Temporal adaptations in the phenylalanine/tyrosine pathway and related factors during nitisinone-induced tyrosinaemia in alkaptonuria

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Highlights

- Tyrosinaemia decreased over time in nitisinone-receiving patients
- No apparent increase in HPLA pathway was seen over time during nitisinone therapy
- A progressive increase in PHE over time potentially limiting tyrosinaemia was seen
- A biochemical state of phenylalaninaemia was seen during nitisinone therapy
- Decreased 24-h urine urea shows adherence to lower protein intake advice
- Decreased 24-h urine creatinine indicated lower muscle mass over time
- Despite decrease in muscle mass there was weight gain over time

Abstract (250)

Background: Adaptations within the phenylalanine (PHE)/tyrosine (TYR) pathway during nitisinone (NIT) are not fully understood.

Objective: To characterise the temporal changes in metabolic features in NIT-treated patients with alkaptonuria.

Patients and methods: Serum (s) and 24-urine (u) homogentisic acid (sHGA, $uHGA_{24}$), TYR (sTYR, $uTYR_{24}$), PHE (sPHE, $uPHE_{24}$), hydroxyphenylpyruvate (sHPPA, $uHPPA_{24}$), hydroxyphenyllactate (sHPLA, $uHPLA_{24}$) and sNIT were measured at baseline (V1) and until month 48 (V6) in 69 NIT-treated patients, recommended to reduce protein intake. The 24-h urine urea ($uUREA_{24}$), creatinine ($uCREAT_{24}$) and body weight were also measured. Amounts of tyrosine metabolites in total body water (TBW) were derived by multiplying the serum concentrations by 60% body weight, and sum of TBW and urine metabolites resulted in combined values (c).

Results: $uUREA_{24}$ and $uCREAT_{24}$ decreased between V1 and V6 during NIT, whereas body weight and sNIT increased. Linear regression coefficient between $uUREA_{24}$ and $uCREAT_{24}$ was extremely strong (R=0.84). sPHE, $_{TBW}$PHE and $cPHE_{24}$ increased gradually from V1 to V6. A decrease in $cTYR_{24}/cPHE_{24}$, sTYR/sPHE and $_{TBW}$TYR/$_{TBW}$PHE was seen from V2 to V6. Serum, 24-urine and combined TYR, HPPA and HPLA either remained stable or decreased from V2 to V6.

Discussion: The gradual increase in PHE suggests adaptation to increasing TYR during NIT therapy. The decrease in protein intake resulted in decreased muscle mass and increased weight gain.

Conclusion: Progressive adaptation by decreasing PHE conversion to TYR occurs over time during NIT therapy. A low protein diet results in loss of muscle mass but also weight gain suggesting an increase in fat mass.

Introduction

Chronic debilitating and mostly irreversible multisystem features which are difficult to treat characterise the advanced stages of alkaptonuria (AKU) (OMIM#203500). The lack of homogentisate 1,2 dioxygenase (HGD) (EC.1.13.11.5) activity from birth in this autosomal recessive disorder results in failure to convert homogentisic acid (HGA) to maleylacetoacetic acid, thus leading to accumulation of HGA and the damaging effects of AKU [1,2]. The conversion of 4-hydroxyphenylpyruvate (HPPA) to HGA is catalysed by the enzyme 4-hydroxyphenylpyruvate 1,2, dioxygenase (HPPD, EC:1.13.11.27). Nitisinone (NIT), an HPPD inhibitor has been approved by the European Medicines Agency as the first disease-modifying therapy for AKU [3,4]. NIT decreases HGA [5] and ameliorates AKU [6]. Unfortunately, inhibition of HPPD also leads to accumulation of metabolites proximal to this inhibition site such as tyrosine (TYR) [7]. Nitisinone induces severe tyrosinaemia associated with unwanted effects such as corneal keratopathy, vitiligo and cataract [8-10]. Cognitive impairment has been observed in children treated with life-saving NIT in hereditary tyrosinaemia type 1 (HT-1) (OMIM#276700) and proposed to be possibly linked to tyrosinaemia [11].

It is not currently known if and how the metabolic pathways adapt to HPPD inhibition induced by NIT over time. Experience in the United Kingdom National Alkaptonuria Centre using low-dose NIT 2 mg daily reveals a rapid onset of HPPD inhibition, with full inhibition occurring over several weeks [6,12]. Inhibition is almost complete at a dose of 2 mg/day, as evidenced by only marginal increases in serum TYR levels when doses were increased up to 8 mg/day [12].

A previous study has shown that NIT inhibits certain cytochrome p450 (CYP450) enzymes as well as renal organic anion transporters [13]. Another publication described time-related increase in circulating NIT possibly due to inhibition of CYP450 enzymes during long-term administration [14]. Activation of enzyme systems can take years to fully manifest as exemplified by the increasing γ-glutamyl transferase during use of anticonvulsant therapy [15].

Overconsumption of dietary protein and therefore also of PHE and TYR is common in many Western countries [16,17]. Like other amino acids, PHE and TYR cannot be stored and have to be catabolised by the tyrosine pathway. Rapid disposal of the daily dietary PHE and TYR load requires an efficient cascade of enzymatic reactions.

Increase in HPPA, HPLA and TYR during NIT therapy has been reported previously [18]. NIT-induced accumulation of HPPA can involve either a conversion to TYR by the bidirectional nature of tyrosine aminotransferase (TAT; EC 2.6.1.5) (Figure S1) or to HPLA; although cell

culture studies show the existence of a bidirectional hydroxyphenylpyruvate reductase (HPPR), it is not clear if this exists in human metabolism [19.20]. Additionally, the conversion of PHE to TYR could be decreased during NIT therapy but the nature of any such change over time is not known. During chronic NIT therapy adaptations of these routes of subsequent metabolism of the accumulating HPPA could determine the magnitude of tyrosinaemia, but studies of such adaptations have yet to be carried out.

We have studied the adaptations of the PHE/TYR pathway using data from a four-year phase 3 study (Suitability of Nitisinone in Alkaptonuria 2 study; SONIA 2), where a dose of NIT of 10 mg daily was tested in patients with AKU for four years. Patients were recommended to decrease dietary protein in order to reduce tyrosinaemia, but there was no active dietetic intervention [4]. Here we present our investigations on the adaptations in the PHE/TYR pathway during treatment with NIT in the SONIA 2 study.

Methods

Study design and patients: SONIA 2 was a four-year, open-label, evaluator-blinded, multicentre, randomised, no-treatment controlled, parallel-group study aiming to recruit 140 patients aged 25 years or older, with a confirmed diagnosis of AKU and any clinical manifestation in addition to increased HGA (Figure S2). Liverpool (UK), Paris (France) and Piešťany (Slovakia) were the three investigational sites, with the Independent Ethics Committee at each centre approving the study. All patients provided a written informed consent prior to inclusion.

Treatment

70 patients were to be randomised to oral NIT 10 mg (Orfadin®) daily and 70 to a control (no-treatment) group. *In this paper, only the NIT group was analysed.* NIT was withdrawn in patients who developed signs of ocular tyrosine-related adverse events. If feasible, once the ocular symptoms had resolved (minimum 2 months after temporary withdrawal), NIT was reintroduced at a lower dose (2 mg daily). Alternatively, the patient was withdrawn from the study. If ocular tyrosine-related symptoms reappeared on the lower dose, NIT was permanently withdrawn and the patient was monitored until the symptoms resolved. There were no restrictions regarding concomitant medications. Patients in both groups could freely use e.g. analgesics, anti-inflammatory drugs and others as needed to treat symptoms of AKU. More details of the design of the study, including various procedures and assessments have been

previously published [4]. A recommendation, reinforced at each visit, was made to patients receiving NIT to decrease dietary protein consumption to minimise the risk of ocular adverse events, without any further active intervention.

