Dietary restriction of tyrosine and phenylalanine lowers tyrosinaemia associated with nitisinone therapy of alkaptonuria.

Juliette H. Hughes[1+], Peter J.M. Wilson[1], Hazel Sutherland[1], Shirley Judd[2], Andrew T. Hughes[1,3], Anna M. Milan[1,3], Jonathan C. Jarvis[4], George Bou-Gharios[1], Lakshminarayan R. Ranganath[1,3], James A. Gallagher[1].

[1] Department of Musculoskeletal Biology I, Institute of Ageing and Chronic Disease, University of Liverpool, Liverpool, L7 8TX, UK

[2] Department of Nutrition and Dietetics, Royal Liverpool University Hospital Trust, Liverpool, L7 8XP, UK

[3] Liverpool Clinical Laboratories, Department of Clinical Biochemistry and Metabolic Medicine, Royal Liverpool and Broadgreen University Hospitals Trust, Liverpool, L7 8XP, UK

[4] School of Sport and Exercise Sciences, Faculty of Science, Liverpool John Moores University, Liverpool, L3 3AF, UK

[+] Corresponding author. Email: hljhugh4@liverpool.ac.uk. Address: William Henry Duncan Building, 6 West Derby Street, Liverpool, L7 8TX, UK.

Abstract

Background: Alkaptonuria (AKU) is caused by homogentisate 1,2-dioxygenase deficiency that leads to homogentisic acid (HGA) accumulation, ochronosis and severe osteoarthropathy. Recently, nitisinone treatment, which blocks HGA formation, has been effective in AKU patients. However, a consequence of nitisinone is elevated tyrosine that can cause keratopathy. The effect of tyrosine and phenylalanine dietary restriction was investigated in nitisinone-treated AKU mice, and in an observational study of dietary intervention in AKU patients.

Methods: Nitisinone-treated AKU mice were fed tyrosine/phenylalanine-free and phenylalanine-free diets with phenylalanine supplementation in drinking water. Tyrosine metabolites were measured pre-nitisinone, post-nitisinone, and after dietary restriction. Subsequently an observational study was undertaken in 10 patients attending the National Alkaptonuria Centre (NAC), with tyrosine >700µmol/L who had been advised to restrict dietary protein intake and where necessary, to use tyrosine/phenylalanine-free amino acid supplements.

Results: Elevated tyrosine (813µmol/L) was significantly reduced in nitisinone-treated AKU mice fed a tyrosine/phenylalanine-free diet in a dose responsive manner. At 3 days of restriction, tyrosine was 389.3µmol/L, 274.8µmol/L and 144.3µmol/L with decreasing phenylalanine doses. In contrast, tyrosine was not effectively reduced in mice by a phenylalanine-free diet; at 3 days tyrosine was 757.3µmol/L, 530.2µmol/L and 656.2µmol/L, with no dose response to phenylalanine supplementation. In NAC patients, tyrosine was

significantly reduced (p=0.002) when restricting dietary protein alone, and when combined with tyrosine/phenylalanine-free amino acid supplementation; 4 out of 10 patients achieved tyrosine <700µmol/L.

Conclusion: Tyrosine/phenylalanine dietary restriction significantly reduced nitisinone-induced tyrosinaemia in mice, with phenylalanine restriction alone proving ineffective. Similarly, protein restriction significantly reduced circulating tyrosine in AKU patients.

Take home message: Reducing dietary intake of tyrosine/phenylalanine reduces nitisinone-induced tyrosinaemia.

Author contributions: JHH, PJMW, HS, SJ, ATH and AMM collected and analysed data. Mouse data was collected under the project licence of JCJ and GBG. LRR and JAG were involved with study concept design and supervised the work. JHH wrote the paper, with contributions from all authors. All authors approved the final version.

Competing interest statement: JHH, PJMW, HS, SJ, ATH, AMM, JCJ, GBG, LRR and JAG declare that they have no conflict of interest.

Ethics approval: The data collected from the NAC was approved by the Institutional Audit Committee (Audit No:ACO3836).

Animal rights: All institutional and national guidelines for the care and use of laboratory animals were followed.

6 keywords: Alkaptonuria; nitisinone; tyrosinaemia; tyrosine; phenylalanine; protein

Introduction

Alkaptonuria (AKU; OMIM #203500) is an autosomal recessive, multisystem disease caused by mutations in the homogentisate 1,2-dioxygenase (HGD) enzyme (EC 1.13.11.5; La Du et al. 1958). HGD deficiency leads to homogentisic acid (HGA) accumulation (figure 1A) in the blood and tissues. Over time, excess HGA forms a dark brown ochronotic pigment that deposits in connective tissues causing them to become stiff and brittle (Gaines and Pai 1987; Janocha et al. 1994; Helliwell et al. 2008; Taylor et al. 2011), leading to early-onset and severe osteoarthropathy.

