



Characterization of changes in the tyrosine pathway by 24-h profiling during nitisinone treatment in alkaptonuria

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ARTICLE INFO

Keywords:

Alkaptonuria
Homogentisic acid
Tyrosine
Hydroxyphenylpyruvate
Hydroxyphenyllactate
Phenylalanine
Nitisinone
24-h sampling

ABSTRACT

Background: Although changes in the tyrosine pathway during nitisinone therapy are known, a complete characterization of the induced tyrosinaemia is lacking to improve disease management.

Patients and methods: Our research aims were addressed by 24-h blood sampling. 40 patients with alkaptonuria (AKU), treated with 0, 1, 2, 4 and 8 mg nitisinone daily ($n = 8$), were studied over four weeks. Serum homogentisic acid (sHGA), tyrosine (sTYR), phenylalanine (sPHE), hydroxyphenylpyruvate (sHPPA), hydroxyphenyllactate (sHPLA) and nitisinone (sNIT) were measured at baseline and after four weeks.

Results: sNIT showed a clear dose-proportional response. sTYR increased markedly but with less clear-cut dose responses after nitisinone. Fasting and average 24-h (C_{av}) sTYR responses were similar. Individual patient sTYR 24-h profiles showed significant fluctuations during nitisinone therapy. At week 4, sTYR, sHPPA and sHPLA all showed dose-related increases compared to V0, with the greatest difference between 1 and 8 mg nitisinone seen for HPLA, while there was no change from V0 in sPHE. sHGA decreased to values around the lower limit of quantitation.

Discussion: There was sustained tyrosinaemia after four weeks of nitisinone therapy with significant fluctuations over the day in individual patients. Diet and degree of conversion of HPPA to HPLA may determine extent of nitisinone-induced tyrosinaemia.

Conclusion: A fasting blood sample is recommended to monitor sTYR during nitisinone therapy. Adaptations in HPPA metabolites as well as the inhibition of tyrosine aminotransferase could be contributing factors generating tyrosinaemia during nitisinone therapy.

1. Introduction

Humans consume phenylalanine (PHE) and tyrosine (TYR) as dietary protein in excess of requirement. The excess PHE and TYR is metabolized fully via the tyrosine catabolic pathway. Alkaptonuria (AKU) is a disorder of the tyrosine pathway caused by the lack of homogentisate 1,2 dioxygenase (HGD) (EC.1.13.11.5) enzymatic activity, interrupting the normal catabolism of TYR and resulting in failure to convert

homogentisic acid (HGA) to maleylacetoacetic acid. This leads to accumulation of HGA and the damaging effects of AKU [1,2]. Inhibiting the enzyme hydroxyphenylpyruvate 1,2, dioxygenase (HPPD) (EC:1.13.11.27) by nitisinone (NIT) decreases HGA [3] and ameliorates AKU [4]. The European Medicines Agency approved NIT 10 mg daily as the first disease-modifying therapy for the rare disease alkaptonuria (AKU) (OMIM#203500) [5], following successful outcomes in the phase 3 Suitability of Nitisinone in Alkaptonuria 2 study (SONIA 2) [6].

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<https://doi.org/10.1016/j.ymgmr.2022.100846>

Received 9 January 2022; Received in revised form 19 January 2022; Accepted 26 January 2022

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Inhibition of HPPD however, not only prevents conversion of hydroxyphenylpyruvate (HPPA) to HGA but also leads to accumulation of metabolites proximal to this inhibition [7]. Massive tyrosinaemia results from HPPD inhibition leading to deleterious effects such as corneal keratopathy [6,8,9]. Further, tyrosinaemia is possibly associated with the cognitive impairment observed in children when nitisinone is used as life-saving therapy in hereditary tyrosinaemia type 1 (HT-1) (OMIM#276700), although the causative mechanisms of this impairment are not yet fully understood [10,11].

HPPA, PHE, TYR, and hydroxyphenyllactate (HPLA) are among the main tyrosine metabolites proximal to HPPD inhibition. Knowledge of how these metabolites change over a 24-h period during NIT therapy has not been established. Although changes in the tyrosine pathway during NIT therapy have been described, a more complete understanding may optimise the management of NIT-induced tyrosinaemia further [7,12]. In addition to the lack of data to characterise the changes in proximal metabolites, information on the influence of meals and physical exercise on diurnal variations of TYR concentrations in adults with AKU on NIT therapy is presently unavailable. Conditions of blood sampling, such as the time of day, fasting or non-fasting and other factors, are not elucidated, and intermittent variations of sTYR levels between two measurements remain to be fully characterised.

In a randomised controlled trial called SONIA 1, conducted between 2013 and 2014, 24-h blood sampling was carried out before any administration of different doses of NIT and at four weeks after NIT treatment. We have earlier presented sTYR 24-h profiles before treatment, as well as the 24-h sNIT profiles at V2, but not the profiles of sPHE, sHPPA and sHPLA during nitisinone treatment [13]. The objective of the present analysis of these data was to determine whether the outcomes of the 24-h sampling could guide better practice in AKU and NIT therapy. A specific aim of the present analysis was to determine whether a fasting or random single blood sample can better reflect the control of tyrosinaemia in adult NIT-treated AKU patients for the purposes of monitoring. A further aim was to examine the effect of meals on tyrosine and other metabolite 24-h concentrations. Further, we wanted to explore the relationships between the metabolites proximal to the site of action of NIT to better characterise the tyrosinaemia during NIT therapy.

2. Methods

2.1. Patients and treatment

SONIA 1 was a phase 2, open-label, multicentre, randomised, no-treatment controlled, parallel-group dose-response study described further in the supplementary file (Table S1, Figs. 1, S1). The study sites were Liverpool (UK) and Piešťany (Slovakia). The aim was to recruit 40 patients with diagnosed AKU, aged 18 years or older, randomised to five groups of 0, 1, 2, 4 and 8 mg of NIT groups, stratified by each study centre using randomly permuted blocks. The visits were designated as V0, V1, and V2, to denote baseline, 2 and 4 weeks in the study. V1 will not be discussed further as the 24-h sampling, the focus of this manuscript, was carried out only at V0 and V2. The duration of the treatment period was 4 weeks, during which the study drug, NIT, was administered once daily in the morning. Further details of the SONIA 1 study have been previously published [14]. Patients were requested to maintain stable dietary habits during the 4-week study period in order not to change their dietary protein intake and to maintain a stable weight. Ethical aspects of the study are discussed further in the supplementary file.