Procedures: Patients visited the study sites at baseline (V1), 3 months (V2), and then annually up to month 48 (V3-V6); a close-out phone call took place at month 49. A physical examination of the patients including measurement of height and weight was carried out at each visit. At each visit, 24-hour urine was collected into 2.5 L bottles containing 30 mL of 5 N H_2SO4 and stored away from direct sunlight, and urea, creatinine and PHE/TYR metabolites were determined. The weight of the collected urine was recorded and used as the volume in the calculations of amounts of PHE/TYR metabolites, urea and creatinine excreted assuming a density of 1 g/mL. An aliquot of the collected urine was frozen and kept at −80°C until analysis. A physical examination of the patients including measurement of height and weight was carried out at each visit. 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), [12] to stabilise the HGA, and kept frozen at −80 °C until analysis. Samples from Piešťany and Paris were transported frozen to Liverpool and all biochemical analyses were performed in the Department of Clinical Biochemistry, Liverpool Clinical Laboratories, Liverpool University Hospital NHS Trust.

Chemical analyses: Measurement of NIT, HGA, TYR, PHE, HPPA, and HPLA, in serum (indicated as sNIT, sHGA, sTYR, sPHE, sHPPA, and sHPLA) and 24-h urine (indicated as $uHGA_{24}$, $uTYR_{24}$, $uPHE_{24}$, $uHPPA_{24}$, and $uHPLA_{24}$) were carried out on all samples collected at the described sampling points.

The concentrations of as sNIT, sHGA, sTYR, sPHE, sHPPA, and sHPLA as well as $uHGA_{24}$, $uTYR_{24}$, $uPHE_{24}$, $uHPPA_{24}$, and $uHPLA_{24}$ were measured by liquid chromatography tandem mass spectrometry based on previously published methods [7,18]; in addition, the fully validated methodologies are UKAS 15189 accredited. 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 (100mm×3.0mm, 3µm, Waters); initial chromatographic conditions of 80:20 water:methanol with 0.1% formic acid (v/v) increased linearly to 10:90 over 5 minutes. 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 $^{13}C_6$-nitisinone, $^{13}C_6$-HGA, d_4-TYR and d_5-PHE in 0.1 % formic acid (v/v) in deionised water. No internal standard was available for HPPA and HPLA at time of analysis and $^{13}C_6$-HGA was therefore validated for use as the internal standard.

Urine urea and creatinine were photometrically assayed 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 [21,22]. Urine creatinine was measured using a validated Jaffe reaction.

Total body water ($_{TBW}$) metabolites of PHE, TYR, HPPA, HPLA and HGA ($_{TBW}$PHE, $_{TBW}$TYR, $_{TBW}$HPPA, $_{TBW}$HPLA, $_{TBW}$HGA): Since PHE and TYR and their metabolites are small molecules that are distributed in total body water [23,24], the concentrations of circulating metabolites were multiplied by the factor of 0.6 times body weight in kilograms to derive the amounts of total body water metabolites [23,24].

Total urinary metabolites: Amounts of the urinary TYR and PHE and their metabolites were similarly calculated by multiplying urine concentrations with the 24-hour urine volumes.

Combined metabolites of PHE, TYR, HPPA, HPLA and HGA ($cPHE_{24}$, $cTYR_{24}$, $cHPPA_{24}$, $cHPLA_{24}$, $cHGA_{24}$): The whole-body water metabolites and 24-h urine metabolites were summed to obtain combined metabolites ($cHGA_{24}$, $cTYR_{24}$, $cPHE_{24}$, $cHPPA_{24}$, and $cHPLA_{24}$) generated daily in the liver and kidney.

Statistical analysis: Only data from NIT-treated patients were used. This includes data from those patients who completed study visits and were found to comply with NIT treatment. Both 2- and 10-mg dose data were included in the data analyses of TYR/PHE pathway metabolites, provided compliance was satisfactory as evidenced by sNIT (appropriately increased) and $uHGA_{24}$ (appropriately decreased).

All statistical analyses were post-hoc. Continuous variables are presented using mean and standard deviation (SD). All visits in all nitisinone-treated patients were analysed with regard to metabolite relationships both by ANOVA (Tukey-Kramer for multiple comparisons) and simple linear regression. Analyses were performed using Graphpad InStat 3 software (version number 3.1); p values <0.05 were considered statistically significant.

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Results

Disposition of patients: Between May 2014 and February 2015, 69 patients were randomised to each of the NIT group, with 55 patients completing the study.

Demographic data and other baseline characteristics: The majority of patients were Caucasian (134 patients, 97.1%) and male (45 patients, 65.2%). The mean age (SD) was 49.0 (11.3) years (Table 1 & S1).

Comparison of visits

HGA measurements: $cHGA_{24}$, $_{TBW}HGA$, sHGA, and $uHGA_{24}$ at visits V2-V6 were significantly lower than at V1 as expected ($p<0.0001$) but showed steady increases from V2 to V6. Mean sHGA increased about 4-fold from V2 to V6, and mean $uHGA_{24}$ increased by a factor of 9.5 (Table 2). Despite this trend, the differences between V2 and V6 were not statistically significant (Tables 2, S2, Figure S2 & S11).

TYR measurements: $cTYR_{24}$, $_{TBW}TYR$, sTYR, and $uTYR_{24}$ at visits V2-V6 were significantly higher than at V1 as expected due to the on-target HPPD inhibition ($p<0.0001$). There was a consistent decrease in all TYR values from V2 to V6. Mean sTYR decreased by 11% and mean $uTYR_{24}$ by 33% from V2 to V6. The differences between V2 and V6 were statistically significant. (Tables 2 & S2, Figure 1).

PHE measurements: Mean sPHE, TBWPHE and cPHE24 where all higher at V2 than at V1, i.e., after introduction of NIT, and showed a consistent further increase at the following visits ($p<0.01$, V6 vs V1), whereas $uPHE_{24}$ decreased from V1 to V2, with a further decrease to V6 ($p<0.05$ for V3 and V4 vs. V1). The highest values for $cPHE_{24}$, $_{TBW}PHE$, and sPHE were at V5 and V6. The lowest value for $uPHE_{24}$ was seen at V4. The statistical significance of all comparisons is shown in Tables 2 & S2 and Figure 2.

HPPA measurements: HPPA measurements in serum (and also $_{TBW}HPPA$) were below the lower limit of quantification (LLOQ) at V1 and were therefore not displayed in the figures. $cHPPA_{24}$ and $uHPPA_{24}$ at visits V2-V6 were all quantifiable. Comparison of values between

V2 and V6 were not significantly different for TBWHPPA and sHPPA, but $cHPPA_{24}$ and $uHPPA_{24}$ at V2 were the highest. The degree of statistical significance of these comparisons can be found in the Tables 2 & S2, Figure S3.