Recently, a drug called nitisinone (2-(2-nitro-4-(trifluoromethyl)benzoyl) cyclohexane-1,3-dione; NTBC) that blocks 4-hydroxyphenylpyruvic acid dioxygenase (HPPD; EC 1.13.11.27) which converts 4-hydroxyphenylpyruvic acid (HPPA) into HGA was shown to prevent ochronosis in AKU mice (Preston et al. 2014; Keenan et al. 2015), leading to a series of human clinical trials assessing nitisinone in AKU (Ranganath et al. 2014). The SONIA-1 trial concluded that nitisinone effectively reduces HGA to a level likely to prevent ochronosis (Ranganath et al. 2014). However, increased serum tyrosine was seen with nitisinone treatment (Ranganath et al. 2014).

Elevated serum tyrosine (tyrosinaemia) causes eye and skin keratopathy in nitisinone-treated hereditary tyrosinaemia type 1 (HT-1; OMIM #276700)(Masurel-Paulet et al. 2008), resembling the dermal and ocular symptoms seen in hereditary tyrosinaemia type II (OMIM #276600)(Macsai et al. 2001). Low-dose nitisinone (0.5mg daily) in an AKU patient has induced ocular irritation and photophobia that presented before corneal crystal deposition (White and Tchan 2018). More worryingly, a study reported mild corneal keratopathy in an AKU patient on 2mg of nitisinone daily with no ocular symptoms or pain (Khedr et al. 2018). This is concerning as untreated keratopathy could be sight-threatening if not detected.

Moreover, the association of tyrosinaemia with neural cognition is another important consideration, as tyrosine is a precursor to neurotransmitters such as dopamine, adrenaline

and noradrenaline. Metabolites of these neurotransmitters have previously been shown to significantly alter with nitisinone treatment in AKU patients (Davison et al. 2018a). Similarly, numerous reports and studies document impaired cognitive function in HT-1 patients that have been treated long-term with nitisinone and protein restriction (Masurel-Paulet et al. 2008; Thimm et al. 2012; van Ginkel et al. 2016). These neurodevelopmental effects however could be due to either nitisinone-induced tyrosinaemia or severe liver failure experienced before diagnosis and subsequent initiation of nitisinone treatment. With the cause of impaired cognition in HT-1 remaining to be elucidated, the off-licence use of nitisinone has been restricted to ≥16 years in AKU.

A tyrosine-lowering strategy that can prevent keratopathy, such as protein/tyrosine restriction, is implemented in conjunction with nitisinone treatment in HT-1 (de Laet et al. 2013; Mayorandan et al. 2014). However, poor adherence to protein supplements coupled with an inadequate low protein diet, can lead to essential amino acid and micronutrient deficiency (Acosta and Matalon 2010; van Spronsen et al. 2017). Although advised, no clinical study has investigated the effectiveness of dietary restriction at reducing nitisinone-induced tyrosinaemia in AKU.

Here, dietary restriction of tyrosine and phenylalanine was investigated in nitisinone-treated AKU mice to establish its effectiveness at reducing tyrosinaemia. Additional observational

data of dietary intervention in AKU patients attending the National Alkaptonuria Centre (NAC; Liverpool, UK), where patients receive 2mg nitisinone daily, is presented. At the NAC, serum tyrosine increases to a mean level of approximately 600-700µmol/L (Milan et al. 2017; Davison et al. 2018b) with nitisinone, therefore patients are advised to restrict protein intake with guidance from a specialist dietician. We provide proof-of-concept that mechanisms reducing uptake of dietary tyrosine into the bloodstream would be effective.

Methods

Mice

BALB/c Hgd-/- mice (Preston et al. 2014), referred to as AKU, were all housed and maintained within the University of Liverpool Biological Services Unit in specific pathogen free conditions in accordance with Home Office UK guidelines. Food and water were available *ad libitum*.

Dietary Restriction Studies

To investigate tyrosine and phenylalanine dietary restriction on nitisinone-induced tyrosinaemia and on other tyrosine pathway metabolites, AKU mice (n=6; 3 male, 3 female) were treated with nitisinone for 1 week on normal diet, then switched to either tyrosine/phenylalanine-free or phenylalanine only-free diets with phenylalanine supplemented into the drinking water to investigate dose response, whilst still on nitisinone.

Controls remained on normal diet. Blood samples were taken pre-nitisinone, 1 week post-nitisinone and then during dietary restriction.

<u>Diets</u>

The normal control mouse diet (CRM(P), product code 801722, Special Diet Services (SDS), UK), contained 0.88% phenylalanine and 0.59% tyrosine. Restricted diets were synthetically made by SDS in pellet form. Synthetic raw materials were used to produce an amino acid defined diet which has an amino acid profile similar to the normal CRM(P) diet, with tyrosine and/or phenylalanine removed and balanced on maize starch.

<u>Nitisinone and phenylalanine provision in drinking water</u>

Nitisinone was added to the drinking water at 4mg/L (0.8mg/kg; based on 30g mouse drinking approximately 6ml/day) and was freely available. Phenylalanine was added to the drinking water at various doses, between 0-5mg/ml as indicated in the results.