2.2. 24-h blood sampling

On Day-2 of the three-day visit, in a hospital in-patient setting, the 24-h blood sampling was carried out at V0, before administration of the first dose of NIT. The schedule of events is described in Table S1 and Fig. S1. Five millilitres of venous blood were obtained through an

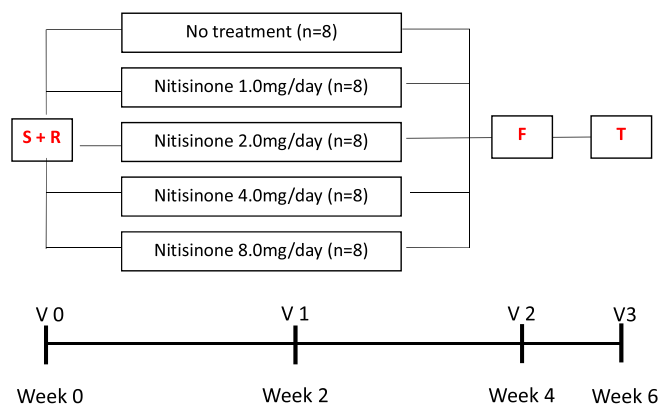


Fig. 1. Suitability Of Nitisinone In Alkaptonuria 1 (SONIA 1) study design. The study consisted of two main periods: treatment and follow-up. After screening, patients were randomised at baseline (V0) (1:1:1:1:1) to no treatment (control), and oral daily doses of nitisinone of 1 mg, 2 mg, 4 mg and 8 mg. The treatment period consisted of 4 weeks, during which study drug was administered. At 6 weeks a follow-up telephone call concluded the study. Visits are shown as V0, V1, V2 and V3 for screening baseline, two weeks, four weeks and telephone close-out (Abbreviations: S + R = screening, baseline and randomisation visit; F = final treatment visit; T = telephone follow-up visit).

indwelling forearm cannula 0 h pre-dose (fasting), and another pre-dose sample (non-fasting) at time 0.5 h, just after completion of breakfast and before intake of NIT. Further samples were collected at 1, 1.5, 2.5, 3.5, 4.5, 6.5, 8.5, 10.5, 12.5, 15.5, 18.5, and 24.5 h after starting breakfast. Breakfast, lunch, and dinner were administered at approximately 0, 5 and 11 h respectively. These meal intakes were not controlled for their protein or calorie content. Patients were resting during the 24-h period, either watching television or simply sitting or sleeping.

At V2, the procedure at V0 was repeated. The last dose of NIT was administered at the time point 0.5 h after start of breakfast at V2.

2.3. Urine sample collection and handling

At V0 and V2, urine was collected for the measurement of urea over 24-h into 2.5 L bottles containing 30 mL of 5 N H2SO4 and stored away from direct sunlight. The weight of the collected urine was recorded and used as the volume in the calculations of amount of urea excreted assuming a density of 1 g/mL. An aliquot of the collected urine was frozen and kept at -20°C until analysis.

2.4. Chemical analyses

Measurement of NIT, HGA, TYR, PHE, HPPA, and HPLA, in serum (indicated as sNIT, sHGA, sTYR, sPHE, sHPPA, and sHPLA) was carried out on all samples collected at the described sampling points. Blood samples were collected in plain serum tubes (Sarstedt, Germany). An aliquot of serum was immediately acidified using perchloric acid (10% v/v 5.8 M) [14], to stabilise the HGA, and kept frozen at -80°C until analysis. Samples from Piešťany were transported frozen by courier to Liverpool and all biochemical analyses were performed in the Department of Clinical Biochemistry, Liverpool Clinical Laboratories, Liverpool University Hospital NHS Trust.

The concentrations of as sNIT, sHGA, sTYR, sPHE, sHPPA, and sHPLA were measured by liquid chromatography tandem mass spectrometry based on previously published methods [7,15]. All analyses were performed on an Agilent 6490 Triple Quadrupole mass spectrometer with Jet-Stream electrospray ionisation coupled with an Agilent 1290 Infinity II Ultra-High Performance Liquid Chromatography system. Briefly, this method incorporates reverse-phase chromatographic separation on an Atlantis dC18 column (100 mm \times 3.0 mm, 3 μm , Waters); initial chromatographic conditions of 80:20 water:methanol with 0.1%

formic acid (v/v) increased linearly to 10:90 over 5 min. Matrix-matched calibration standards and quality controls were used with appropriate isotopic-labelled internal standards with quantification in multiple reaction mode (NIT, PHE and TYR in positive ionisation and HPPA, HPLA and HGA in negative ionisation). Sample preparation was by dilution in a combined internal standard solution containing $^{13}\text{C}_6$ -nitroisone, $^{13}\text{C}_6$ -HGA, d_4 -TYR and d_5 -PA in 0.1% formic acid (v/v) in deionised water. No internal standard was available for HPPA and HPLA at time of analysis and $^{13}\text{C}_6$ -HGA was therefore validated for use as the internal standard.

Urine urea was photometrically assayed in the 24-h urine collection on a Roche Cobas 701 using an automated assay (hydrolysis with urease and subsequent oxidation of NADH). Urine urea was used to objectively estimate dietary protein intake in keeping with other studies [16,17].

2.5. Derived data

The areas under the 24-h concentration vs. time curve (AUC_{24}) for sNIT, sHGA, sTYR, sPHE, sHPPA, and sHPLA were calculated using the trapezoidal rule. AUC_{24} divided by 24 then gave the average 24-h serum values (C_{av}) for each analyte.

2.6. Statistical analysis

All statistical analyses were post-hoc. Descriptive statistics (mean and standard deviations [SD]) have been calculated for each analyte at each time point, as well as for C_{av} and AUC_{24} .