HPLA measurements: HPLA measurements in serum (and also TBWHPLA) were below the LLOQ at V1 and were therefore not displayed in the figures. $cHPLA_{24}$ and $uHPLA_{24}$ at visits V2-V6 were all quantifiable. Comparison of values between V2 and V6 were not significantly different for $cHPLA_{24}$, TBWHPLA and sHPLA, but $uHPLA_{24}$ was increased at V2 compared to other visits. The degree of statistical significance of these comparisons can be found in the Tables 2 & S2, Figure S4.

HGA/TYR: $cHGA_{24}/cTYR_{24}$, TBWHGA/TBWTYR, sHGA/sTYR, and $uHGA_{24}/uTYR_{24}$ at visits V2-V6 were significantly lower than at V1 ($p<0.0001$). Comparison of values between V2 and V6 were not significantly different (Tables 2 & S2, Figure S5 & S12).

TYR/PHE: $cTYR_{24}$ /$cPHE_{24}$, TBWTYR/$TBWPHE_{24}$, sTYR/sPHE, and $uTYR_{24}/uPHE_{24}$ at visits V2-V6 were significantly higher than at V1 ($p<0.0001$). Values for V2 and V3 were the highest for $cTYR_{24}$ /$cPHE_{24}$, TBWTYR/$TBWPHE_{24}$, and sTYR/sPHE. The degree of statistical significance of these comparisons shown in the Tables 2 & S2, Figure 3.

HPPA/TYR: Since the HPPA measurements in serum (and also TBWHPPA) were below the LLOQ at V1, the ratios could not be calculated and are not displayed in the figures. $cHPPA_{24}/cTYR_{24}$ and $uHPPA_{24}/uTYR_{24}$ at visits V2 to V6 were significantly higher than at V1 ($p<0.0001$). Comparison of values between V2 and V6 were not significantly different for TBWHPPA/TBWTYR, sHPPA/sTYR and $uHPPA_{24}/uTYR_{24}$, but $cHPPA_{24}/cTYR_{24}$ at V2 was significantly higher than at V3 to V6 (Tables 2 & S2, Figure S6).

HPPA/HPLA: Since both HPPA and HPLA measurements in serum (and also TBWHPPA) were below the LLOQ at V1, the ratios could not be calculated and are not displayed in the figures. $cHPPA_{24}/cHPLA_{24}$ and $uHPPA_{24}/uHPLA_{24}$ at visits V1 to V6 were all similar. Likewise, comparison of values between V2 to V6 were not significantly different for TBWHPPA/TBWHPLA, and sHPPA/sHPLA were similar between V2 to V6. Details can be found in Tables 2 & S2, Figure S7.

HPLA/TYR: Since HPLA measurements in serum (and also TBWHPLA) were below the LLOQ at V1, therefore the ratios could not be calculated and are not displayed in the figures. $cHPLA_{24}/cTYR_{24}$ and $uHPLA_{24}/uTYR_{24}$ at visits V2 to V6 were significantly higher than at

V1 ($p<0.0001$). Comparison of values between V2 to V6 were not significantly different for $cHPLA_{24}/cTYR_{24}$, $_{TBW}HPLA/_{TBW}TYR$, sHPLA/sTYR and $uHPLA_{24}/uTYR_{24}$ (Tables 2 & S2, Figure S8).

Miscellaneous: Measurements of NIT in serum were below the LLOQ at V1 as expected (samples collected before first dose of NIT administered) and therefore data from V1 are not shown in the figure 4. There was an increase in sNIT over time so that V6 values were the highest ($p<0.01$) (Figure 4). sNIT increased by 41% from V2 to V6.

$uCREAT_{24}$ decreased from V2 onwards with lowest values at V6 ($p<0.0001$); comparison of values at V1-V6 is shown in Figure 4. $uUREA_{24}$ as well as $uUREA_{24}$/kg decreased over time between V1 and V6 ($p<0.0001$); the lowest values were found at V5 and V6 and statistical significance of comparisons are shown in Figure 4. The change in total body weight from V1 to V6 was statistically significant ($p<0.0001$) (Figure 4).

Relationships between UREA and CREAT data during nitisinone treatment, with advice to restrict protein intake: The following relationships were observed namely, $uUREA_{24}$ vs body weight ($R=0.3$); $uCREAT_{24}$ vs body weight ($R=0.28$); $uCREAT_{24}$ vs $uUREA_{24}$ ($R=0.84$); and $uCREAT_{24}$ vs $uUREA_{24}$/kg ($R=0.68$) (Figure 5).

Discussion

The $uUREA_{24}$ decreased over time with lowest at V5 and V6 visits suggesting that consumption of protein in SONIA 2 decreased during the study. This is despite diet not being actively managed in SONIA 2 beyond instructing patients at each visit regarding the dangers of high-protein intake during NIT treatment. This decrease in protein intake in NIT-receiving patients in SONIA 2 has been published previously, but only with data for year one [25]. The decrease in protein intake also influenced metabolite changes. Urea, or urea nitrogen, is a product of protein metabolism. Amino acids surplus to daily requirements, are deaminated to produce ammonia, with the ammonia then converted in the liver to urea. The quantity of urea produced is therefore proportional to the protein intake at steady state, when the body's capacity to catabolize protein, and adequately excrete urea in the urine is intact [26].

A recent publication analysing the NIT-induced tyrosinaemia in a 4-week study in AKU (SONIA 1) concluded that the degree of conversion of accumulating HPPA to HPLA could determine the magnitude of the tyrosinaemia [18]. In the present manuscript, the unique opportunity the SONIA 2 study provides namely the ability to monitor metabolic trends in patients with AKU receiving NIT over a long period is investigated. The $uTYR_{24}$ decreased

between V2 and V6, with no statistical difference in sTYR, TBWTYR and $cTYR_{24}$ and is consistent with a reduction in dietary protein as demonstrated by decrease in $uUREA_{24}$; however, the relative proportions of TYR increased compared to HPPA suggesting that the metabolic adaptation in terms of conversion to HPLA had not improved from V2 to V6. There was a significant decrease in $cHPPA_{24}$ and $uHPPA_{24}$, with a similar pattern in $cHPLA_{24}$ and $uHPLA_{24}$; these changes are in keeping with the progressive decrease in dietary protein intake from V2 to V6. These changes of TYR, HPPA and HPLA could be interpreted as increased HPPA conversion to TYR over time from V2 to V6. While there was a reduction in absolute amounts of conversion of HPPA to HPLA in keeping with the decreasing protein intake from V2 to V6, the relative proportions were similar suggesting no further decrease in formation of HPLA from HPPA over time. Overall these findings suggest an increase in TYR but no adaptive induction of HPLA formation from V2 to V6.