<u>Calculating dietary restriction</u>

Daily food and water intake is approximately 5g and 6ml respectively, per 30g bodyweight for the BALB/cByJ strain (Bachmanov et al. 2002). A 30g mouse on the normal CRM(P) diet therefore consumes approximately 44mg/day phenylalanine and 29.5mg/day tyrosine. In

the tyrosine/phenylalanine-free diet study, drinking water was supplemented with 5mg/ml, 2.5mg/ml and 1.25mg/ml phenylalanine, equating to approximately 68% phenylalanine/0% tyrosine, 34% phenylalanine/0% tyrosine and 17% phenylalanine/0% tyrosine, respectively. In the phenylalanine free diet study, drinking water was supplemented with 2.5mg/ml, 0.625mg/ml and 0mg/ml phenylalanine, equating to approximately 34% phenylalanine/100% tyrosine, 8.5% phenylalanine/100% tyrosine and 0% phenylalanine/100% tyrosine, respectively.

Blood Collection from mice

Acidified plasma from venous tail bleeds was collected to analyse tyrosine pathway metabolites via HPLC tandem mass spectrometry (Hughes et al. 2015).

National Alkaptonuria Centre (NAC) patients

NAC patients receive off-licence treatment of 2mg nitisinone daily, dietary advice and have serum tyrosine monitored at yearly visits (Milan et al. 2017). The NAC aims to achieve tyrosine <500µmol/L. Tyrosine thresholds of 500-700µmol/L and 700-900µmol/L are used as guidleines for reducing dietary protein consumption to 0.9g and 0.8g protein/kg bodyweight/day respectively. Patients with tyrosine >700µmol/L are more closely monitored with additional blood sampling if required. Fasting blood samples were taken in the morning. Ten anonymised NAC patients with tyrosine >700µmol/L are reported here. All

patients received advice to reduce dietary protein intake to a recommended minimum level of 0.75g protein/kg bodyweight/day, to reduce circulating tyrosine whilst meeting minimum nutritional requirements. A TYR cooler® (Vitaflo, UK) protein substitute (free in tyrosine/phenylalanine) drink was used by 7/10 patients. TYR cooler dosage was determined by the dietician for each individual; protein removed from the diet, below the recommended minimum, was exchanged for the equivalent amount of protein in the cooler. These patients had been attending the NAC for a mean of 36.5 months [range 29-48] at the initial tyrosine concentration, with a mean age of 56 years [range 29-71]. The gender of patients was 5 male, 5 female. 8 were white British, 1 was Indian and 1 was Pakistani.

Statistical analysis

Statistical analysis was performed using Stats Direct 3 statistical software (England, UK). Means are represented as mean±standard error on the mean (SEM). Statistical significance, using $\alpha=0.05$; $p<0.05$ *, $p<0.01$ **, $p<0.001$ ***. Error bars represent SEM.

Results

Effect of nitisinone treatment on tyrosine pathway metabolites

Tyrosine pathway metabolites were measured in the plasma of 24 AKU mice pre- and post-nitisinone treatment (4mg/L in drinking water for 7 days) (figure 2B). HGA was significantly

decreased 8-fold from 255.1±43.2μmol/L to 32.9±3.2μmol/L whilst all other metabolites increased; all changes were significant (p<0.001, two-tailed paired t-test). Tyrosine increased 11-fold from 72.9±3.6μmol/L to 813.3±37.6μmol/L. Phenylalanine increased 1.5-fold from 67.3±2.4μmol/L to 100.8±4.9μmol/L. HPPA increased 14.5-fold from 10.5±0.4μmol/L to 151.8±12.9μmol/L. 4-hydroxyphenyllactic acid (HPLA) increased 19-fold from 2.0±0.2μmol/L to 38.6±3.0μmol/L.

Tyrosine/phenylalanine dietary restriction

Nitisinone-treated AKU mice were fed a tyrosine and phenylalanine-free diet, with phenylalanine supplemented into the drinking water (5-1.25mg/ml). The control group remained on a normal diet. Blood samples were taken according to the scheme in figure 2A. Three days after dietary restriction, tyrosine was reduced from 813.3±37.6μmol/L (all groups, n=24; figure 2B) in a dose responsive manner to 389.3±42.3μmol/L, 274.8±6.3μmol/L, 144.3±13.0μmol/L in the 5mg, 2.5mg and 1.25mg restricted groups, respectively. After 14 days of restriction, these reductions were maintained. All comparisons to the control group at both 3 and 14 days were significant (p<0.001).

After 3 days of dietary restriction, significant reductions in phenylalanine, HGA, HPPA and HPLA were present, however the dose response was not as clear in these metabolites

(figure 2B). After 14 days of dietary restriction, only HGA and HPLA showed significant reductions compared to the control group.

