JIMD Reports
DOI 10.1007/8904_2018_109

RESEARCH REPORT

Serum Amino Acid Profiling in Patients with Alkaptonuria Before and After Treatment with Nitisinone

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Received: 12 February 2018 / Revised: 28 March 2018 / Accepted: 13 April 2018

Abstract *Background:* Alkaptonuria (AKU) is a rare inherited disorder of the tyrosine metabolic pathway. Our group is evaluating the use of the homogentisic acid-lowering agent nitisinone in patients with AKU. A major biochemical consequence of this treatment is hypertyrosinaemia. Herein we report the concentration of 20 serum amino acids over a 36-month period pre- and post-treatment with nitisinone.

Methods: Fasting serum samples were collected at baseline (pre-nitisinone), 3 (2 mg nitisinone every other day), 6, 12, 24 and 36 (2 mg nitisinone daily) months. Amino acids were measured using the Biochrom 30 high-performance liquid chromatography cation exchange system with ninhydrin detection.

Results: Fifty patients [21 female, mean age (±standard deviation) 54.1 (15.6) years (range 25–75); 29 male, mean age 49.3 (11.6) years (range 22–70 years)] were included. Following treatment mean tyrosine concentrations increased seven- to eight-fold (baseline, 69.8 μmol/L; 3 months, 670.7 μmol/L; 6 months, 666.4 μmol/L; 12 months, 692.9 μmol/L; 24 months, 649.4 μmol/L; 36 months, 724.8 μmol/L, $p = <0.001$ for all visits compared to baseline).

At baseline mean phenylalanine, aspartic acid and arginine were outside the normal reference range. Following treatment the ratios of phenylalanine/tyrosine, phenylalanine/large neutral amino acids, arginine/branched chain amino acids and branched chain/aromatic amino acids decreased ($p = <0.05$), and the tyrosine/large neutral amino acid ratio increased ($p = <0.0001$).

Conclusions: Marked hypertyrosinaemia was observed following treatment with nitisinone. Noteworthy changes were also observed in the ratio of several amino acids following treatment with nitisinone suggesting that the availability of amino acids for neurotransmitter biosynthesis and liver function may be altered following treatment with nitisinone.

Communicated by: Brian Fowler, PhD

Electronic supplementary material: The online version of this article (https://doi.org/10.1007/8904_2018_109) contains supplementary material, which is available to authorized users.

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Abbreviations

AAA	Aromatic amino acids
AKU	Alkaptonuria
BCAA	Branched chain amino acids
HGA	Homogentisic acid
HGD	Homogentisate-1,2-dioxygenase
HPPD	Hydroxyphenylpyruvic acid dioxygenase
HT1	Tyrosinaemia type 1
LAT-1	Large neutral amino acid transporter
LNAA	Large neutral amino acids
NAC	National Alkaptonuria Centre

Introduction

Alkaptonuria (AKU, OMIM: 203500) is a rare autosomal recessive disorder of the tyrosine metabolic pathway, resulting from a congenital deficiency in the enzyme homogentisate-1,2-dioxygenase (HGD, E.C.1.12.11.5). AKU occurs in 1 in 250,000 of the general population (Phomphutkul et al. 2002), but in certain countries it is observed more commonly; for instance, in Slovakia it is estimated to occur in 1 in 19,000 of the population (Zatkova 2011; Milch 1960).

One of the major metabolic implications of AKU is that the circulating concentration of homogentisic acid (HGA) significantly increases. This pathognomonic finding is thought to be causally responsible for a number of abnormalities observed in the disease. These include spondyloarthropathy, characterised by progressive kyphoscoliosis and impaired spinal and thoracic mobility, as well as renal and prostate stones, aortic valve stenosis, osteoporosis, fractures and ruptures of tendons, ligaments and muscle (Ranganath et al. 2013).

Supportive medical management of AKU is the mainstay of treatment and includes a low-protein diet, analgesia and arthroplasty (Ranganath et al. 2013). A newer unlicensed treatment for AKU is the drug nitisinone, a competitive reversible inhibitor of the enzyme hydroxyphenylpyruvic acid dioxygenase (HPPD, E.C. 1.13.11.27). It reduces the formation of HGA and thus has the potential to prevent or slow the complications observed in patients with AKU. Previous studies (Phomphutkul et al. 2002; Suwannarat et al. 2005; Introne et al. 2011; Ranganath et al. 2016; Milan et al. 2017) have demonstrated the clear biochemical impact of nitisinone treatment, all demonstrating a $> 94\%$ reduction in urinary HGA. However none have shown improvement in clinical parameters. This may be for several reasons, including the following: (1) AKU is a slow progressive disorder, and thus study duration may not have been long enough to demonstrate improvement; (2) the optimal dose of nitisinone may not have been used; and (3) clinical outcomes measured may not have been appropriate.