3. Results

3.1. Baseline demographics and other parameters in SONIA 1

Forty AKU patients, 15 from the Liverpool and 25 from the Piešťany study sites, were screened and randomised into five groups (no treatment, 1, 2, 4 and 8 mg NIT), with all randomised patients completing the 4-week study. Most study patients (67.5%) were male, and the mean age for all 40 patients was 47.2 years, ranging from 19 years to 63 years (Table 1). There were three Asian patients and the rest were Caucasian. All patients had normal renal function (eGFR). Demographic and metabolite data were collected in all patients with no missing values. 24-h blood sampling was carried out as planned in all patients at V0 and again at V2. Analyses for sNIT, sHGA, sTYR, sPHE, sHPPA, and sHPLA

Table 1
Demographic and baseline characteristics in SONIA 1.

	Untreated	1 mg	2 mg	4 mg	8 mg
Age (years) ^a	45.9 (15.3)	44.4 (10.9)	43.9 (13.7)	47.3 (10.7)	54.4 (7.3)
Body weight (week 0) (kg) ^a	69.9 (22.8)	88 (15.6)	74.6 (10.9)	76.9 (14.3)	81.1 (13.7)
Body weight (week 4) (kg) ^a	70.4 (22.9)	88 (16.6)	75.6 (11.7)	77.1 (15)	81.5 (13)
Height (cm) ^a	165.3 (12.1)	170.6 (7.1)	167.1 (9.4)	168.4 (5.9)	165.9 (6.7)
Sex n (%)	Female 4 (50.0%)	1 (12.5%)	3 (37.5%)	3 (37.5%)	2 (25.0%)
	Male 4 (50.0%)	7 (87.5%)	5 (62.5%)	5 (62.5%)	6 (75.0%)
Serum creatinine $\mu\text{mol/L}$ ^a	55.7 (13.5)	61.3 (10.2)	50.4 (10.7)	63.2 (13.3)	60.4 (11.2)
eGFR mL/min/ m^2 ^a	127 (30)	141 (24)	157 (27)	119 (22)	132 (35)
Urine Urea (week 0) (mmol/day) ^a	338 (74)	441 (139)	348 (100)	414 (132)	308 (63)
Urine Urea (week 4) (mmol/day) ^a	360 (101)	385 (75)	343 (87)	389 (148)	328 (91)

^a Values expressed as Mean (SD); n = 8 in each dose group.

were performed with acceptable intra-assay and inter-assay coefficients of variation of less than 6% and 7%, respectively, as previously published [7]. The body weight, and 24-h urine urea were not statistically different at V0 and V2.

3.2. sNIT

At V2, the pre-dose and C_{av} sNIT increased with increasing doses (Table 2, Fig. S2). sNIT increased following NIT administration reaching a peak at 3 h before stabilising over the rest of the 24 h (Fig. 2). The pharmacokinetic parameters have been described in a previous publication [13].

3.3. sHGA

At V0, all sHGA values were similar in all groups (Table 2). Before the first dose of NIT was administered, the sHGA in all patients increased from fasting levels to an initial peak, after breakfast, at around 2 h, before declining. It then showed a further larger increase after lunch and dinner, peaking at around 12 h before reaching trough values at 24 h (Table S3, Fig. S3).

At V2 the no-NIT group showed similar results compared to V0 (Table 2). In the treated patients, many values were below the limit of quantification (LLOQ) of the assay of 3.1 $\mu\text{mol/L}$, especially at higher NIT doses, and no calculations of mean values could be performed. There was a dramatic decrease compared to V0 even at the lowest dose of NIT as has previously been described [13,14].

3.4. sTYR

At V0, all sTYR values in all treatment groups were similar. At V2, the results for the no-NIT group were similar to the V0 results (Table 2).

sTYR increased from fasting levels to an initial peak after breakfast at around one hour, before declining. It then showed a further larger increase after lunch and dinner, peaking at around 12 h before reaching trough values at 24 h (Table S4, Fig. S3).

At V2, results for the no-NIT group were similar to those at V0 (Table 2). All NIT dose-groups, including the lowest one, showed markedly increased sTYR responses; mean AUC_{24} increased by 27% from 1 mg to 8 mg NIT. The 24-h profiles showed an initial peak at around one hour followed by a second peak at around 12 h (Table 2, Table S4, Fig. 3). C_{av} for sTYR was similar to pre-dose, fasting values (Table 2).

In individual patients (Fig. 4), significant fluctuations of sTYR were observed during NIT therapy, apparently showing larger absolute excursions in NIT dosed patients (maximum/fasting values range: 11–378 $\mu\text{mol/L}$; maximum/minimum values range: 91–387 $\mu\text{mol/L}$) than in those not receiving NIT (maximum/fasting values range: 0–32 $\mu\text{mol/L}$; maximum/minimum values range: 16–42 $\mu\text{mol/L}$). Table S5 shows that mean variation of the two fasting values in individual patient profiles (time 0 and time 24.5 h) was less than the variation between the minimum and maximum values.

3.5. sPHE

At V0 and V2, the sPHE values in all groups were similar, and the C_{av} for sPHE was similar to fasting, pre-dose values (Table 2). sPHE did not change from V0 to V2.

sPHE values increased to an initial peak between 1 and 2 h after breakfast, followed by a further larger peak at around 12 h before declining to fasting levels at 24 h, both at V0 (Table S5, Fig. S3) and V2 (Table S5, Fig. 3).

3.6. sHPPA

At V0, all sHPPA values were below the LLOQ of the assay (19 $\mu\text{mol/L}$)

Table 2

showing mean (SD) fasting (or pre-dose for sNIT), average 24-h serum concentrations (Cav) and total area under the curve (AUC₂₄) for nitisinone, tyrosine and its metabolites in SONIA 1.