A second tyrosine/phenylalanine dietary restriction study (data not shown) was carried out, with lower doses of phenylalanine supplementation in the water (0.625mg/ml, 0.3125mg/ml and 0mg/ml). Further restriction of phenylalanine however did not result in lower plasma tyrosine concentrations than those shown in figure 2, at both 3 and 4 days of restriction. No significant weight changes were seen between any of the groups at 4 days of restriction.

Phenylalanine only dietary restriction

Nitisinone-treated AKU mice were fed a phenylalanine-free diet with normal tyrosine, with phenylalanine supplemented in drinking water (2.5-0mg/ml). The control group remained on a normal diet. Blood samples were taken according to the scheme in figure 3A. Plasma tyrosine was 63.5±2.7µmol/L pre-nitisinone and increased to 748.9±26.8µmol/L post-nitisinone (n=24, all groups, figure 3B). After 3 days of phenylalanine restriction, tyrosine levels were 757.3±54.9µmol/L, 533.2±28.8µmol/L and 656.2±69.6µmol/L, with only the latter two groups, (0.625mg/ml and phenylalanine-free), being significantly different to control (p<0.001 and p=0.014, respectively). After 7 days of dietary restriction, none of the restricted groups had significantly lower tyrosine compared with the control group.

Significant reductions in plasma phenylalanine were seen at both 3 days (all groups p<0.001 compared with control) and after 7 days (0.625mg/ml and phenylalanine-free, both p<0.001 compared with control; 2.5mg/ml not significantly different, p=0.065) of phenylalanine dietary restriction. HPLA demonstrated significant differences after 3 days in the 0.625mg/ml and phenylalanine-free groups however these significant differences were not maintained after 7 days. HGA showed significant differences after 7 days of dietary restriction in all the restricted groups however these differences were not seen after 3 days. HPPA was not significantly reduced in the restricted groups compared with control, at neither 3 nor 7 days of dietary restriction.

Body weight

The extent of tyrosine/phenylalanine dietary restriction (%) in these studies was based upon daily food and water intake values for mice weighing 30g. At day 7 when dietary restriction was implemented, the mean body weight of all mice (n=24) was 29.8g (23.5–35.7g) and 30.0g (23.7–37.1g) in the tyrosine/phenylalanine-free and phenylalanine only-free diet studies respectively.

Individual body weight was monitored over the duration of dietary restriction. At 14 days of restriction in the tyrosine/phenylalanine-free diet study, the restricted groups showed no significant change in body weight (figure 2C) compared to the control group (p=0.841).

Figure 3C shows the changes in body weight after 7 days of restriction in the phenylalanine only diet study. One-way ANOVA demonstrated a significant difference in weight change between the groups (p=0.014), with differences found between the 2.5mg/ml group compared with the 0.625mg/ml (p=0.23) and phenylalanine-free (p=0.021) groups (post-hoc Tukey test). None of the restricted groups however showed weight changes significantly different to the control group.

NAC dietary intervention in AKU patients

Average estimated protein intake from 7-day food diaries in all AKU patients attending the NAC was 1.1g protein/kg bodyweight/day at baseline visit 1 (unpublished data) before any intervention, which is comparable with the general population (NDNS2016). With nitisinone, dietary protein intake is restricted to a recommended minimum level of 0.75g protein/kg bodyweight/day (SACN/COMA2008/2017), with additional metabolic requirements provided by prescribed tyrosine/phenylalanine-free amino acid supplements in some patients.

Overall, significant reductions in serum tyrosine (figure 4) were seen in the 10 NAC patients observed (p=0.002; two-tailed Wilcoxon's signed rank test), with 4 patients reducing serum tyrosine <700µmol/L. Three patients (figure 4, dashed lines) reduced serum tyrosine (mean[range]) by 22[18-30]% by advised protein restriction alone; the other 7 patients required a combination of reduced protein intake with tyrosine/phenylalanine-free supplements to achieve a 33[8-62]% reduction. The initial tyrosine value for patients using amino acid supplements was with dietary restriction alone, with subsequent values using a combination of reduced protein intake and amino acid supplementation. Across all 10 patients and all sample time points, phenylalanine (mean[range]) was 57[38-108]µmol/L.

Discussion

The treatment of AKU with the HGA-lowering drug nitisinone has proven to be effective at reducing HGA (Ranganath et al. 2018a). However, data from clinical trial (Ranganath et al. 2014) and off-label prescription use at the NAC (Milan et al. 2017) clearly demonstrate concomitant elevation of tyrosine. Tyrosinaemia can cause eye and skin keratopathy in a minority of patients requiring a lower dose or cessation of nitisinone, or a strict low protein diet, to resolve symptoms. Low-dose nitisinone, ranging from 0.5-2mg daily, has caused eye complications in 5 reported AKU patients to date (Introne et al. 2011; Stewart et al. 2014; Khedr et al. 2018; White and Tchan 2018). Attempting to restrict dietary tyrosine and

phenylalanine consumption, whilst taking nitisinone to keep HGA low, is the most logical approach to combat tyrosinaemia. A protein restricted diet however proves very difficult to maintain and often causes keratopathy to return when not adhered to (Khedr et al. 2018). Therefore, finding an alternative strategy to reduce dietary uptake of tyrosine and/or phenylalanine would allow nitisinone-treated AKU patients to consume a normal protein diet. Before such strategies are investigated, the findings of this paper has showed that reducing dietary tyrosine/phenylalanine is effective at reducing tyrosine in AKU mice, with similar observational evidence that lowering tyrosine via dietary intervention in patients is achievable, therefore establishing a proof of principle for targeting dietary tyrosine/phenylalanine in patients who are on nitisinone.