At the National Alkaptonuria Centre (NAC) in Liverpool, nitisinone is being used off licence (patients are given 2 mg of nitisinone daily, off licence) for treatment of AKU for all patients in the UK, as it is recognised that nitisinone is a potential treatment for this debilitating disease. At the NAC it has also been demonstrated in a real-life setting that this treatment can reduce urinary HGA by 94% (Milan et al. 2017).

In contrast nitisinone is already licensed for the treatment of hereditary tyrosinaemia type 1 (HT1) (HT I, OMIM 276700) and has proved to be a very efficacious mode of treatment (McKiernan 2013; McKiernan et al. 2015).

One of the major metabolic consequences of treating patients with nitisinone in AKU and HT1 is that circulating tyrosine concentrations markedly increase (Lindstedt et al. 1992; Phomphutkul et al. 2002; Suwannarat et al. 2005; Introne et al. 2011; Ranganath et al. 2016; Olsson et al. 2015; McKiernan et al. 2015; van Ginkel et al. 2016, 2017; Milan et al. 2017; Davison et al. 2017).

Hypertyrosinaemia has been observed in patients with AKU that attended the NAC in Liverpool and during the Suitability Of Nitisinone In Alkaptonuria 1 (SONIA-1) clinical trial (Ranganath et al. 2016), which evaluated the efficacy of different daily doses of nitisinone over a 4-week period. Beyond this there is nothing reported on the wider impact of nitisinone on amino acid metabolism.

For many years there has been keen interest in amino acid metabolism in health and disease, beyond the traditional setting of inborn errors of metabolism. This is because amino acids are not only basic metabolites but are also key regulators in many metabolic pathways. Examples of areas studied include cognitive function and Alzheimer's disease (Ravaglia et al. 2004), gastroesophageal cancer (Crotti et al. 2017), diabetes (Bi and Henry 2017) and aortic dissection (Wang et al. 2017).

In addition, while there have been several studies evaluating serum and plasma amino acid concentrations both in men and women of a variety of ages (Armstrong and Stave 1973a, 1973b, 1973c; Rudman et al. 1989; Caballero et al. 1991; Pitkänen et al. 2003; Chan et al. 1999; Sarwar et al. 1991; Kouchiwa et al. 2012; Davison et al. 2015), none have reported fasting serum amino acid concentrations in patients with AKU pre- and post-nitisinone therapy.

For the first time, this longitudinal survey reports the concentration of essential amino acids (valine, leucine, isoleucine, threonine, methionine, tryptophan, phenylalanine, lysine and histidine) and non-essential amino acids (aspartic acid, asparagine, glutamic acid, glutamine, alanine, glycine, cystine, arginine, tyrosine, serine and proline) in patients that have attended the NAC with AKU pre- and post-nitisinone therapy over a 36-month period.

Patients and Analytical Methods

Patients

Protocol for Patients Who Attended the NAC for Treatment with Nitisinone

The protocol for treatment at the NAC is that patients with confirmed AKU are commenced on a 2 mg dose of nitisinone, on alternative days for the first 3 months, which is then increased to 2 mg daily thereafter. Assessments are repeated on an annual basis to monitor response to therapy.

Nitisinone is given off licence to investigate its safety and efficacy. It is hypothesised that if HGA levels are reduced before the onset of overt ochronosis, this might prevent or stop the development of the debilitating features observed in AKU.

Inclusion criteria are that individuals must have the diagnosis of AKU, are residents of England or Scotland and are over the age of 16 years. Confirmed diagnosis of AKU is based upon increased urinary excretion of HGA [urine HGA excretion in healthy volunteers has been demonstrated to be <2.91 μmol/day (Davison et al. 2015)] and is mandatory for referral to the NAC. Exclusion criteria are individuals must not be pregnant and/or lactating. All patients are provided with written information about the scope of the centre and the assessments they will receive. All patients at the NAC have biochemical measurements with clinical assessments performed at baseline, day 4 (2 days post-nitisinone), 3 months, 6 months and 12 months, with annual monitoring thereafter.