Nitisinone (mg)	V0					V2				
	0	1	2	4	8	0	1	2	4	8
sNIT										
Pre-dose ^a	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	0.5 (0.25)	0.93 (0.4)	1.94 (0.52)	4.79 (2.07)
Cav ^a	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	0.52 (0.17)	1.07 (0.26)	1.97 (0.43)	5.39 (1.91)
AUC ₂₄ ^b	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	12.47 (4.05)	25.79 (6.21)	47.3 (10.4)	129.3 (45.8)
sHGA										
Fasting ^a	28 (9)	28 (11)	30 (8)	32 (7)	28 (8)	31 (12)	<LLOQ	<LLOQ	<LLOQ	<LLOQ
Cav ^a	37 (14)	34 (7)	37 (11)	39 (8)	37 (8)	36 (12)	<LLOQ	<LLOQ	<LLOQ	<LLOQ
AUC ₂₄ ^b	883 (336)	820 (168)	879 (256)	924 (196)	879 (196)	860 (279)	<LLOQ	<LLOQ	<LLOQ	<LLOQ
sTYR										
Fasting ^a	54 (15)	68 (20)	62 (11)	60 (9)	55 (5)	56 (15)	653 (106)	715 (171)	803 (155)	813 (145)
Cav ^a	60 (5)	56 (5)	59 (6)	63 (9)	61 (3)	61 (10)	672 (109)	731 (120)	800 (126)	856 (106)
AUC ₂₄ ^b	1431 (244)	1342 (99)	1525 (284)	1440 (247)	1449 (245)	1457 (236)	16,131 (2618)	17,551 (2874)	19,189 (3012)	20,545 (2555)
sPHE										
Fasting ^a	55 (9)	57 (8)	62 (11)	63 (11)	58 (5)	60 (9)	59 (10)	60 (11)	58 (18)	60 (15)
Cav ^a	60 (5)	56 (5)	59 (6)	63 (9)	61 (3)	62 (5.7)	56.5 (8)	57 (8)	59 (14)	63 (9)
AUC ₂₄ ^b	1428 (122)	1344 (118)	1419 (147)	1517 (1517)	1461 (76)	1485 (136)	1357 (186)	1377 (196)	1409 (329)	1504 (213)
sHPPA										
Fasting ^a	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	48 (15)	45 (20)	59 (18)	53 (28)
Cav ^a	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	49 (8)	53 (18)	57 (8)	65 (14)
AUC ₂₄ ^b	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	1175 (191)	1260 (424)	1369(198)	1570 (338)
sHPLA										
Fasting ^a	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	50 (29)	43 (8)	75 (39)	80 (18)
Cav ^a	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	41 (10)	48 (9)	64 (18)	84 (19)
AUC ₂₄ ^b	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	987 (229)	1150 (216)	1536 (421)	2013 (454)

<LLOQ = measurements below limit of quantification.

ND = Not determined due to several values <LLOQ.

^a μmol/L.

^b μmol/L/h; n = 8 in each dose group; values expressed as mean (SD).

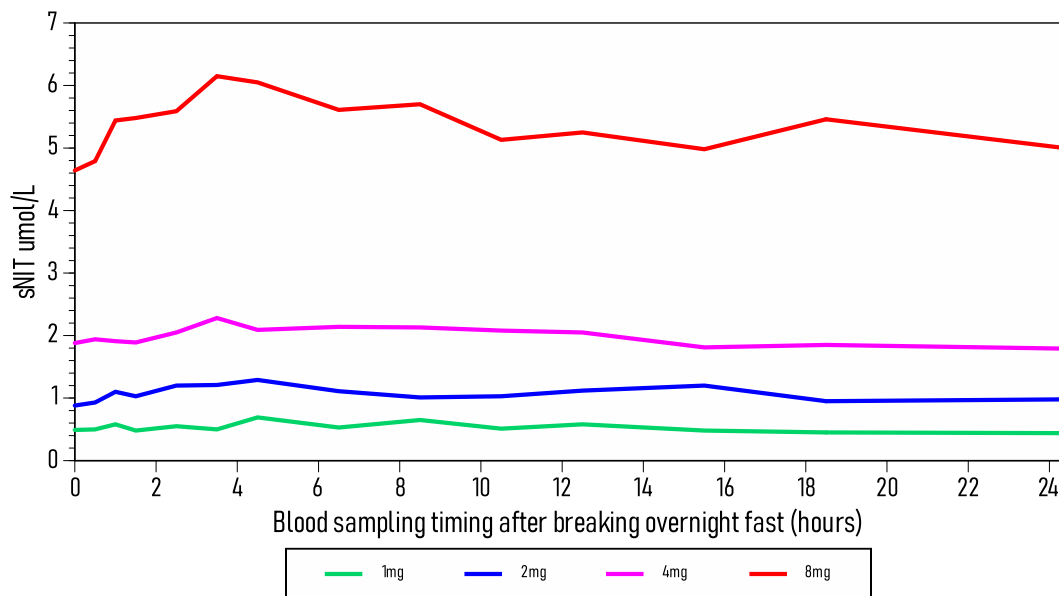


Fig. 2. sNIT 24-h profiles shown at week 4. The legend indicates the various dose groups of nitisinone in SONIA 1 (n = 8 in each dose group). The time points are 0, 0.5, 1, 1.5, 2.5, 3.5, 4.5, 6.5, 8.5, 10.5, 12.5, 15.5, 18.5, and 24.5 h after breaking overnight fast. Breakfast, lunch and dinner were given at 0, 4.5 and 10.5 h in the time course approximately. Error bars are not shown for the purposes of clarity and can be found instead in Tables S2 and S3. Dose 0 mg removed from sNIT figure as values below lower limit of quantification (LLOQ).

L), and no calculations or graphical presentations could be made. The same was the case for the no-NIT group at V2.

At V2, HPPA concentrations were quantifiable in all samples from

NIT-treated patients and. Showed a small progressive increase with increasing doses. Mean AUC₂₄ increased by about 33% from 1 to 8 mg NIT (Table 2). From the fasting values, sHPPA increased to a small peak