Tyrosine threshold recommendations with nitisinone treatment, which are often arbitrary, vary in the literature. In HT-1 children <12 years, the recommended tyrosine level is 200-400µmol/L, with concentrations after this age allowed to rise, with eye complications experienced >800µmol/L (de Laet et al. 2013). Another HT-1 review of 22 centres treating patients aged 0-24 years found maximum acceptable tyrosine levels to be 200-800µmol/L, with 400µmol/L determined to be a safe and feasible target (Mayorandan et al. 2014). A report of an adult AKU patient who experienced eye keratopathy whilst receiving low-dose nitisinone (0.5-1.5mg daily) suggests a target tyrosine of <600µmol/L (White and Tchan 2018), whilst a more recent NAC publication suggests <500µmol/L is acceptable (Ranganath

et al. 2018b). NAC tyrosine thresholds are designed to fit a dietetic care plan, that aims to reduce serum tyrosine in order to prevent symptomatic keratopathy, with systematic removal of dietary protein, whilst taking into account compliance difficulites experienced in patients.

In this study, the mouse tyrosine/phenylalanine-free diet clearly showed a dose-responsive reduction in plasma tyrosine, with all groups reaching <400µmol/L. The least restricted group (5mg/ml) reduced tyrosine to 389µmol/L at 3 days, achieved with approximately 68% phenylalanine/no tyrosine. We also showed evidence that human patients on nitisinone can reduce serum tyrosine with dietary intervention. Tyrosine was significantly lowered either by a low protein diet alone or in combination with prescribed tyrosine/phenylalanine-free amino acid supplementation. Although 4/10 patients achieved tyrosine <700µmol/L, tyrosine was greatly reduced in some patients, notably from 1084µmol/L to 762µmol/L, and from 1217µmol/L to 859µmol/L. Of the 4 patients that reduced tyrosine below 700µmol/L, only 1 patient achieved this with dietary advice alone; the other 3 patients used amino acid supplementation. Prior to nitisinone, most adult AKU patients will have consumed a normal protein diet throughout their life, therefore adaption to dietary changes can be difficult, especially in the absence of tyrosine-related keratopathy where no benefit is perceived.

The NAC patient data was not collected as a controlled trial, but as an observational study where patients are only advised to restrict their protein intake, therefore strict monitoring of compliance and protein intake was not carried out. After specialist advice, patients were responsible for restricting dietary protein in their daily lives, with guidance from the dietician if needed. Progress with dietary compliance is a long-term iterative process, since achieving long-term, sustained behaviour change in adults is a recognised national dilemma. Compliance is the most likely reason that only 1/10 NAC patients achieved <500µmol/L tyrosine and is why mice were used for the restriction experiments reported here. The diets of mice are easy to control, in addition to eliminating other confounding factors such as lifestyle, exercise, mobility and disease severity differences. The mouse data provides evidence that dietary restriction of tyrosine/phenylalanine can effectively reduce nitisinone-induced tyrosinaemia, providing the rationale to carry out a controlled trial in human AKU patients, where lower tyrosine levels than those presented here would be expected.

In mice, the dose response observed due to phenylalanine supplementation, in the absence of tyrosine (figure 2), suggests that tyrosine derived from phenylalanine is important to target. However, phenylalanine only restriction was ineffective at lowering nitisinone-induced tyrosinaemia, even with complete phenylalanine restriction (figure 3). This is an important consideration as emerging experimental and dietary phenylalanine-lowering strategies for the metabolic disease phenylketonuria (OMIM #261600) may have been

beneficial for nitisinone-induced tyrosinaemia if phenylalanine restriction alone had proven effective. Such strategies include enzymatic phenylalanine degradation by exogenous phenylalanine ammonia lyase enzyme, diets supplemented with naturally occurring glycomacropeptide (GMP) protein that is naturally low in phenylalanine instead of synthetic amino acid foods, and genetically modified probiotics that target phenylalanine-degradation in the intestine (Al Hafid and Christodoulou 2015; Bell et al. 2017).