Ethical Approval

Data collection and analyses at the NAC have approval from the Royal Liverpool and Broadgreen University Hospital Trust Audit Committee (Audit no. ACO3836). This is not a clinical trial, and therefore ethical approval was not required. Data obtained is following standard clinical assessments during the course of providing a service upon referral to the NAC. Patients are informed verbally and through patient information leaflets about the activities of the NAC. Patients are also explicitly informed that data may be used for publication, and within the NAC patient information leaflet, the following paragraph is included:

> We could publish results from the study but if we do, we will make sure that you cannot be identified in anyway. All data used for publicity or for other research purposes will ensure total anonymity. Please let us know when you are visiting the NAC that you understand and have no objections to this.
>
> No patient has objected to the use of their data.

Subjects Included in the Study

To date (November 2017), 62 patients with AKU have been enrolled at the NAC for treatment with nitisinone.

Twelve of the sixty-two patients were excluded from this survey; seven were Welsh patients, and therefore funding was not available for treatment with nitisinone; three patients were on nitisinone prior to enrolment at the NAC; and two patients were not receiving a standard nitisinone treatment regimen due to corneal keratopathy (Khedr et al. 2017).

Fifty patients [21 female, mean age (±standard deviation) 54.1 (15.6) years (range 25–75); 29 male, mean age 49.3 (11.6) years (range 22–70 years)] were included in this 36-month longitudinal survey reporting the biochemical data obtained from monitoring 20 serum amino acids. This is an ongoing service, and at the time of preparing this manuscript, results were not available for all 50 patients at each time point, apart from baseline. Serum amino acid results are included from baseline (pre-nitisinone), 3 (2 mg nitisinone every other day), 6 (2 mg nitisinone daily), 12 (2 mg nitisinone daily), 24 (2 mg nitisinone daily) and 36 (2 mg nitisinone daily) months. Results are not included after baseline in all patients as individuals have attended the NAC for different durations and not all patients have attended planned visits to the NAC.

Analytical Methods

Sample Collection

Serum samples were collected from patients (S-Monovette, Sarstedt, Germany), centrifuged (10 min at 3,000 rpm) and stored at −20°C until analysis. All serum samples were collected following an overnight fast (at least 8 h). Patients' dietary intake of protein was not restricted during this study.

Measurement of Serum Amino Acids

Serum amino acid concentrations were determined using Biochrom 30 high-performance liquid chromatography cation exchange system with ninhydrin detection (Biochrom, Cambridge, UK). For details of this widely adopted method for measurement of serum amino acids, see instructions for use of Biochrom 30+ Amino Acid Analyser (2018) (version 41 56 1783 IVD instruction for use English is 16.doc http://www.biochrom.co.uk/user_downloads/?c=17).

Calibration and Internal Standards

Calibration was performed using a series of aqueous standards purchased from Sigma (Dorset, UK) [amino acid basic standard (product code, A6282); amino acid acidic/neutral standard (product code, A6407); glutamine (product code, G8540). Stock calibration standards were made to 2.0 mmol/L, except glutamine which was made to 2.5 mmol/L. All working calibrator concentrations were

then diluted with loading buffer (Biochrom, product code: 80-2038-70) to a final concentration of 100 μmol/L, except for cystine which was 50 μmol/L. The diluted working calibrator also contained the internal standards S-2-aminoethyl-L-cysteine hydrochloride (product code, A2636) and norleucine (product code, N6877) at a final concentration of 200 μmol/L and 5-sulphosalicylic acid at a final concentration of 91.7 mmol/L.

Internal Quality Control

ClinCheck® plasma control (level 1) for amino acids was used as internal quality control material (Recipe, Germany). This contained all amino acids measured, apart from cystine, which was purchased from Sigma (Dorset, UK) to make in-house internal quality control material. Inter-assay coefficient of variation for all 20 amino acids was less than 4%, as detailed below. [Supplementary data for individual amino acids – data are summarised as mean amino acid concentration (μmol/L) (±standard deviation), coefficient of variation (%). Aspartic acid, 12 (0.5), 3.9; threonine, 109.3 (1.9), 1.8; serine, 96.7 (1.5), 1.5; asparagine, 45 (1.1), 2.3; glutamic acid, 98 (1.2), 1.3; glutamine, 408.9 (9.1), 2.2; glycine, 185.3 (3.0), 1.6; alanine, 405.9 (8.6), 2.1; valine, 233.2 (5.1), 2.2; cystine, 13.2(0.3), 2.4; methionine, 27.9 (0.9), 3.1; isoleucine, 76.8 (2.3), 3.0; leucine, 139.4 (3.1), 2.2; tyrosine, 68.3 (1.7), 2.5; phenylalanine, 78.1 (1.8), 2.3; lysine, 171.8 (3.0), 1.7; histidine, 73.5 (1.7), 2.3; tryptophan, 53.5 (1.0), 1.8; arginine, 30.4 (0.8), 2.8; proline, 236.2 (3.7), 3.7].