The situation with regard to PHE is different with $cPHE_{24}$, TBWPHE and sPHE all increasing from V2 to V6, and with $uPHE_{24}$ decreasing suggesting that not only is there an increase in absolute amounts of PHE over time, but also a reciprocal decrease in conversion of PHE to TYR. There was a 24.5% increase in mean $cPHE_{24}$ between V1 and V6. The relative proportions of PHE and TYR (the decreasing TYR/PHE ratio) over time also indicate more PHE accumulation compared to TYR. With decreasing protein intake from V2 to V6 it is suggested that this may have resulted in more efficient renal reabsorption of both PHE and TYR, thus explaining the lower $uPHE_{24}$ and $uTYR_{24}$. All these findings with regard to PHE indicates a *progressively adaptable* pathway change, unlike the HPPA to HPLA conversion. *These changes in PHE are consistent with a relative chemical phenylalaninaemia while remaining within the usual reference interval for sPHE(Figure 6).*

In terms of absolute molar amounts of tyrosine pathway metabolites handled by the HPLA conversion and the PHE accumulation, the HPLA pathway seems to be quantitatively more important. However, the increase in PHE during NIT therapy requires further investigation. Phenylketonuria (PKU; OMIM # 261600), an autosomal recessive disorder of PHE metabolism, presents with high PHE concentrations in the tissues and in the circulation due to total or partial deficiency of phenylalanine hydroxylase (PAH; EC # 1.14.16.1) activity, and is associated with low TYR concentrations [27]. In PKU, the inability to convert PHE to TYR results in the increase in PHE which is an order of magnitude higher than the increase in PHE reported in the present dataset during NIT therapy. Alternative pathways to eliminate PHE, involving metabolites such as phenylpyruvate, phenyllactate, phenylacetate and others are

found to become active in PKU [27,28]; investigations are underway to clarify if such metabolites are also increased in NIT-induced tyrosinaemia. Further, in PKU the formation of HPPA from PHE bypassing TYR has been described (Figure S9) [28]; it is not known if such a pathway makes a contribution to metabolic adaptation during NIT treatment.

Tyrosinaemia is an inevitable consequence of NIT treatment. In routine clinical practice this is anticipated and managed by means of a low-protein diet employing an algorithm-based approach depending upon the sTYR concentrations. NIT therapy has only been approved for adults with AKU, and not in children, and in the adult group habituated to consume a 'normal' diet, pragmatic thresholds for lower dietary protein are used in the United Kingdom National Alkaptonuria Centre. These include reducing protein intake to 0.9 and 0.8 g/kg body weight for sTYR values between 501-700, and 701-900 µmol/L respectively, whereas in those with values greater than 900 µmol/L, additional amino acid supplements deficient in PHE/TYR are also employed. These thresholds are used because sTYR has been implicated in the increase in ocular TYR, and to exhibit a known relationship to it [29,30].

Urine creatinine was also measured in all samples collected in SONIA 2, where it was noticed that $uCREAT_{24}$ decreased significantly from V2 to V6. Total daily excretion of creatinine has been widely accepted as a technique to assess muscle mass in humans since almost all of the body's creatine is found in skeletal muscle and that creatine is converted non-enzymatically at a constant rate to creatinine [31]. Therefore, total daily urinary excretion of creatinine at steady state would be proportional to total-body creatine content and total body skeletal muscle mass in those with normal renal function [31]. It was confirmed that all SONIA 2 patients had normal renal function. Many recent studies including large prospective clinical studies have employed $uCREAT_{24}$ as a surrogate marker of muscle mass and related a decrease in $uCREAT_{24}$ to higher all-cause and cardiovascular mortality [32,33]. The decrease in $uCREAT_{24}$ observed in our study is consistent with a decrease in muscle mass from V2 to V6.

The decrease in $uCREAT_{24}$ is even more significant since an increase in body weight in NIT-receiving SONIA 2 patients was seen between V2 and V6, a finding that has been reported recently [25]. It was suggested that this was due to a compensatory increased intake of fats and carbohydrates However, the previous report [25] did not include data on $uCREAT_{24}$ and the present analysis suggests that despite the increase in body weight, there was a decrease in $uCREAT_{24}$ and therefore muscle mass. Our resulting conclusion is that body composition is adversely impacted in NIT-receiving patients recommended a low-protein diet, with a decrease

in muscle mass and an increase in fat mass. Further, a significant finding is that a strong association between $uUREA_{24}$ and $uCREAT_{24}$ (R=0.84) was found, indicating that decreased protein intake resulted in muscle mass reduction (Figure 5).

In routine clinical practice, the amounts of creatinine in 24-h urine have been used to determine completeness of urine collections over a 24-h period but we do not believe that incomplete 24-h urine collections could account for our findings given the gradual change in these urine parameters over time [34].

A recent report on AKU patients prior to starting NIT found presence of malnutrition and sarcopenia attributed to previous attempts at protein restriction aimed at reducing HGA accumulation as well as due to spondyloarthropathy and relative immobility of the condition [35]. Therefore, we face a challenge as the intervention to mitigate NIT-induced tyrosinaemia with low-protein diets could potentiate sarcopenia and be detrimental to overall patient well-being. The dual impact of lower muscle mass compounded by increasing body weight is undesirable in a rheumatological condition where every kilogram of body mass increases the stresses and strains on the locomotor system [36]. Moving forward, approaches to mitigating tyrosinaemia should include minimising weight gain and maintaining muscle mass.

The finding of an increase in sNIT between V2 and V6 has been previously reported and attributed to a NIT-induced inhibition of the CYP450 oxidases for which NIT is also a substrate [14]. Interestingly CYP450 inhibition can also evolve over time as observed with sNIT in our study, similar to enzyme induction observed with anticonvulsant usage [37]. It should also be pointed out that despite the increase in sNIT over time, neither sHGA nor the $uHGA_{24}$ decreased over the same period. The previous report [15] attributed the higher HGA and higher NIT over the treatment period to inhibition of renal organic anion transporters by NIT [13]. This can however only explain the increase in sHGA and TBWHGA but not the lack of decrease in $cHGA_{24}$ and $uHGA_{24}$ (Figure S10). The lower protein intake evidenced by lower $uUREA_{24}$ also does not explain this discordance, as this should also result in decrease in HGA. We suggest that HPPD-resistance to NIT over time together with inhibition of renal organic anion transporters may explain these findings. Resistance to herbicides of the HPPD-inhibitor class has been described and one explanation is that of a more efficient degradation of these HPPD-inhibitors such as NIT; however, this cannot be the explanation for our data since sNIT increased rather than decreased [38]. The studies into HPPD-herbicide resistance found no abnormalities in the HPPD gene itself [38]; a change in the HPPD gene in somatic cells is also

unlikely in the human setting. Careful follow-up and studies of patients on long-term therapy with NIT will be necessary to understand this better.

There were limitations in the current study. The relatively small numbers of patients studied were due to the rarity of AKU. Indirect measures of dietary protein intake and muscle mass were employed, even though these are well accepted and validated measures which are frequently used in clinical outcome studies due to their practicability. The chemical analyses including uUREA were made during the study site visits and may not fully reflect the situation when patients are in their usual home environment. Further, the use of these simple urine biomarkers allowed important observations to be made and thereby easily justifiable. Metabolite measurements in a single blood sample were used to calculate TBW values as it is impractical to carry out 24-h blood sampling; however, a recent publication showed that 24-h serum averaged values are close to the single fasting values [19].

In summary, we describe how a decrease in dietary protein intake led to reduced muscle mass. This coupled with body weight gain indicated a potential detrimental body compositional change. The increase in HGA, despite increasing sNIT, is consistent with a low-degree of resistance to nitisinone over time. A very strong association possibly causal between lower protein intake and lower muscle mass was shown. The increase in PHE over time could to be a beneficial progressive adaptation of the tyrosine pathway to NIT-induced tyrosinaemia; a state of relative chemical hyperphenylalaninaemia. There appeared to be no increase in HPLA pathway adaptation over time.