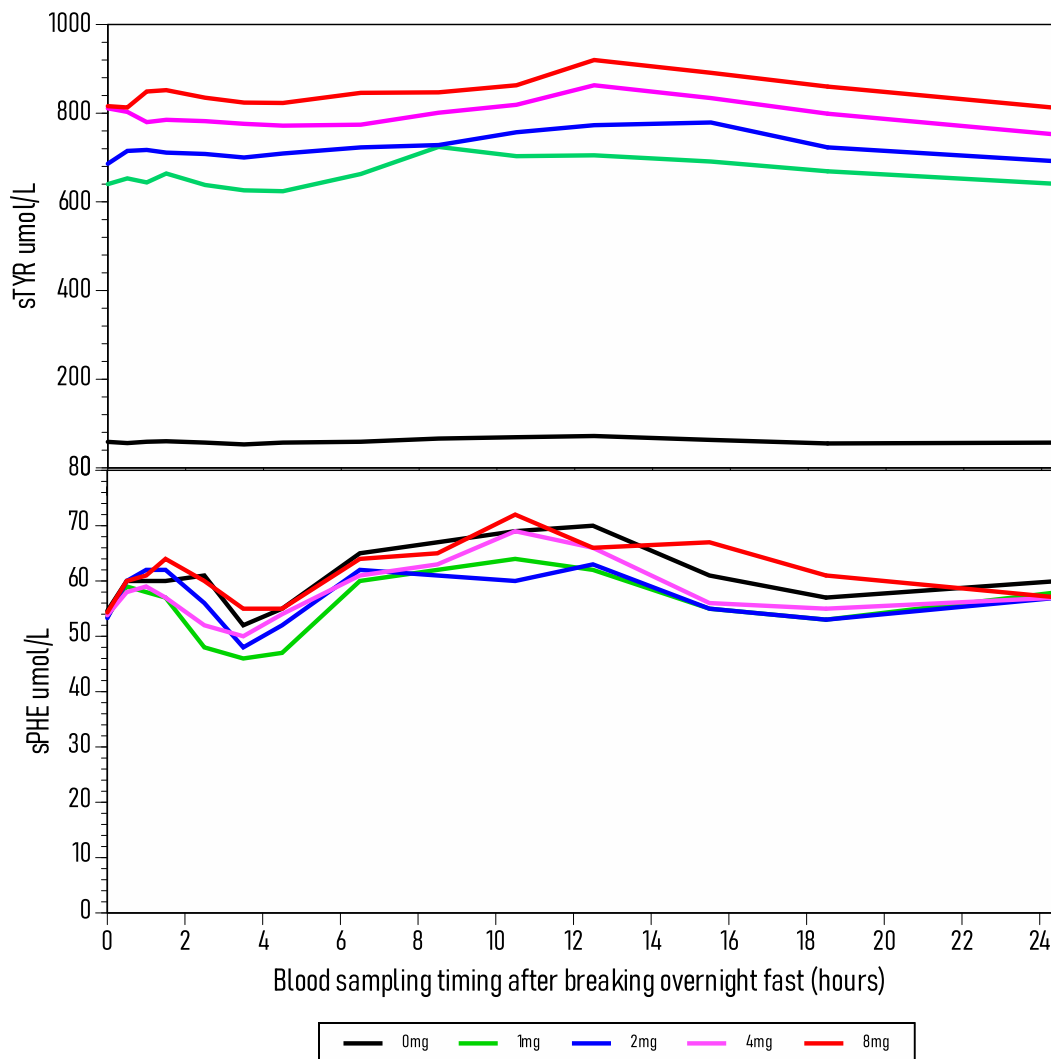


Fig. 3. sTYR and sPHE 24-h profiles shown at week 4. The legend indicates the various dose groups of nitisinone in SONIA 1 ($n = 8$ in each dose group). The time points are 0, 0.5, 1, 1.5, 2.5, 3.5, 4.5, 6.5, 8.5, 10.5, 12.5, 15.5, 18.5, and 24.5 h after breaking overnight fast. Breakfast, lunch and dinner were given at 0, 4.5 and 10.5 h in the time course approximately. Error bars are not shown for the purposes of clarity and can be found instead in Tables S4 and S5.

at around 1 h after breakfast before declining, and then increasing to a larger peak at between 15 and 18 h before declining back to fasting levels at 24 h (Table S6, Fig. 5). C_{av} for sHPPA was similar to pre-dose, fasting values (Table 2).

3.7. sHPLA

At V0, all sHPLA values were below LLOQ of the assay ($4 \mu\text{mol/L}$), and no calculations or graphical presentations could be made. The same was the case for the no-NIT group at V2.

At V2, HPPA concentrations were quantifiable in all samples from NIT-treated patients and showed a small increase with increasing doses. Mean AUC_{24} increased by about 50% from 1 to 8 mg NIT (Table 2). From the fasting values, sHPLA did not show a consistent initial or later peak after meals (Table S7, Fig. 5). C_{av} for sHPLA was similar to pre-dose, fasting values (Table 2).

4. Discussion

The results of the temporal profiles over the 24-h period as well as the C_{av} values, suggest that there is minimal diurnal variation in sTYR, when values are examined of the group as a whole. However, when

individual patient profiles are examined, there can be more than 300 $\mu\text{mol/L}$ fluctuations in sTYR in NIT-dosed patients both when maximum values are compared against fasting and against minimum values (Fig. 4, Table 3). This suggests that aiming to achieve tyrosine targets (Table S8) in the management of tyrosinaemia during NIT therapy could be compromised if a single random blood sample was employed to reflect the tyrosine status during NIT treatment. For example, a sTYR value could vary as much as 267–296 $\mu\text{mol/L}$ simply due to the randomness of the timing of the sample (Table 3). On the other hand, the variation in the two fasting samples over the 24-h interval of the study namely time 0 and 24.5 h show less variability than the minimum/maximum variability of a random sample (Table S5). The data therefore suggests that a standardised blood sample such as a fasting morning sample, may better guide clinical decisions regarding sTYR management during NIT therapy. Our conclusions are different from those of a 24-h blood sampling study carried out in PKU, where the authors concluded that based on minimal diurnal variation in sPHE a single blood sample could reliably reflect the metabolic control in adult PKU patients, a condition resulting in persistently elevated sPHE circulating concentrations [18].

Those patients who did not receive NIT, on the other hand, showed much lower absolute fluctuations of sTYR over the 24-h (Fig. 4), less than 50 $\mu\text{mol/L}$ excursion both when maximum values were compared