Sample Preparation

100 μL of calibrator/serum/internal quality control material was mixed with 100 μL of a solution containing sulfosalicylic acid (183.4 mmol/L) to precipitate proteins and internal standards (S-2-aminoethyl-L-cysteine hydrochloride and norleucine both at 100 μmol/L). Samples were then put in the fridge at 4°C for 1 h and then centrifuged at 13,000 rpm for 10 min. 60 μL of supernatant was injected onto the analytical column for analysis.

Data Analysis and Calculations

Amino acid concentrations were calculated offline using EZCHrom Elite software (version 3.2.1, Agilent, USA).

All statistical analyses were performed using GraphPad Instat (version 3.10, 2009, CA, USA) and Analyse-it for Microsoft Excel (version 2.20 Analyse-it Software, Ltd., Leeds, UK).

Kolmogorov-Smirnov testing was performed to assess if serum amino concentrations were normally distributed. For non-parametric data a Kruskal-Wallis test with a Dunn's multiple comparisons post hoc test was used to assess significant differences in serum amino acid concentrations pre- and post-treatment with nitisinone. For parametric data a one-way ANOVA with Tukey post hoc test was applied. A p value <0.05 was deemed significant.

Amino Acid Ratios

All amino acid concentrations used in calculations were in μmol/L. Ratios were calculated to assess dietary adequacy of protein intake (calculation a) (Antener et al. 1981); to predict phenylalanine, tyrosine and arginine availability to the brain for neurotransmitter synthesis (calculations b–d) (Lieberman 1999); and to assess liver metabolism, hepatic functional reserve and the severity of liver damage (calculation e) (Fischer et al. 1976).

(a) Phenylalanine to tyrosine = [phenylalanine]/[tyrosine]
(b) Tyrosine to large neutral amino acids ([tyrosine]:[LNAA]) = [tyrosine]/([tyrosine] + [phenylalanine] + [tryptophan] + [leucine] + [isoleucine] + [valine])
(c) Phenylalanine to large neutral amino acids ([phenylalanine]/[LNAA]) = [phenylalanine]/([tyrosine] + [phenylalanine] + [tryptophan] + [leucine] + [isoleucine] + [valine])
(d) Arginine to branched chain amino acids ([arginine]/[BCAA]) = [arginine]/([leucine] + [isoleucine] + [valine])
(e) Branched chain amino acids to aromatic amino acids ([BCAA]/[AAA]) = ([leucine] + [valine] + [isoleucine])/[phenylalanine] + [tyrosine])

Results

Serum Amino Acid Concentrations Pre-nitisinone Treatment

Table 1 (and Fig. S1, supplementary data) summarises mean (±standard deviation) fasting serum amino acid concentrations from all patients included in this longitudinal survey. Fifty patients had amino acids measured at baseline (Table 1, Fig. S1, supplementary data). After baseline amino acids were not measured in all patients as

Table 1 Serum amino acid concentrations (mean ± standard deviation, μmol/L) in patients attending the National Alkaptonuria Centre over a 36-month period