Due to phenylalanine being an essential amino acid, its restriction alone could lead to inadequate protein turnover, with the balance of catabolism/anabolism disrupted, causing a loss of lean mass (Yannicelli 2015). Although not significantly different to the control group in the phenylalanine only restriction study, there was a significant body weight reduction in the phenylalanine-free and 0.625mg/ml groups compared to the 2.5mg/ml group (figure 3) at 7 days. This weight loss was seen in the presence of tyrosine, suggesting that phenylalanine restriction could lead to catabolism, therefore targeting tyrosine would be more desirable. At 14 days of restriction, no significant weight changes were seen in figure 2 in which tyrosine was absent and phenylalanine was partially present.

It is essential to balance lowering protein intake in order to lower tyrosine, with maintaining a healthy weight and muscle mass, since catabolism itself provides residual tyrosine. During routine NAC visits, anthropometric data such as such as grip, mid upper arm circumference

and bioimpedance data such as lean mass and fat mass, is collected to provide sequential evidence of body composition trends to minimise risk of catabolism. These parameters were not investigated in the AKU patients presented in figure 4, as less than half of the tyrosine concentrations were measured at a routine (yearly) NAC visit, where such anthropometric data is collected. These parameters are worthy of investigation in both mice and humans in the future.

In addition to decreased tyrosine, other metabolic improvements were observed with dietary restriction in mice. HGA, which is lowered with nitisinone treatment, was then significantly lowered in the restricted groups compared to the control in the tyrosine/phenylalanine-free study. Both HPPA and HPLA in mice, which were elevated with nitisinone as recently shown in human AKU patients (Milan et al. 2019), were significantly reduced compared to the control with restriction. Although the effect of elevated HPPA and HPLA is unknown, reducing them back towards normal physiological levels would be desirable. Phenylalanine in mice was significantly reduced after 3 days in the tyrosine/phenylalanine restriction study, but this was not maintained to 14 days. In human NAC patients, phenylalanine levels were within the normal reference range.

Nitisinone is a lifelong treatment for AKU and is associated with hypertyrosinaemia. Removal of tyrosine/phenylalanine, but not phenylalanine alone, from the diet of mice is

effective at reducing nitisinone-induced tyrosinaemia. Protein restriction coupled with tyrosine/phenylalanine-free amino acid supplementation was able to reduce tyrosine levels in patients observed at the NAC, although these reductions were greater in mice, most likely owing to patient compliance. A controlled trial in AKU patients would likely see results similar to the mouse data shown here. We suggest that strategies reducing tyrosine uptake from the intestine, such as degradation of tyrosine by an exogenous enzyme or a tyrosine-specific binder that prevents uptake into the circulation, that allow a normal protein diet to be consumed, could be investigated not only for nitisinone-treated AKU patients, but also for nitisinone-treated HT-1 patients.

References

Acosta PB, Matalon KM (2010) Nutrition management of patients with inherited disorders of aromatic amino acid metabolism. In: Acosta PB (ed) Nutrition Management of Patients with Inherited Metabolic Disorders. Jones and Bartlett Publishers, Sudbury, pp 119–174.

Al Hafid N, Christodoulou J (2015) Phenylketonuria: a review of current and future

treatments. Transl Pediatr 4:304–317.

Bachmanov AA, Reed DR, Beauchamp GK, Tordoff MG (2002) Food intake, water intake, and drinking spout side preference of 28 mouse strains. Behav Genet 32:435–443.

Bell SM, Wendt DJ, Zhang Y, et al (2017) Formulation and PEGylation optimization of the therapeutic PEGylated phenylalanine ammonia lyase for the treatment of phenylketonuria. PLoS One 12:e0173269.

Davison AS, Norman B, Milan AM, et al (2018a) Assessment of the effect of once daily nitisinone therapy on 24-h urinary metadrenalines and 5-hydroxyindole acetic acid excretion in patients with alkaptonuria after 4 weeks of treatment. JIMD Rep 41:1–10.

Davison AS, Norman BP, Smith EA, et al (2018b) Serum amino acid profiling in patients with alkaptonuria before and after treatment with nitisinone. JIMD Rep 41:109–117.

de Laet C, Dionisi-Vici C, Leonard J V, et al (2013) Recommendations for the management of tyrosinaemia type 1. Orphanet J Rare Dis 8:8.

Gaines JJ, Pai GM (1987) Cardiovascular ochronosis. Arch Pathol Lab Med 111:991–994.

Helliwell TR, Gallagher JA, Ranganath L (2008) Alkaptonuria - a review of surgical and autopsy pathology. Histopathology 53:503–512.

Hughes AT, Milan AM, Davison AS, et al (2015) Serum markers in alkaptonuria: simultaneous analysis of homogentisic acid, tyrosine and nitisinone by liquid chromatography tandem mass spectrometry. Ann Clin Biochem 52:597–605.

Introne WJ, Perry MB, Troendle J, et al (2011) A 3-year randomized therapeutic trial of nitisinone in alkaptonuria. Mol Genet Metab 103:307–314.

Janocha S, Wolz W, Srsen S, et al (1994) The human gene for alkaptonuria (AKU) maps to chromosome 3q. Genomics 19:5–8.