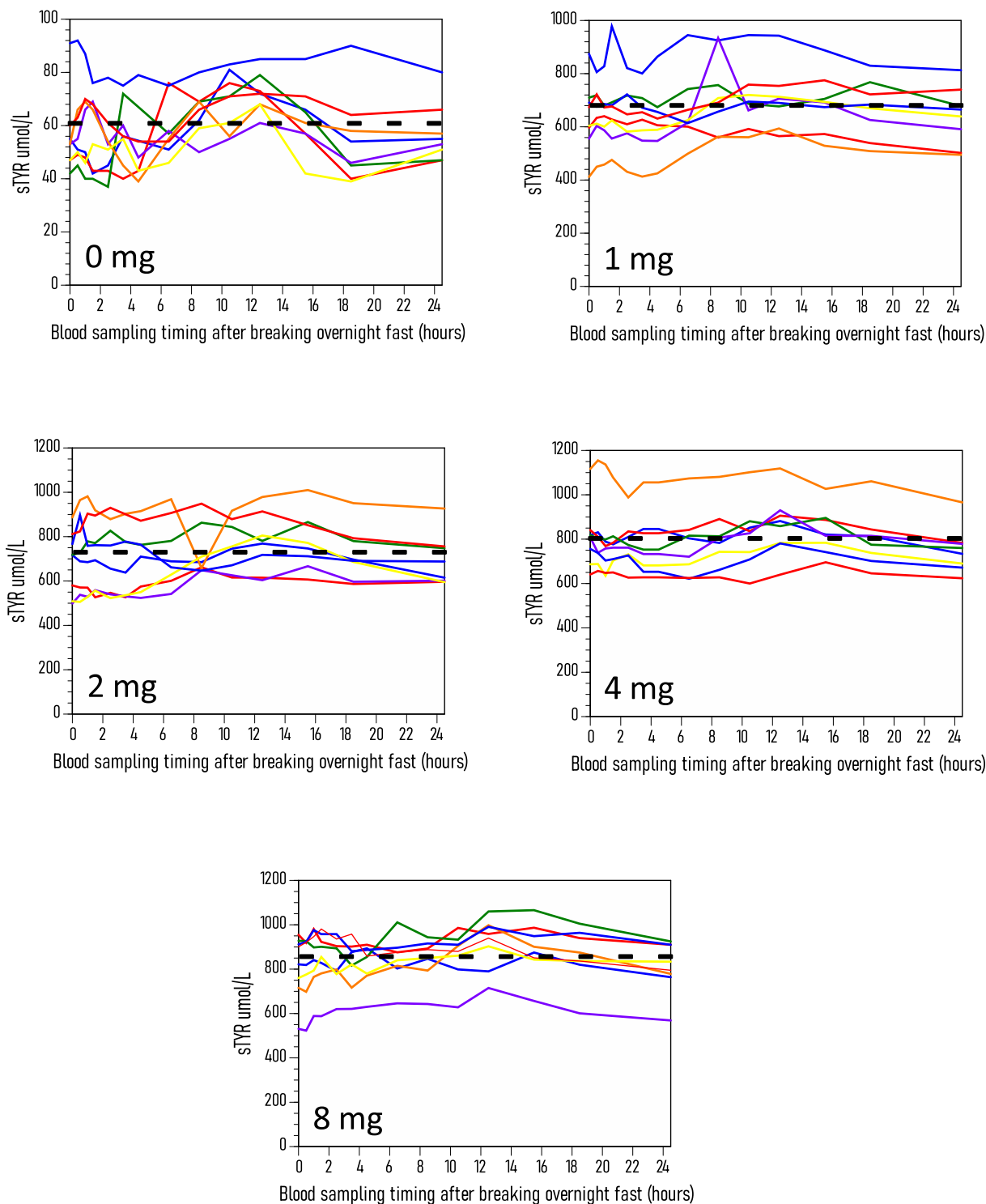


Fig. 4. Individual patient sTYR 24-h profiles showing the variability between minimum and maximum values at the different doses of nitisinone (0, 1, 2, 4, 8 mg). Each dose had same number of patients ($n = 8$). The time points are 0, 0.5, 1, 1.5, 2.5, 3.5, 4.5, 6.5, 8.5, 10.5, 12.5, 15.5, 18.5, and 24.5 h after breaking overnight fast. Breakfast, lunch and dinner were given at 0, 4.5 and 10.5 h in the time course approximately. Different scales were used to better indicate the fluctuations in sTYR for the doses shown. Dotted black lines of the C_{av} for each dose show how this can misinform the fluctuations seen over the day.

against fasting as well as the minimum. This is consistent with the idea that the HGA accumulation in untreated AKU does not inhibit the proximal parts of the tyrosine pathway, and the daily dietary flux of tyrosine appears to continue as in non-AKU subjects, without leading to accumulation of tyrosine. The lack of reversibility of conversion of HPPA to HGA, and the efficient HGA urinary elimination, are also likely important factors in the relatively normal tyrosine flux in untreated AKU

patients. In patients not treated with NIT, the changes in circulating HGA corresponds to the timing and amounts of food consumed, namely a smaller transient response after breakfast and a larger sustained response after lunch and dinner. Despite the accumulating HGA, changes attributable to product (HGA) inhibition on more proximal steps in the pathway are not observed. Apart from sHGA, no changes are observed in sPHE, sTYR, sHPPA and sHPLA in HGD-deficient AKU