Amino acid (reference range, μmol/L)	Baseline ($n = 50$)	3 months ($n = 37$)	6 months ($n = 29$)	12 months ($n = 37$)	24 months ($n = 34$)	36 months ($n = 20$)
Phenylalanine (30–76)[E,a]	87.42 (13.9)	84.4 (18.3)	91.5 (22.5)	94.5 (13.5)	92.9 (13.6)	97.6 (15.0)
Tyrosine (29–92)[a]	69.8 (15.7)	670.7 (155.0)	666.4 (162.3)	692.9 (119.8)	649.4 (183.6)	724.8 (116.8)
Tryptophan (40–79)[E,a]	51.8 (11.5)	50.9 (9.9)	48.5 (11.9)	48.1 (11.1)	51.1 (10.2)	53.5 (14.7)
Glutamine (326–800)[b]	571.2 (108.0)	476.2 (79.4)	495.1 (81.1)	527.6 (86.0)	525.4 (77.8)	469.4 (103.5)
Asparagine (30–70)[b]	72.1 (15.1)	64.7 (14.6)	65.3 (17.9)	69.2 (11.0)	61.3 (11.8)	61.4 (9.1)
Aspartic acid (2–5)[c]	39.8 (9.7)	38.2 (10.9)	37.9 (9.9)	38.1 (6.7)	39.5 (8.7)	45.4 (17.0)
Glutamic acid (26–151)[c]	128.9 (40.4)	129.2 (56.6)	141.1 (63.5)	151.8 (37.4)	137.5 (32.4)	149.5 (49.2)
Cystine (36–61)[d]	11.1 (9.3)	6.9 (4.6)	8.3 (8.4)	9.6 (9.1)	13.9 (9.1)	15.7 (13.5)
Methionine (10–41)[E,d]	25.5 (6.6)	21.8 (4.5)	23.7 (5.9)	25.8 (5.4)	24.4 (4.3)	25.2 (5.3)
Glycine (120–436)[e]	316.7 (77.6)	299.5 (60.6)	330.5 (61.6)	343.2 (63.2)	329.9 (75.1)	332.6 (74.4)
Isoleucine (20–91)[E,e]	69.7 (17.6)	63.2 (15.5)	64.5 (15.7)	64.8 (12.1)	66.6 (13.5)	72.5 (18.0)
Leucine (44–169)[E,e]	148.8 (27.8)	132.1 (28.4)	137.1 (30.7)	139.4 (21.6)	139.7 (20.7)	134.6 (33.1)
Proline (66–330)[e]	204.3 (89.6)	183.3 (76.3)	207.9 (108.9)	180.4 (63.5)	182.3 (53.3)	182.5 (75.8)
Valine (79–313)[E,e]	240.4 (43.4)	222.9 (44.6)	226.7 (45.4)	234.2 (36.0)	241.9 (40.2)	250.3 (52.7)
Alanine (112–529)[e]	462.0 (96.9)	444.8 (92.0)	485.1 (124.8)	427.8 (83.3)	448.5 (82.6)	450.6 (79.5)
Histidine (43–111)[E,f]	84.1 (12.3)	85.0 (12.8)	86.7 (16.5)	80.7 (9.5)	85.2 (11.7)	79.3 (10.0)
Lysine (66–242)[E,f]	191.3 (32.2)	180.5 (33.8)	180.45 (38.8)	177.8 (27.9)	183.8 (28.0)	185.6 (27.4)
Arginine (14–102)[f]	105.2 (23.4)	113.9 (31.1)	116.2 (26.9)	98.1 (13.8)	96.6 (15.9)	108.2 (18.0)
Serine (69–206)[g]	162.3 (28.7)	149.8 (21.9)	155.5 (29.6)	161.3 (18.6)	157.9 (30.5)	162.4 (25.6)
Threonine (43–218)[E,g]	132.2 (27.5)	114.5 (25.5)	112.7 (27.0)	112.5 (21.3)	113.4 (23.6)	124.5 (27.8)
Phenylalanine/tyrosine (0.80–1.0)	1.28 (0.21)	0.13 (0.04)	0.13 (0.03)	0.14 (0.03)	0.16 (0.1)	0.13 (0.04)
Tyrosine/LNAA (0.0–0.14)	0.10 (0.01)	0.55 (0.07)	0.54 (0.10)	0.54 (0.05)	0.52 (0.09)	0.54 (0.06)
Phenylalanine/LNAA (0.09–0.13)	0.13 (0.01)	0.07 (0.01)	0.07 (0.02)	0.07 (0.01)	0.08 (0.02)	0.07 (0.01)
Arginine/BCAA (0.16–0.23)	0.23 (0.05)	0.27 (0.07)	0.27 (0.07)	0.23 (0.04)	0.22 (0.05)	0.24 (0.04)
BCAA/AAA (Fisher's ratio) (2.7–3.5)	2.93 (0.37)	0.56 (0.14)	0.71 (0.86)	0.56 (0.09)	0.71 (0.49)	0.56 (0.13)