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Author contributions

LRR, JAG, GBG – pioneered the idea for SONIA 2, secured funding, and managed the study, drafting manuscript and final approval of the manuscript.

MK assessed SONIA 2 patients and edited the manuscript.

AMM, ATH, ASD, BPN – carried out the metabolic analyses, drafting manuscript and final approval of the manuscript

RI, JBA – Assisted with conduct of the SONIA 2 study in Liverpool, Piešťany and Paris respectively, drafting manuscript and final approval of the manuscript

MR, BO – assisting SONIA 2 data analyses as well as drafting manuscript and final approval of the manuscript

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Data sharing statement for SONIA 2

SONIA 2 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.

Declaration of interests

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

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

None of the other authors have anything to declare

Legend to main and supplementary tables and figures

Main tables

Table 1. Data at baseline and subsequent visits in nitisinone receiving AKU patients in the SONIA 2. The visits range from V1 (baseline), V2 (month 3), V3 (month 12), V4 (month 24), V5 (month 36) to V6 (month 48).

Table 2. Measured metabolic data shown according to visits in nitisinone receiving AKU patients in the SONIA 2. The visits range from V1 (baseline), V2 (month 3), V3 (month 12), V4 (month 24), V5 (month 36) to V6 (month 48).

Main figures

Figure 1. Changes in sTYR, $uTYR_{24}$, $_{TBW}TYR$, and $cTYR_{24}$ in the nitisinone group of the SONIA 2. (p values only indicated for between-visit comparisons where statistical significance was achieved). The visits range from V1 (baseline), V2 (month 3), V3 (month 12), V4 (month 24), V5 (month 36) to V6 (month 48).

Figure 2. Changes in sPHE, $uPHE_{24}$, $_{TBW}PHE$, and $cPHE_{24}$ in the nitisinone group of the SONIA 2. (p values only indicated for between-visit comparisons where statistical significance was achieved). The visits range from V1 (baseline), V2 (month 3), V3 (month 12), V4 (month 24), V5 (month 36) to V6 (month 48).

Figure 3. Changes in sTYR/sPHE, $uTYR_{24}/uPHE_{24}$, $_{TBW}TYR/_{TBW}PHE$, and $cTYR_{24}/cPHE_{24}$ in the nitisinone group of the SONIA 2. (p values only indicated for between-visit comparisons where statistical significance was achieved). The visits range from V1 (baseline), V2 (month 3), V3 (month 12), V4 (month 24), V5 (month 36) to V6 (month 48).

Figure 4. Changes in $uCREAT_{24}$, sNIT, total body weight, and $uUREA_{24}$ in the nitisinone group of the SONIA 2. (p values only indicated for between-visit comparisons where statistical significance was achieved). The visits range from V1 (baseline), V2 (month 3), V3 (month 12), V4 (month 24), V5 (month 36) to V6 (month 48).

Figure 5. Linear regression graphs of relationships between (a) $uCREAT_{24}$ and $uUREA_{24}$, and (b) $uUREA_{24}$ and total body weight.

Figure 6. A, B. A cartoon representation of changing relationships between PHE, TYR, HPPA and HPLA during NIT therapy. In A, the figure highlights the state at the early stage of NIT therapy showing conversion of PHE to TYR (solid black arrow) inhibited less by TYR (less

prominent curved red arrow), and similarly conversion of HPPA to HPLA (a straight solid red arrow) as well as lower conversion between HPPA and TYR (a straight black arrow). In B, the figure highlights an adapted state after longer NIT therapy showing lesser conversion of PHE to TYR (thin solid black arrow) inhibited more by TYR (more prominent curved red arrow), and stable conversion of HPPA to HPLA (a straight solid red arrow) as well as stable conversion between HPPA and TYR (a straight black arrow).

Supplementary table

Table S1. Demographic and related data in the SONIA 2 showing all data in the nitisinone and no-nitisinone groups. The visits range from V1 (baseline), V2 (month 3), V3 (month 12), V4 (month 24), V5 (month 36) to V6 (month 48).

Table S2. Derived metabolic data shown according to visits in the nitisinone receiving AKU patients in the SONIA 2. The visits range from V1 (baseline), V2 (month 3), V3 (month 12), V4 (month 24), V5 (month 36) to V6 (month 48).

Supplementary figures

Figure S1. The PHE.TYR metabolic pathway is shown highlighting the site of the enzyme defect observed in AKU and the site of action of nitisinone, a reversible competitive inhibitor of 4-hydroxyphenylpyruvate dioxygenase. The pathway also highlights the dynamic relationships between HPPA, TYR and HPLA, a key relationship after introduction of nitisinone. (HPPR – 4- hydroxyphenylpyruvate reductase)

Figure S2. Changes in sHGA, $uHGA_{24}$, $_{TBW}HGA$, and $cHGA_{24}$ in the nitisinone group of the SONIA 2. (p values only indicated for between-visit comparisons where statistical significance was achieved). The visits range from V1 (baseline), V2 (month 3), V3 (month 12), V4 (month 24), V5 (month 36) to V6 (month 48).

Figure S3. Changes in sHPPA, $uHPPA_{24}$, $_{TBW}HPPA$, and $cHPPA_{24}$ in the nitisinone group of the SONIA 2. (p values only indicated for between-visit comparisons where statistical significance was achieved). The visits range from V1 (baseline), V2 (month 3), V3 (month 12), V4 (month 24), V5 (month 36) to V6 (month 48).

Figure S4. Changes in sHPLA, $uHPLA_{24}$, $_{TBW}HPLA$, and $cHPLA_{24}$ in the nitisinone group of the SONIA 2. (p values only indicated for between-visit comparisons where statistical

significance was achieved). The visits range from V1 (baseline), V2 (month 3), V3 (month 12), V4 (month 24), V5 (month 36) to V6 (month 48).

Figure S5. Changes in sHGA/sTYR, $uHGA_{24}/uTYR_{24}$, $_{TBW}HGA/_{TBW}TYR$, and $cHGA_{24}/cTYR_{24}$ in the nitisinone group of the SONIA 2. (p values only indicated for between-visit comparisons where statistical significance was achieved). The visits range from V1 (baseline), V2 (month 3), V3 (month 12), V4 (month 24), V5 (month 36) to V6 (month 48).

Figure S6. Changes in sHPPA/sTYR, $uHPPA_{24}/uTYR_{24}$, $_{TBW}HPPA/_{TBW}TYR$, and $cHPPA_{24}/cTYR_{24}$ in the nitisinone group of the SONIA 2. (p values only indicated for between-visit comparisons where statistical significance was achieved). The visits range from V1 (baseline), V2 (month 3), V3 (month 12), V4 (month 24), V5 (month 36) to V6 (month 48).

Figure S7. Changes in sHPPA/sHPLA, $uHPPA_{24}/uHPLA_{24}$, $_{TBW}HPPA/_{TBW}HPLA$, and $cHPPA_{24}/cHPLA_{24}$ in the nitisinone group of the SONIA 2. (p values only indicated for between-visit comparisons where statistical significance was achieved). The visits range from V1 (baseline), V2 (month 3), V3 (month 12), V4 (month 24), V5 (month 36) to V6 (month 48).

