschini N, Cambra FJ, Campistol J, Vilaseca M (2001) Is there a relationship between plasma phenylalanine and cholesterol in phenylketonuric patients under dietary treatment? Clin Biochem 34(5):373–376
9. Couce ML, Vitoria I, Aldámiz-Echevarría L, Fernández-Marmiesse A, Roca I, Llarena M, Sánchez-Pintos P, Leis R, Hermida A (2016) Lipid profile status and other related factors in patients with hyperphenylalaninaemia. Orphanet J Rare Dis 11(1):123
10. Daelman L, Sedel F, Tourbah A (2014) Progressive neuropsychiatric manifestations of phenylketonuria in adulthood. Rev Neurol (Paris) 170(4):280–287
11. Donlon J, Sarkissian C, Levy H, Scriver CR (2014) Hyperphenylalaninemia: phenylalanine hydroxylase deficiency. In *The online metabolic and molecular bases of inherited disease* (eds Valle D, Beaudet AL, Vogelstein B, Kinzler KW, Antonarakis SE, Ballabio A, Gibson K, Mitchell G) (McGraw-Hill, New York, NY, USA) http://ommbid.mhmedical.com/content.aspx?bookid=971&Sectionid=62673211 [accessed January 23, 2017]
12. Feillet F, Agostoni C (2010) Nutritional issues in treating phenylketonuria. J Inherit Metab Dis 33(6):659–664
13. Finkelstein JD (1998) The metabolism of homocysteine: pathways and regulation. Eur J Pediatr 157:S40–S44
14. Hermida-Ameijeiras A, Crujeiras V, Roca I, Calvo C, Leis R, Couce ML (2017) Arterial stiffness assessment in patients with phenylketonuria. Medicine (Baltimore) 96(51):e9322
15. Huemer M, Simma B, Mayr D, Möslinger D, Mühl A, Schmid I, Ulmer H, Bodamer OA (2012) Free asymmetric dimethylarginine (ADMA) is low in children and adolescents with classical phenylketonuria (PKU). J Inherit Metab Dis 35(5):817–821
16. Kanzelmeyer N, Tsikas D, Chobanyan-Jürgens K, Beckmann B, Vaske B, Illsinger S, Das AM, Lücke T (2012) Asymmetric dimethylarginine in children with homocystinuria or phenylketonuria. Amino Acids 42(5):1765–1772
17. Loscalzo J (2004) L-arginine and atherothrombosis. J Nutr 34(10 Suppl):2798S–2800S discussion 2818S-2819S
18. Nijveldt RJ, Teerlink T, van Guldener C, Prins HA, van Lambalgen AA, Stehouwer CD, Rauwerda JA, van Leeuwen PA (2003) Handling of asymmetrical dimethylarginine and symmetrical dimethylarginine by the rat kidney under basal conditions and during endotoxaemia. Nephrol Dial Transpl 18:2542–2550
19. Özcan Ö, Ipcioglu OM, Gultepe M (2012) Unexpectedly low asymmetric dimethylarginine (ADMA) and homocysteine levels in patients with phenylketonuria (PKU). J Inherit Metab Dis 35(6):1153
20. Rocha JC, Almeida MF, Carmona C, Cardoso ML, Borges N, Soares I, Salcedo G, Lima MR, Azevedo I, van Spronsen FJ (2010) The use of prealbumin concentration as a biomarker of nutritional status in treated phenylketonuric patients. Ann Nutr Metab 56(3):207–211
21. Rocha JC, van Spronsen FJ, Almeida MF, Soares G, Quelhas D, Ramos E, Guimarães JT, Borges N (2012) Dietary treatment in phenylketonuria does not lead to increased risk of obesity or metabolic syndrome. Mol Genet Metab 107(4):659–663
22. Schulpis KH, Karakonstantakis T, Bartzeliotou A, Karikas GA, Papassotiriou I (2004) The association of serum lipids, lipoproteins and apolipoproteins with selected trace elements and minerals in phenylketonuric patients on diet. Clin Nutr 23(3):401–407
23. Schulpis KH, Papassotiriou I, Tsakiris S, Vounatsou M, Chrousos GP (2005) Increased plasma adiponectin concentrations in poorly controlled patients with phenylketonuria normalize with a strict diet: evidence for catecholamine-mediated adiponectin regulation and a complex effect of phenylketonuria diet on atherogenesis risk factors. Metabolism 54(10):1350–1355
24. Singh RH, Cunningham AC, Mofidi S, Douglas TD, Frazier DM, Hook DG, Jeffers L, McCune H, Moseley KD, Ogata B, Pendyal S, Skrabal J, Splett PL, Stembridge A, Wessel A, Rohr F (2016) Updated, web-based nutrition management guideline for PKU: an evidence and consensus based approach. Mol Genet Metab 118(2): 72–83
25. Sirtori LR, Dutra-Filho CS, Fitarelli D, Sitta A, Haeser A, Barschak AG, Wajner M, Coelho DM, Llesuy S, Belló-Klein A et al (2005) Oxidative stress in patients with phenylketonuria. Biochim Biophys Acta 15 1740(1):68–73

26. Stühlinger MC, Tsao PS, Her JH, Kimoto M, Balint RF, Cooke JP (2001) Homocysteine impairs the nitric oxide synthase pathway: role of asymmetric dimethylarginine. Circulation 104:2569–2575
27. Ueland PM (1983) Pharmacological and biochemical aspects of S-adenosylhomocysteine and S-adenosylhomocysteine hydrolase. Pharmacol Rev 34:223–253
28. Vallance P, Leiper J (2004) Cardiovascular biology of the asymmetric dimethylarginine:dimethylarginine dimethylaminohydrolase pathway. Arterioscler Thromb Vasc Biol 24(6):1023–1030
29. van Spronsen FJ, van Wegberg AM, Ahring K, Belanger-Quintana A, Blau N, Bosch AM, Burlina A, Campistol J, Feillet F, Giżewska M et al (2017) Key European guidelines for the diagnosis and management of patients with phenylketonuria. Lancet Diabetes Endocrinol 5(9):743–756
30. van Wegberg AMJ, MacDonald A, Ahring K, Bélanger-Quintana A, Blau N, Bosch AM, Burlina A, Campistol J, Feillet F, Giżewska M, Huijbregts SC, Kearney S, Leuzzi V, Maillot F, Muntau AC, van Rijn M, Trefz F, Walter JH, van Spronsen FJ (2017) The complete European guidelines on phenylketonuria: diagnosis and treatment. Orphanet J Rare Dis 12(1):162
31. Verduci E, Banderali G, Moretti F, Lassandro C, Cefalo G, Radaelli G, Salvatici E, Giovannini M (2016) Diet in children with phenylketonuria and risk of cardiovascular disease: a narrative overview. Nutr Metab Cardiovasc Dis 26(3):171–177