All samples were collected in a fasted state (overnight fast >8 h). Baseline refers to pre-nitisinone therapy; at 3 months, patients received 2 mg nitisinone every other day; after 3 months, patients received 2 mg nitisinone daily. Essential[E] amino acids. Amino acid reference ranges were determined in-house. Amino acid ratio reference ranges were adopted from (Antener et al. 1981; Lieberman 1999; Kouchiwa et al. 2012). *LNAA* large neutral amino acids, *BCAA* branched chain amino acids, *AAA* aromatic amino acid

[a] Aromatic
[b] Amidic
[c] Acidic
[d] Sulphur containing
[e] Aliphatic
[f] Basic
[g] Hydroxylic

individuals have attended the NAC for different durations and not all patients attended planned visits to the NAC.

Mean amino acid concentrations were within the normal reference range at baseline (pre-nitisinone treatment) apart from phenylalanine, aspartic acid, arginine and cystine (Table 1), the latter being the only amino acid below the lower reference range, despite there being a large spread of concentrations (Fig. S1, supplementary data). All other amino acids detailed were outside the upper reference range. Following treatment with nitisinone, these amino acids did not change significantly over the 36-month period studied.

Impact of Nitisinone Treatment on Serum Amino Acid Concentrations

Mean serum tyrosine and tryptophan concentrations were within the normal reference range pre-nitisinone treatment,

and phenylalanine was marginally outside the normal reference range (Table 1, Fig. S1, supplementary data). Following treatment with 2 mg nitisinone, every other day (3-month visit) and daily (all visits after 3 months), tyrosine concentrations increased to seven- to eight-fold ($p = <0.001$, at all visits) (Fig. S1, supplementary data).

Phenylalanine and tryptophan concentrations were not significantly different over the 36-month period studied when compared to baseline. Phenylalanine concentrations did however show an increasing concentration.

Several amino acid ratios were calculated (calculations a–e) (Table 1). All amino acid ratios were within the normal range pre-nitisinone treatment, except the phenylalanine to tyrosine ratio, which was increased. Following treatment with nitisinone, all amino acid ratios showed a significant decrease (see Table 2 for p values; all were <0.05), apart from the tyrosine to LNAA ratio which showed a significant increase ($p = <0.0001$).

Amino Acid Concentrations Showing Differences After Nitisinone Treatment Started

Table 2 summarises significant differences in amino acid concentrations that were observed between visits after nitisinone treatment was started. Apart from tyrosine, the amino acids asparagine and glutamine showed significant decreases in concentration post-nitisinone treatment at 24 and 36 months ($p = 0.004$) and 3, 6 and 36 months, respectively ($p = <0.0001$).

Table 2 Serum amino acid concentrations that were significantly different in patients attending the National Alkaptonuria Centre over a 36-month period

Amino acid	p	Change in amino acid concentration observed compared to baseline	Change observed
Asparagine	0.004	24 and 36 months	Decrease
Glutamine	<0.0001	3, 6 and 36 months	Decrease
Tyrosine	<0.0001	3, 6, 12, 24 and 36 months	Increase
Phenylalanine/tyrosine	<0.0001	3, 6, 12, 24 and 36 months	Decrease
Tyrosine/LNAA	<0.0001	3, 6, 12, 24 and 36 months	Increase
Phenylalanine/LNAA	<0.0001	3, 6, 12, 24 and 36 months	Decrease
Arginine/BCAA	<0.01 <0.05	3 months 6 months	Decrease
BCAA/AAA (Fisher's ratio)	<0.0001	3, 6, 12, 24 and 36 months	Decrease

Baseline refers to pre-nitisinone therapy; at 3 months, patients received 2 mg nitisinone every other day; after 3 months, patients received 2 mg nitisinone daily. *LNAA* large neutral amino acids, *BCAA* branched chain amino acids, *AAA* aromatic amino acid

Discussion

Herein for the first time, we report the concentration of 20 serum amino acids over a 36-month period in a large cohort of patients with AKU before and after treatment with nitisinone. This highly unique longitudinal survey examines the impact of AKU and its treatment on amino acid metabolism in a real-life healthcare setting. Traditionally the focus has been on the tyrosine metabolic pathway when studying AKU, and several reports have shown that patients treated with nitisinone have marked hypertyrosinaemia (Phomphutkul et al. 2002; Suwannarat et al. 2005; Introne et al. 2011; Ranganath et al. 2016; Olsson et al. 2015; Milan et al. 2017; Davison et al. 2017). In line with previous authors we too observed a significant increase in serum tyrosine following treatment with nitisinone. This occurs because nitisinone is a reversible competitive inhibitor of HPPD and essentially creates a new metabolic defect upstream of its site of action. The biochemical pattern observed is that which is seen in type 3 tyrosinaemia (OMIM: 276710), so-called pseudo-type 3 tyrosinaemia.