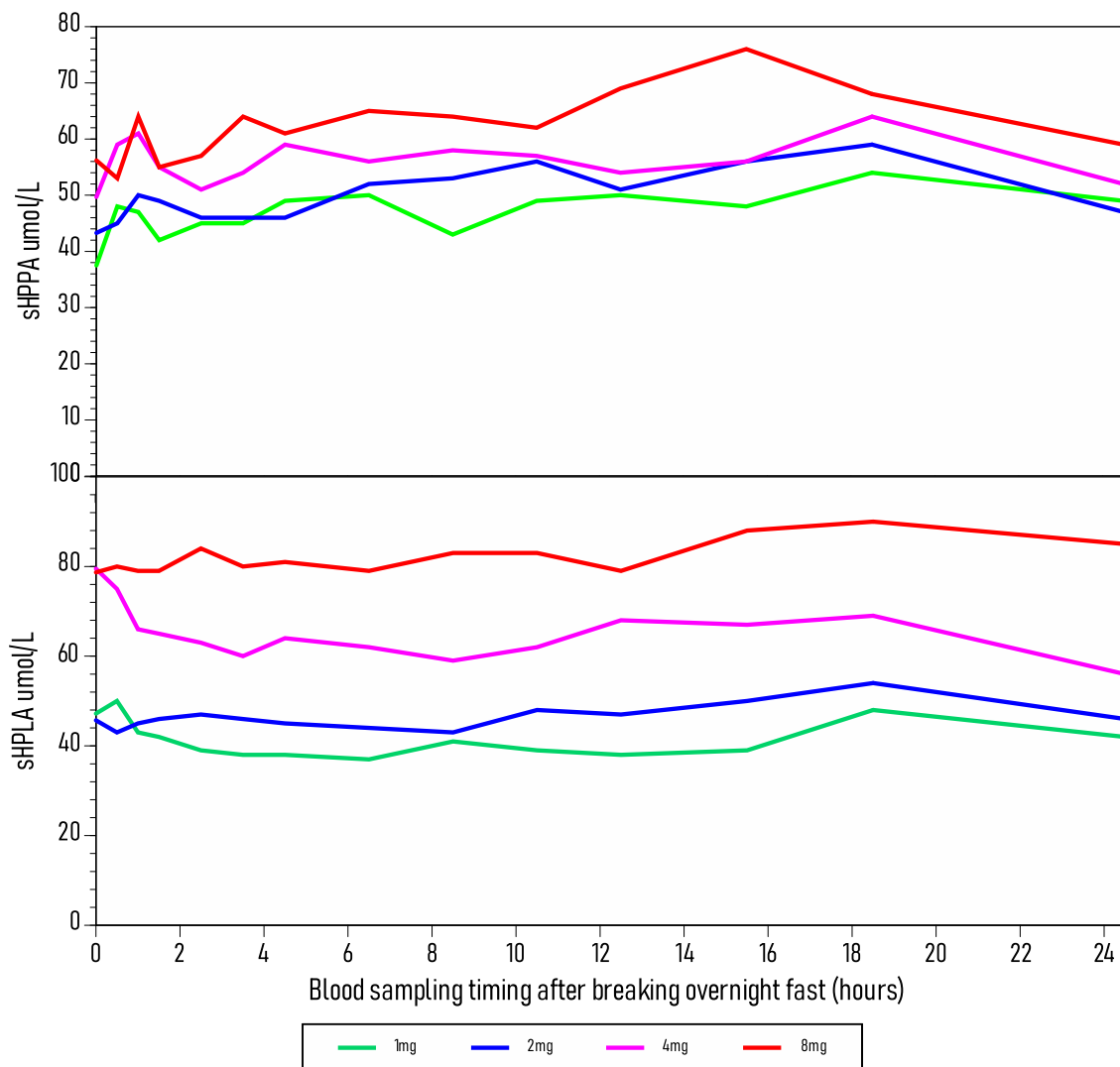


Fig. 5. sHPPA and HPLA 24-h profiles shown at week 4. The legend indicates the various dose groups of nitisinone in SONIA 1 ($n = 8$ in each dose group). The time points are 0, 0.5, 1, 1.5, 2.5, 3.5, 4.5, 6.5, 8.5, 10.5, 12.5, 15.5, 18.5, and 24.5 h after breaking overnight fast. Breakfast, lunch and dinner were given at 0, 4.5 and 10.5 h in the time course approximately. Error bars are not shown for the purposes of clarity and can be found instead in Tables S6 and S7. Dose 0 mg removed as values below lower limit of quantification (LLOQ).

Table 3

Individual patient comparisons of sTYR maximum versus fasting as well as maximum versus minimum during the 24-h profiling at V2 ($n = 8$ in each group; values expressed as mean (SD)).

	0 mg	1 mg	2 mg	4 mg	8 mg
Maximum/fasting ^a	17.5 (11.9)	123 (114)	127 (86)	67 (32)	125 (79)
Maximum/ minimum ^b	29 (9.2)	166 (93)	167 (58)	154 (34)	178 (68)

^a Maximum refers to largest value over the 24-h and the fasting to time 0.

^b Maximum refers to largest and minimum to smallest value over the 24-h.

patients not receiving NIT, unlike HPPD inhibition described below.

The similarity in body weight and the urine urea between the V0 and V2 is not inconsistent with the idea that patients adhered to a stable lifestyle during the study period. A fuller study of the effect of lifestyle factors over a 24-h period, such as meals, is necessary in order to better understand the tyrosinaemia. It has been suggested that exercise can increase circulating amino acids such as tyrosine due to release from muscle. The effect of exercise could, however, not be studied in SONIA 1

as it was carried out with resting patients [19,20]. In the patients not receiving NIT, a rise in sTYR in the first 2–4 h could be due to initial absorption of protein ingested at breakfast at time 0.5 h, followed by release of insulin due to carbohydrate in the breakfast meal, causing amino acid transport from the circulation into cells enabling protein anabolism to replace overnight catabolism. The larger sustained increase in sTYR following lunch and dinner is consistent with greater protein intakes compared to breakfast. Intake of PHE and TYR during meals permits these amino acids to be utilised for normal metabolic processes such as synthesis of protein, catecholamines, thyroid hormones and melanin.

HPPD is mainly expressed in the liver and kidney in humans, and nitisinone has a strong affinity for hepatic HPPD. The first-pass effect of gut-absorbed nitisinone in the liver via the portal blood allows NIT access to the hepatic HPPD before the remaining unbound NIT reaches the systemic circulation via the hepatic veins. The NIT-mediated inhibition of HPPD in vitro has a long half-life of around 63 h due to the very strong affinity of NIT for HPPD [21,22]. NIT is not irreversibly bound to HPPD but it dissociates slowly from the HPPD-inhibitor complex; rodent studies showed selective retention of radiolabelled NIT in the liver, and kidneys [21,22]. In SONIA 1, even with the lowest NIT doses, daily

administration is likely to have resulted in progressive hepatic HPPD retention of gut-absorbed NIT until steady state was reached, as evidenced by the excellent decrease in sHGA even on the 1 mg dose.

In our NIT-treated patients, sTYR, sHPPA and sHPLA all increased with NIT dosing; mean C_{av} for sTYR increased by about 27%, for HPPA by about 33%, and for HPLA by about 50% from the 1 mg to the 8 mg dose. HPPA is formed from tyrosine and metabolized to HPLA but also converted back to tyrosine by the bidirectional and rate-limiting tyrosine aminotransferase (TAT) (Fig. S8). A possible explanation for the less pronounced increase in sTYR with increasing NIT dose may be that increasing HPPA inhibits its own conversion back to TYR. The more consistent dose-dependent increase in sHPLA during NIT therapy suggests a preferential conversion of HPPA to HPLA, rather than to TYR; it can be hypothesised that inter-patient variability of HPLA conversion could influence the degree of tyrosinaemia; those who generate less HPLA during NIT may have higher sTYR.