Keenan CM, Preston AJ, Sutherland H, et al (2015) Nitisinone arrests but does not reverse ochronosis in alkaptonuric mice. JIMD Rep 24:45–50.

Khedr M, Judd S, Briggs MC, et al (2018) Asymptomatic corneal keratopathy secondary to hypertyrosinaemia following low-dose nitisinone and a literature review of tyrosine keratopathy in alkaptonuria. JIMD Rep 40:31–37.

La Du BN, Zannoni VG, Laster L, Seegmiller JE (1958) The nature of the defect in tyrosine metabolism in alcaptonuria. J Biol Chem 230:250–261.

Macsai MS, Schwartz TL, Hinkle D, et al (2001) Tyrosinemia type II: nine cases of ocular signs and symptoms. Am J Ophthalmol 132:522–527.

Masurel-Paulet A, Poggi-Bach J, Rolland M-O, et al (2008) NTBC treatment in tyrosinaemia type I: long-term outcome in french patients. J Inherit Metab Dis 31:81–87.

Mayorandan S, Meyer U, Gokcay G, et al (2014) Cross-sectional study of 168 patients with hepatorenal tyrosinaemia and implications for clinical practice. Orphanet J Rare Dis 9:107.

Milan AM, Hughes AT, Davison AS, et al (2019) Quantification of the flux of tyrosine pathway

metabolites during nitisinone treatment of alkaptonuria. Sci Reports 2019 91 9:10024.

Milan AM, Hughes AT, Davison AS, et al (2017) The effect of nitisinone on homogentisic acid and tyrosine: a two-year survey of patients attending the National Alkaptonuria Centre, Liverpool. Ann Clin Biochem 54:323–330.

Preston AJ, Keenan CM, Sutherland H, et al (2014) Ochronotic osteoarthropathy in a mouse model of alkaptonuria, and its inhibition by nitisinone. Ann Rheum Dis 73:284–289.

Ranganath LR, Khedr M, Milan AM, et al (2018a) Nitisinone arrests ochronosis and decreases rate of progression of Alkaptonuria: Evaluation of the effect of nitisinone in the United Kingdom National Alkaptonuria Centre. Mol Genet Metab 125:127–134.

Ranganath LR, Khedr M, Milan AM, et al (2018b) Nitisinone arrests ochronosis and decreases rate of progression of alkaptonuria: evaluation of the effect of nitisinone in the United Kingdom National Alkaptonuria Centre. Mol Genet Metab 125:127–134.

Ranganath LR, Milan AM, Hughes AT, et al (2014) Suitability Of Nitisinone In Alkaptonuria 1 (SONIA 1): an international, multicentre, randomised, open-label, no-treatment controlled, parallel-group, dose-response study to investigate the effect of once daily nitisinone on 24-h urinary homogentisic acid. Ann Rheum Dis 1:1–6.

Stewart RMK, Briggs MC, Jarvis JC, et al (2014) Reversible keratopathy due to hypertyrosinaemia following intermittent low-dose nitisinone in alkaptonuria: a case report. In: JIMD rep 17:1-6.

Taylor AM, Boyde A, Wilson PJ, et al (2011) The role of calcified cartilage and subchondral bone in the initiation and progression of ochronotic arthropaththy in alkaptonuria. Arthritis Rheum 63:3887–3896.

Thimm E, Richter-Werkle R, Kamp G, et al (2012) Neurocognitive outcome in patients with hypertyrosinemia type I after long-term treatment with NTBC. J Inherit Metab Dis 35:263–268.

van Ginkel WG, Jahja R, Huijbregts SCJ, et al (2016) Neurocognitive outcome in tyrosinemia type 1 patients compared to healthy controls. Orphanet J Rare Dis 11:87.

van Spronsen FJ, van Rijn M, Meyer U, Das AM (2017) Dietary considerations in tyrosinemia type I. In: Advances in Experimental Medicine and Biology. pp 197–204.

White A, Tchan MC (2018) Nitisinone-induced keratopathy in alkaptonuria: a challenging diagnosis despite clinical suspicion. JIMD Rep 40:7–9.