Recently Milan et al. (2017) reported tyrosine concentrations in patients treated with nitisinone over a 24-month period. In contrast, this longitudinal survey reports the concentration of 20 serum amino acids, not just tyrosine over a 36-month period, to establish the wider impact of treatment with nitisinone on amino acid metabolism. Moreover data presented herein is in larger cohort of patients.

Concerns exist over the impact of hypertyrosinaemia, as tyrosine is transported across the blood-brain barrier and may result in high brain tyrosine concentrations (Thimm et al. 2011) and thus increased dopamine concentrations in cerebral spinal fluid (CSF) as tyrosine is its precursor. A previous study has demonstrated an increase in homovanillic acid (dopamine metabolite) concentrations in CSF (Thimm et al. 2011), which suggests that dopamine metabolism may be altered. Moreover animal studies have also shown a direct neurotoxic effect of tyrosine, including oxidative stress (Macedo et al. 2013) and altering DNA repair (De Pra et al. 2014).

Changes in other aromatic amino acids were fairly unremarkable. Serum tryptophan remained within the normal reference range over the 36-month period. Previous studies in patients with HT1 treated with nitisinone and in animal models have suggested that hypertyrosinaemia observed following treatment may reduce the biosynthesis of serotonin and thus be responsible for altered cognitive function and behavioural problems observed in HT1

(De Laet et al. 2011; Thimm et al. 2012; Masurel-Paulet et al. 2008; Bendadi et al. 2014; van Ginkel et al. 2016). Evidence of this is sparse, and only one small study has reported decreased CSF concentrations of 5-hydroxyindole acetic acid (serotonin metabolite) (Thimm et al. 2011). Mechanisms that have been proposed suggest (1) that tyrosine may inhibit tryptophan hydroxylase activity, which is the rate-limiting step in serotonin metabolism, and (2) tyrosine may compete for transport into the central nervous system via a common large neutral amino acid transporter (LAT-1) and thus reduce tryptophan uptake required for serotonin biosynthesis (Hillgartner et al. 2016). It is reassuring that tryptophan did not change following nitisinone in the present data set.

Phenylalanine showed an increasing trend over the period studied. This is in contrast to reports in patients with HT1 that have shown reduced concentrations of phenylalanine following nitisinone therapy (Daly et al. 2012; van Vliet et al. 2014). While HT1 patients are on a protein-restricted diet, it is still thought that nitisinone itself may be the cause of the lower phenylalanine observed. The underlying mechanism for this is not fully understood (Harding et al. 2014). It has been postulated that hypertyrosinaemia may also reduce phenylalanine uptake into the brain due to competition for the LAT-1 (van Ginkel et al. 2016). This supposition may be supported as a significant increase in the ratio calculated in equation b (tyrosine to large neutral amino acids) and significant decrease in the ratios calculated in equations c–d (phenylalanine to large neutral amino acids; arginine to branched chain amino acids) were observed after nitisinone treatment implying that amino acid availability to the brain may be reduced, with the exception of tyrosine. The ratios are proposed to be a more reliable measure of the availability of tyrosine, phenylalanine and arginine to the brain instead of individual amino acid concentrations (Lieberman 1999).

While the significant decrease in the ratios implies a reduced availability of the detailed amino acids to the brain for neurotransmitter synthesis, it does not fit with the serum concentrations of phenylalanine observed in the current survey. However the concentration of phenylalanine in serum does not necessarily reflect that which is seen in CSF.

Of note pre-nitisinone therapy, the phenylalanine to tyrosine ratio was increased compared to the normal reference range (Antener et al. 1981). The reason for this is uncertain, as typically this ratio is monitored to assess the flux of phenylalanine to tyrosine and the adequacy of protein intake. One may postulate that phenylalanine hydroxylase activity may be reduced, thus causing a mild increase in the ratio. Reduced enzyme activity has been reported in individuals with increased inflammation (Wannemacher et al. 1976). As AKU is a chronic disease process, it is possible that inflammatory processes are altered; however this was not assessed. Another possibility is that the increase in phenylalanine is an artefact; however this is unlikely as samples were centrifuged immediately and stored at −20°C until they were analysed.