Figure S8. Changes in sHPLA/sTYR, $uHPLA_{24}/uTYR_{24}$, $_{TBW}HPLA/_{TBW}TYR$, and $cHPLA_{24}/cTYR_{24}$ in the nitisinone group of the SONIA 2. (p values only indicated for between-visit comparisons where statistical significance was achieved). The visits range from V1 (baseline), V2 (month 3), V3 (month 12), V4 (month 24), V5 (month 36) to V6 (month 48).

Figure S9. Pathway adapted from KEGG (phenylalanine and tyrosine metabolism) showing alternative route of disposal of PHE to HPPD bypassing TYR. (KEGG - Kyoto Encyclopedia of Genes and Genomes)

Figure S10. Changes in in sHGA, $uHGA_{24}$, $_{TBW}HGA$, and $cHGA_{24}$ in the nitisinone group of the SONIA 2 excluding V1. (p values only indicated for between-visit comparisons where statistical significance was achieved). The visits range from V2 (month 3), V3 (month 12), V4 (month 24), V5 (month 36) to V6 (month 48).

Figure S11. Changes in sHGA, $uHGA_{24}$, $_{TBW}HGA$, and $cHGA_{24}$ in the nitisinone group of the SONIA 2. (p values only indicated for between-visit comparisons where statistical significance was achieved). The visits range from V1 (baseline), V2 (month 3), V3 (month 12), V4 (month 24), V5 (month 36) to V6 (month 48). The Y-axis is shown in log scale.

Figure S12. Changes in sHGA/sTYR, $uHGA_{24}$/$uTYR_{24}$, $_{TBW}$HGA/$_{TBW}$TYR, and $cHGA_{24}$/$cTYR_{24}$ in the nitisinone group of the SONIA 2. (p values only indicated for between-visit comparisons where statistical significance was achieved). The visits range from V1 (baseline), V2 (month 3), V3 (month 12), V4 (month 24), V5 (month 36) to V6 (month 48). The Y-axis is shown in log scale.

Table 1.

Data on age, weight, uUREA and uCREAT					
Visits	Age years	Weight Kg****	$uUREA_{24}$ mmol/day****	uUREA mmol/Kg****	$uCREAT_{24}$ mmol/day****
V1 (n=69)	49.0 (11.3)	74.8 (14.8)	311 (94)	4.23 (10.2)	10.3 (3.0)
V2 (n=69)	49.2 (11.3)	75.7 (15.1)	368 (214)	4.89 (2.75)	14.7 (11.7)
V3 (n=67)	50 (11.3)	78.1 (15)	280 (83)	3.67 (1.1)	10.6 (3.1)
V4 (n=65)	51 (11.3)	78.6 (15.3)	305 (92)	3.98 (1.36)	11 (3.4)
V5 (n=60)	52 (11.3)	78.6 (15.8)	247 (100)	3.14 (1.14)	8.4 (3.3)
V6 (n=56)	53 (11.4)	78.4 (16.1)	261 (137)	3.27 (1.44)	8.5 (4.4)
Variation among visit means is significantly greater than expected by chance with p<: *<0.05; **<0.01; ***<0.001; ****<0.0001; between visit comparisons are shown in figures (main and supplementary). CREAT – creatinine;					

Table 2.

Measured metabolites						
	V1 (n=69)	V2 (n=69)	V3 (n=67)	V4 (n=65)	V5 (n=60)	V6 (n=56)
sHGA µmol/L****	30.4 (11)	0.7 (1.3)	0.7 (1.6)	2.1 (6.5)	2.2 (6.1)	2.9 (7.4)
sTYR µmol/L****	65 (15)	951 (215)	915 (204)	872 (246)	896 (293)	848 (337)
sPHE µmol/L****	56.8 (9.5)	58.8 (12.1)	58.4 (11.7)	64.4 (10.9)	66.6 (11.8)	68.8 (19)*
sHPPA µmol/L		40.8 (32.2)	36.2 (7.6)	39.4 (9.6)	40.2 (11.9)	41.1 (15.2)
sHPLA µmol/L		89.7 (29.4)	87.4 (27)	90.7 (33.2)	90.6 (31.6)	95.4 (40.9)
sNIT µmol/L**		4.19 (1.8)	4·34 (1·86)	5·04 (2·67)	5·28 (2·74)	5·91 (3·92)
$uHGA_{24}$ µmol/day****	34985 (13114)	165 (172)	181 (401)	990 (3870)	1640 (6365)	1569 (6220)
$uTYR_{24}$ µmol/day****	162 (88)	1651 (1099)	1291 (662)	1180 (628)	1271 (690)	1112 (650)
$uPHE_{24}$ µmol/day*	121 (271)	76 (50)	57 (26)	55 (26)	70 (49)	61 (31)
$uHPPA_{24}$ µmol/day****	44 (102)	21058 (13026)	15846 (5086)	14527 (5405)	14669 (6570)	14341 (7019)
$uHPLA_{24}$ µmol/day****	43 (66)	16183 (8748)	13257 (4029)	14622 (5122)	14168 (5563)	12360 (6567)
sHGA/sTYR****	0.48 (0.17)	0.0008 (0.002)	0.0008 (0.002)	0.014 (0.07)	0.018 (0.08)	0.028 (0.11)*
sTYR/sPHE****	1.16 (0.26)	16.7 (4.7)	16.1 (3.9)	13.8 (4.2)	13.7 (4.4)	12.7 (4.6)*
sHPPA/sTYR		0.044 (0.04)	0.041 (0.01)	0.046 (0.01)	0.044 (0.01)	0.047 (0.01)
sHPPA/sHPLA		0.468 (0.32)	0.445 (0.13)	0.478 (0.18)	0.47 (0.15)	0.506 (0.38)
sHPLA/sTYR		0.095 (0.03)	0.096 (0.2)	0.102 (0.03)	0.097 (0.03)	0.108 (0.04)
$uHGA_{24}/uTYR_{24}$****	266 (141)	0.103 (0.08)	0.138 (0.29)	3.1 (16.1)	5.1 (24.9)	7.6 (37.4)
$uTYR_{24}/uPHE_{24}$****	1.78 (0.66)	23.2 (6)	22.5 (5.4)	21.9 (6.8)	21.8 (7.6)	21.4 (6.9)
$uHPPA_{24}/uTYR_{24}$****	0.22 (0.45)	15.2 (7.9)	15.1 (8.3)	15.8 (11.6)	13.7 (9)	15.3 (9)
$uHPPA_{24}/uHPLA_{24}$	0.99 (1.2)	1.29 (0.34)	1.22 (0.29)	1.18 (0.86)	1.06 (0.31)	1.53 (1.94)
$uHPLA_{24}/uTYR_{24}$****	0.25 (0.34)	12.4 (6.7)	13.2 (8.4)	16.5 (15.4)	13.8 (9.2)	13.9 (10.4)

Variation among column means is significantly greater than expected by chance with p<: *<0.05; **<0.01; ***<0.001; ****<0.0001; within sTYR group comparisons are shown in figures (main and supplementary).
S - serum; uX_{24} - 24-h urine measurement; HGA – homogentisic acid; TYR – tyrosine; PHE – phenylalanine; HPPA – 4-hydroxyphenylpyruvate; HPLA – 4-hydroxyphenyllactate; NIT – nitisinone;

Figure 1.