Yannicelli S (2015) Protein requirements in inherited metabolic diseases. In: Bernstein LE, Rohr F, Helm JR (eds) Nutrition Management of Inherited Metabolic Diseases. Lessons from Metabolic University. Springer International Publishing Switzerland, pp 63–73.
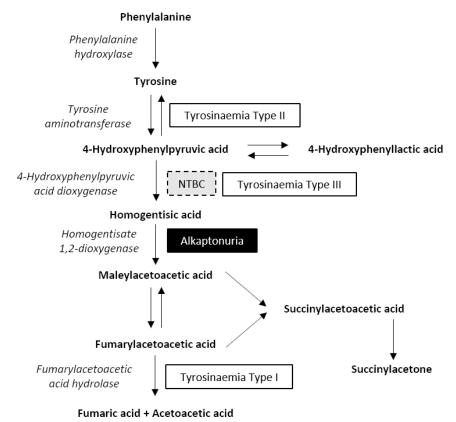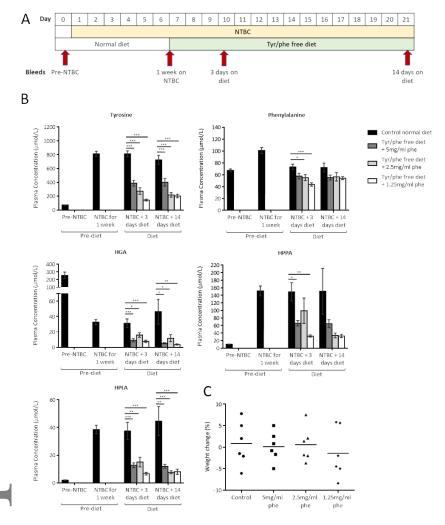
Figure legends

Figure 1. Tyrosine metabolism pathway. Enzymes are in italics. Disorders are in boxes. NTBC (nitisinone) inhibits 4-hydroxyphenylpyruvic acid dioxygenase.

Figure 2. Dietary restriction of tyrosine and phenylalanine in NTBC-treated AKU mice. (A) shows when blood samples were taken and when diet conditions were altered. (B) shows plasma metabolite levels before NTBC treatment (all mice; n=24), after 1 week of NTBC treatment (all mice; n=24), then after 3 (n=6) and 14 days (n=6) of tyrosine/phenylalanine restriction. (C) shows the change in bodyweight of individual mice in each group from day 7 (start of dietary restriction) and day 21 (after 14 days of dietary restriction). NTBC=nitisinone. HGA=homogentisic acid. HPPA=4-hydroxyphenylpyruvic acid. HPLA=4-hydroxyphenyllactic acid. Error bars represent SEM. One-way ANOVA (Tukey post-hoc) significance: $p < 0.05$ *, $p < 0.01$ **, $p < 0.001$ ***.

Figure 3. Dietary restriction of phenylalanine in NTBC-treated AKU mice. (A) shows when blood samples were taken and when diet conditions were altered. (B) shows the plasma metabolite levels before NTBC treatment (all mice; n=24), after 1 week of NTBC treatment (all mice; n=24), then after 3 (n=6) and 14 (n=6) days of diet phenylalanine restriction. (C) shows the change in bodyweight of individual mice in each group from day 7 (start of dietary restriction) and day 14 (after 7 days of dietary restriction). NTBC=nitisinone. HGA=homogentisic acid. HPPA=4-hydroxyphenylpyruvic acid. HPLA=4-hydroxyphenyllactic acid. Error bars represent SEM. One-way ANOVA (Tukey post-hoc) significance: $p < 0.05$ *, $p < 0.01$ **, $p < 0.001$ ***.
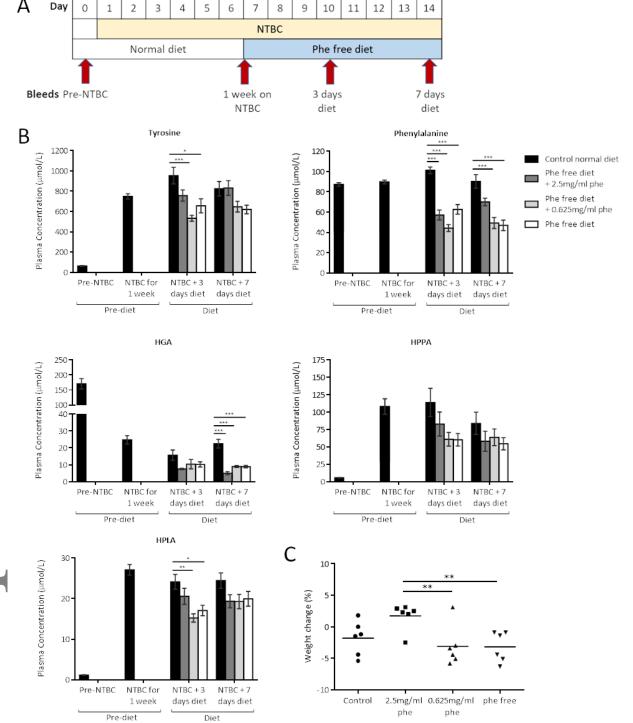
Figure 4. Serum tyrosine from 10 AKU patients receiving nitisinone attending the National Alkaptonuria Centre. All 10 patients had tyrosine >700µmol/L. Both dietary advice (restriction of dietary protein to a recommended minimum level of 0.75g protein/kg bodyweight) (n=3) and dietary advice in combination with tyrosine/phenylalanine-free amino acid supplements (n=7) reduced serum tyrosine. Initial tyrosine concentrations in the group using amino acid supplements were measured after dietary advice alone, with subsequent tyrosine concentrations measured after a combination of dietary advice and amino acid supplement use.