Following the commencement of nitisinone treatment, the BCAA to AAA ratio significantly decreased, potentially misleadingly suggesting that following treatment liver function was altered. The reason for the change in ratio is the inability to degrade tyrosine, an aromatic acid, greatly increasing the denominator of the ratio. However as tyrosine is in such vast quantities from a metabolic defect in the tyrosine metabolic pathway, and not related to liver function, one cannot use this ratio reliably to make an assessment of liver function. Prior to starting treatment, the BCAA to AAA ratio was within the normal range indicating that patients had normal liver function.

A number of changes were observed in several amino acids over the course of this longitudinal survey (Fig. S1), but none appear to be related to treatment with nitisinone and are not consistent at all-time points evaluated. It is thought that these findings are likely to reflect the natural fluctuation one would observe in amino acids, and no reliable conclusions can be drawn from these data.

Of particular interest were the mean amino acid concentrations (Table 1) that were outside the normal reference range independent of nitisinone therapy. Specifically cystine was lower than the lower reference range, and arginine and aspartic acid concentrations were higher.

Decreases in cystine can be observed due to haemolysis, delayed separation and or platelet/leukocyte contamination (Perry and Hansen 1969; Bowron et al. 2012). As discussed previously samples were separated immediately from cells and stored before analysis at −20°C, thus eliminating this as a mechanism for the low cystine concentrations observed. One may postulate that the low cystine observed is a result of its reaction with glutathione, via glutathione-cystine transhydrogenase activity to form cysteine as part of the body's defence against oxidative stress. The rationale for this is that AKU is accompanied by increased oxidative stress (Davison et al. 2016), which, in part, results from the benzoquinone polymers that are produced from the high concentrations of HGA observed in AKU. Cysteine was not measured in this longitudinal survey but should be evaluated to assess the validity of this hypothesis.

It has been proposed that increases in aspartic acid and glutamic acid can be observed from the deamination of asparagine and glutamine, respectively, due to delayed separation and haemolysis (Perry and Hansen 1969, Bowron et al. 2012). Samples did not visually appear to be haemolysed; however no haemolytic indices were measured so this cannot be completely excluded as a contributory factor. Moreover samples were separated

immediately from cells and stored before analysis at −20°C. One possibility is that the proposed increased oxidative stress observed in AKU results in the increased formation of glutamic acid from glutamine. Glutamic acid is the precursor for glutathione which is essential for maintaining the redox state of the cell (Davison et al. 2016).

If deamination of asparagine to aspartic acid was the cause of increased aspartic acid in this survey, one would have expected lower concentrations of asparagine to be observed, which was not the case. The significance of this finding is unknown.

Conclusions

Significant hypertyrosinaemia has been demonstrated following treatment with the HGA-lowering drug nitisinone. The implications of the hypertyrosinaemia are in large unknown. However in this longitudinal survey, it has been demonstrated that amino acid ratios have been significantly altered suggesting that the availability of amino acids for neurotransmitter biosynthesis and liver function may be altered.

Compliance with Ethics Guidelines

All procedures reported in this review were in accordance with the ethical standards of the local hospital ethics committee and with the Helsinki Declaration of 1975, as revised in 2000.

The National Alkaptonuria Centre and all subsequent data analysis have been approved by the Royal Liverpool and Broadgreen University Hospital Trust Audit Committee (Audit no. ACO3836).

Conflict of Interest

Davison AS, Norman BP, Smith EA, Devine J, Usher J, Hughes AT, Khedr M, Milan AM, Gallagher JA and Ranganath LR have no conflict of interest.

Davison AS was the main author who wrote the manuscript. Devine J and Usher J deproteinised samples before analysis. Smith E performed laboratory analysis. Norman BP contributed to data analysis and reviewed and made corrections to the manuscript. Milan AM, Hughes AT, Khedr M and Gallagher J are senior colleagues in the AKU research group; they reviewed and made corrections to the manuscript. Ranganath LR recruited patients and supplied samples for analysis and is the clinical director of the National Alkaptonuria Centre and AKU research group. He reviewed and made corrections to the manuscript.

Funding

This work was funded by the Department of Health's NHS Highly Specialised Services Commissioning Group. Davison AS is funded through a National Institute for Health Research (grant code: HCS DRF-2014-05-009).

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