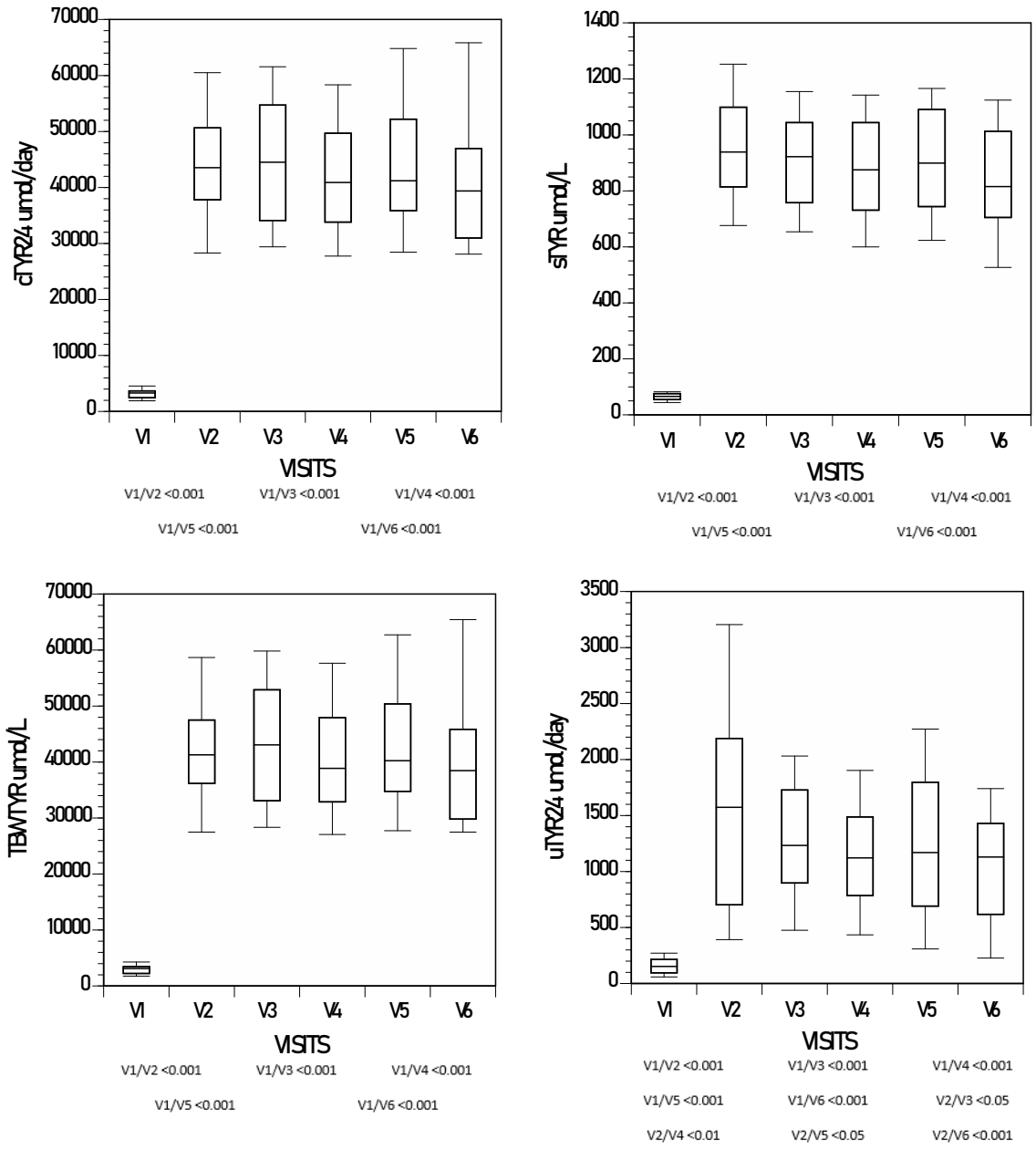

dTYR24 umol/day
70000
60000
50000
40000
30000
20000
10000
0
V1
V2
V3
V4
V5
V6
VISITS
V1/V2 <0.001
V1/V3 <0.001
V1/V4 <0.001
V1/V5 <0.001
V1/V6 <0.001
sTYR umol/L
1400
1200
1000
800
600
400
200
0
V1/V2 <0.001
V1/V3 <0.001
V1/V4 <0.001
V1/V5 <0.001
V1/V6 <0.001
TBWTYR umol/L
70000
60000
50000
40000
30000
20000
10000
0
V1/V2 <0.001
V1/V3 <0.001
V1/V4 <0.001
V1/V5 <0.001
V1/V6 <0.001
uTYR24 umol/day
3500
3000
2500
2000
1500
1000
500
0
V1/V2 <0.001
V1/V3 <0.001
V1/V4 <0.001
V1/V5 <0.001
V1/V6 <0.001
V2/V3 <0.05
V2/V4 <0.01
V2/V5 <0.05
V2/V6 <0.001

Figure 2.