During NIT-induced tyrosinaemia, the adaptations to minimise sTYR could include a decreased conversion of PHE to TYR, reduced conversion of accumulating HPPA to TYR, a lower conversion of TYR to HPPA, and a more efficient conversion of HPPA to HPLA. The magnitude of the adaptations could determine the degree of tyrosinaemia during NIT therapy. In the present dataset, there is no support for a decrease in the conversion of PHE to TYR, even though NIT mice studies showed that sPHE increased after NIT administration suggesting possible TYR-mediated inhibition of phenylalanine hydroxylase [23]. The attenuated increase in TYR in SONIA 1 could indicate an inhibition of the bidirectional flux between TYR and HPPA due to effect on TAT. There is more convincing support for increasing dose-dependent conversion of HPPA to HPLA. Examining ratios of TYR/PHE, HPPA/TYR, HPPA/HPLA and HPLA/TYR in those with and without keratopathy could clarify the metabolic relationships further and help identify those with higher risk of tyrosinaemia-mediated keratopathy.

While the metabolic relationships between PHE, TYR, HPPA and HPLA could influence the degree of tyrosinaemia, the predominant influence is still the amount of dietary protein. A recent study in mice was able to markedly decrease NIT-induced tyrosinaemia by employing a PHE/TYR restricted diet [23]. In the present study, the peak of sHGA and sTYR was delayed compared to sNIT because they are protein intake-mediated, and reflect continuing intestinal PHE and TYR absorption. The increased fluctuations in sTYR during NIT treatment, compared to those before NIT administration, is probably due to HPPD inhibition [8].

An improved understanding of NIT-induced tyrosinaemia could mitigate complications of this condition which include corneal keratopathy, vitiligo and possibly cognitive impairment [6,9,11,24]. TYR thresholds been based on rodent studies are available for the risk of corneal keratopathy development. With sTYR around 900 $\mu\text{mol/L}$, the solubility of TYR is exceeded, and corresponds to ocular TYR of 3500 $\mu\text{mol/L}$ [25], when TYR crystallises in the cornea leading to corneal keratopathy; similar TYR thresholds for cutaneous and possible brain effects have not been described [25]. In HT-1, life-saving NIT therapy is employed at high doses of 1–2 mg/kg body weight daily in childhood and with the knowledge of possible tyrosinaemia-associated cognitive impairment [10,11]; the goal of dietetic management is to keep sTYR between 200 and 400 $\mu\text{mol/L}$ up to the age of about 12 years [26]. sTYR thresholds in NIT-treated adult AKU patients are more pragmatic as most of these patients are habituated to eating normally. In the United Kingdom National Alkaptonuria Centre, a specialist dietician manages the tyrosinaemia, to defined sTYR thresholds (Table S8), employing 7-day food diaries and fasting sTYR monitoring [27]. Daily dietary protein requirements designed to meet the need for limiting amino acids methionine, tryptophan, threonine and lysine [28,29], result in obligate excess consumption of PHE and TYR. The tyrosine catabolic pathway is therefore critical in handling excess PHE and TYR, while ensuring no loss of nutrients by converting to glucogenic fumarate and lipogenic acetoacetate.

The limitations of our data are the small size, with only 8 patients per dose group, and short study period. The duration of SONIA 1 was 4 weeks and it is not known whether the pathway undergoes further changes to catabolise PHE and TYR over the longer term. Furthermore, for several analytes, the concentrations were below the LLOQ, either before or after administration of NIT.

In summary, we conclude that a single fasting sample for sTYR is recommended for the purposes of clinical management of tyrosinaemia during NIT therapy. The fluctuations of 24-h sTYR during NIT therapy do not directly correspond to the NIT serum profile but are instead influenced by meals including dietary protein intake. Excessive dietary protein (tyrosine) consumption as well as the inhibition of TAT could be the main factors generating tyrosinaemia during NIT therapy. Besides diet, the dynamic adaptive relationships between sHPPA, sTYR and sHPLA during NIT therapy could further determine the degree of tyrosinaemia and their clarification could thereby help optimise the management of tyrosinaemia.

Author statement

Ranganath LR – conceived the study, carried it out at the Liverpool site, gathered the data, analysed and drafted the manuscript.

Milan AM, Hughes AT, Davison AS, Norman BP – carried out chemical analyses, and edited the manuscript.

Khedr M – helped to carry out the study at the Liverpool site.

Bou-Gharios G, Gallagher JA – helped conceive the study, and edited the manuscript.

Gornall M, Jackson R, – carried out the statistical aspects of the study.

Imrich R, Rovinsky J, – carried out the study at the Piešťany site.

Rudebeck M; Olsson B – helped conceive the study, and edited the manuscript extensively.

Data-sharing statement for SONIA 1

SONIA 1 data access will be granted in response to qualified research requests. All de-identified individual participant data, for patients with separate consent signed for this purpose, can be made available to researchers. Data will be shared based on: the scientific merit of the proposal – i.e. the proposal should be scientifically sound, ethical, and have the potential to contribute to the advancement of public health as well as the feasibility of the research proposal – i.e. the requesting research team must be scientifically qualified and have the resources to conduct the proposed project. The data files would exclude data dictionaries that require user licenses. Data could be made available following finalized regulatory authority review and end of any data exclusivity periods and ending after 36 months or until corresponding author is able to fulfil this obligation whichever is earlier. Further, the study protocol and statistical analysis plan can be made available. Proposals should be directed to j.a.gallagher@liverpool.ac.uk to gain access. Data requestors will need to sign a data access agreement.

This manuscript is not submitted for review anywhere else at present.

Declaration of Competing Interest

Lakshminarayan Ranganath received fees for lectures and consultations from Swedish Orphan Biovitrum.

Mattias Rudebeck and Olsson B, are share-holders and were employees of Swedish Orphan Biovitrum at the time of the study.

Acknowledgements

We would like to thank the European Commission for the Framework 7 grant award (DevelopAKUre, project number: 304985) that was crucial to allow SONIA 1 to be carried out.

We would like to thank all patients in SONIA 1, a demanding four-week participation, as well as the patient societies supporting the patients in the study and for their immense efforts in successfully recruiting these many patients.

We wish to thank the following people (with their affiliation in brackets) for their invaluable support during SONIA 1: Pam Neagle (administrative support for SONIA 1), and Helen Bygott (AKU Specialist Nurse, RLUH).

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jmgmr.2022.100846>.

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