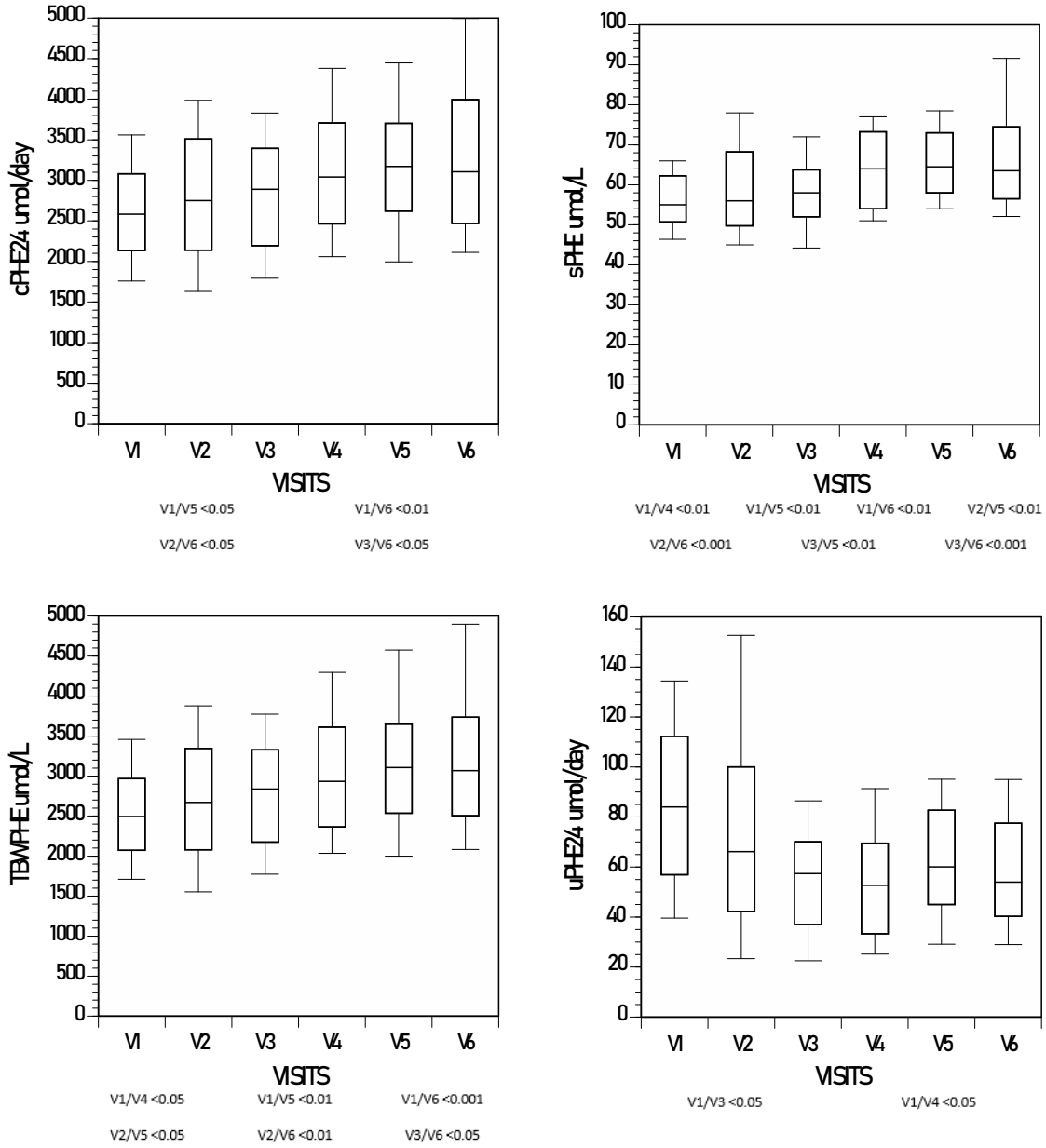

cPHE24 umol/day
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
V1 V2 V3 V4 V5 V6
VISITS
V1/V5 <0.05
V1/V6 <0.01
V2/V6 <0.05
V3/V6 <0.05
sPHE umol/L
100
90
80
70
60
50
40
30
20
10
0
V1 V2 V3 V4 V5 V6
VISITS
V1/V4 <0.01
V1/V5 <0.01
V1/V6 <0.01
V2/V5 <0.01
V2/V6 <0.001
V3/V5 <0.01
V3/V6 <0.001
TBMPHE umol/L
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
V1 V2 V3 V4 V5 V6
VISITS
V1/V4 <0.05
V1/V5 <0.01
V1/V6 <0.001
V2/V5 <0.05
V2/V6 <0.01
V3/V6 <0.05
uPHE24 umol/day
160
140
120
100
80
60
40
20
0
V1 V2 V3 V4 V5 V6
VISITS
V1/V3 <0.05
V1/V4 <0.05

Figure 3.

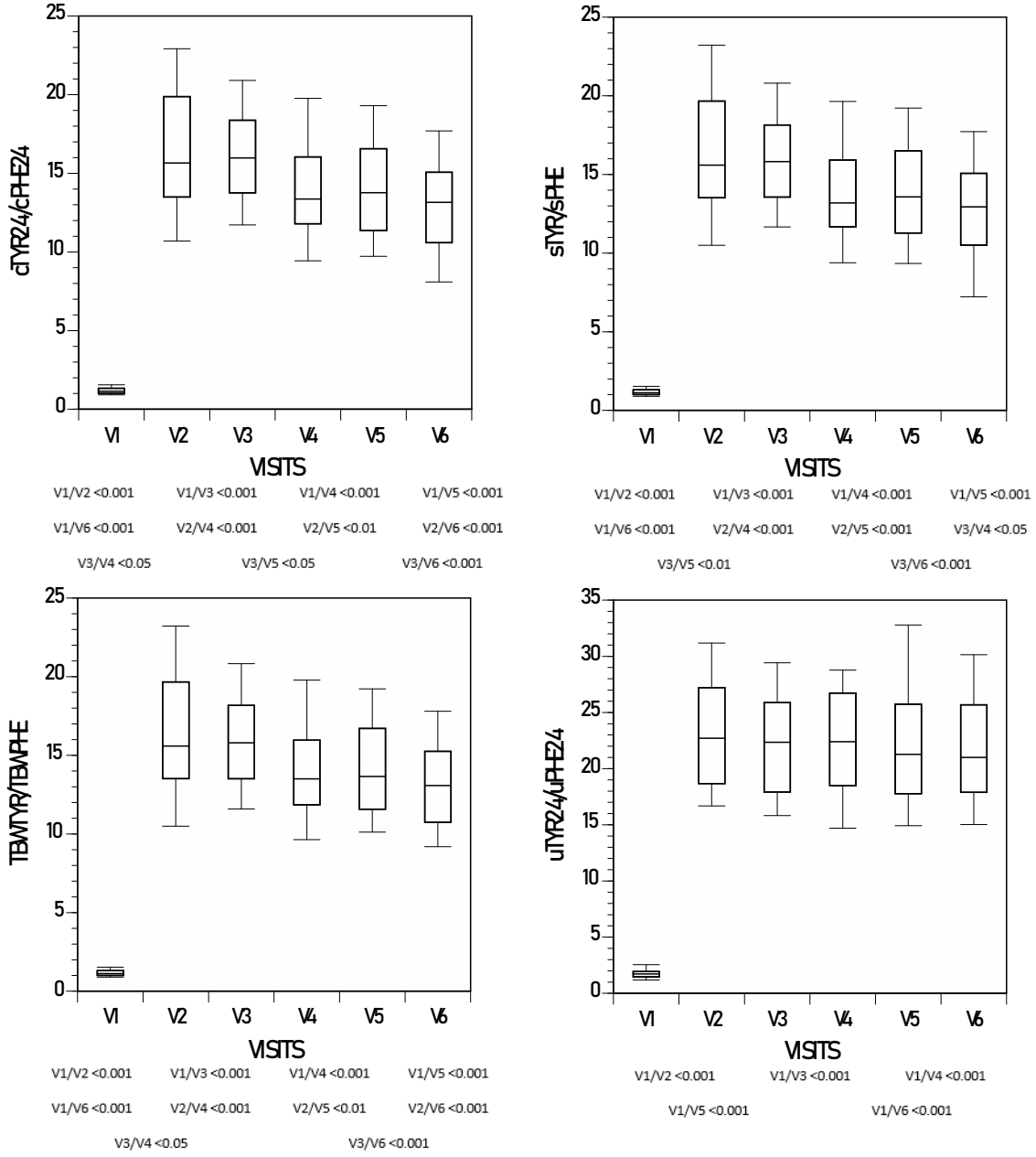

cTYR24/cPHE24
sTYR/sPHE
TBMTYR/TBMPHE
uTYR24/uPHE24
VISITS
V1
V2
V3
V4
V5
V6
V1/V2 <0.001
V1/V3 <0.001
V1/V4 <0.001
V1/V5 <0.001
V1/V6 <0.001
V2/V4 <0.001
V2/V5 <0.01
V2/V6 <0.001
V3/V4 <0.05
V3/V5 <0.05
V3/V6 <0.001
V1/V2 <0.001
V1/V3 <0.001
V1/V4 <0.001
V1/V5 <0.001
V1/V6 <0.001
V2/V4 <0.001
V2/V5 <0.001
V3/V4 <0.05
V3/V5 <0.01
V3/V6 <0.001
V1/V2 <0.001
V1/V3 <0.001
V1/V4 <0.001
V1/V5 <0.001
V1/V6 <0.001
V2/V4 <0.001
V2/V5 <0.01
V2/V6 <0.001
V3/V4 <0.05
V3/V6 <0.001
V1/V2 <0.001
V1/V3 <0.001
V1/V4 <0.001
V1/V5 <0.001
V1/V6 <0.001

Figure 4.

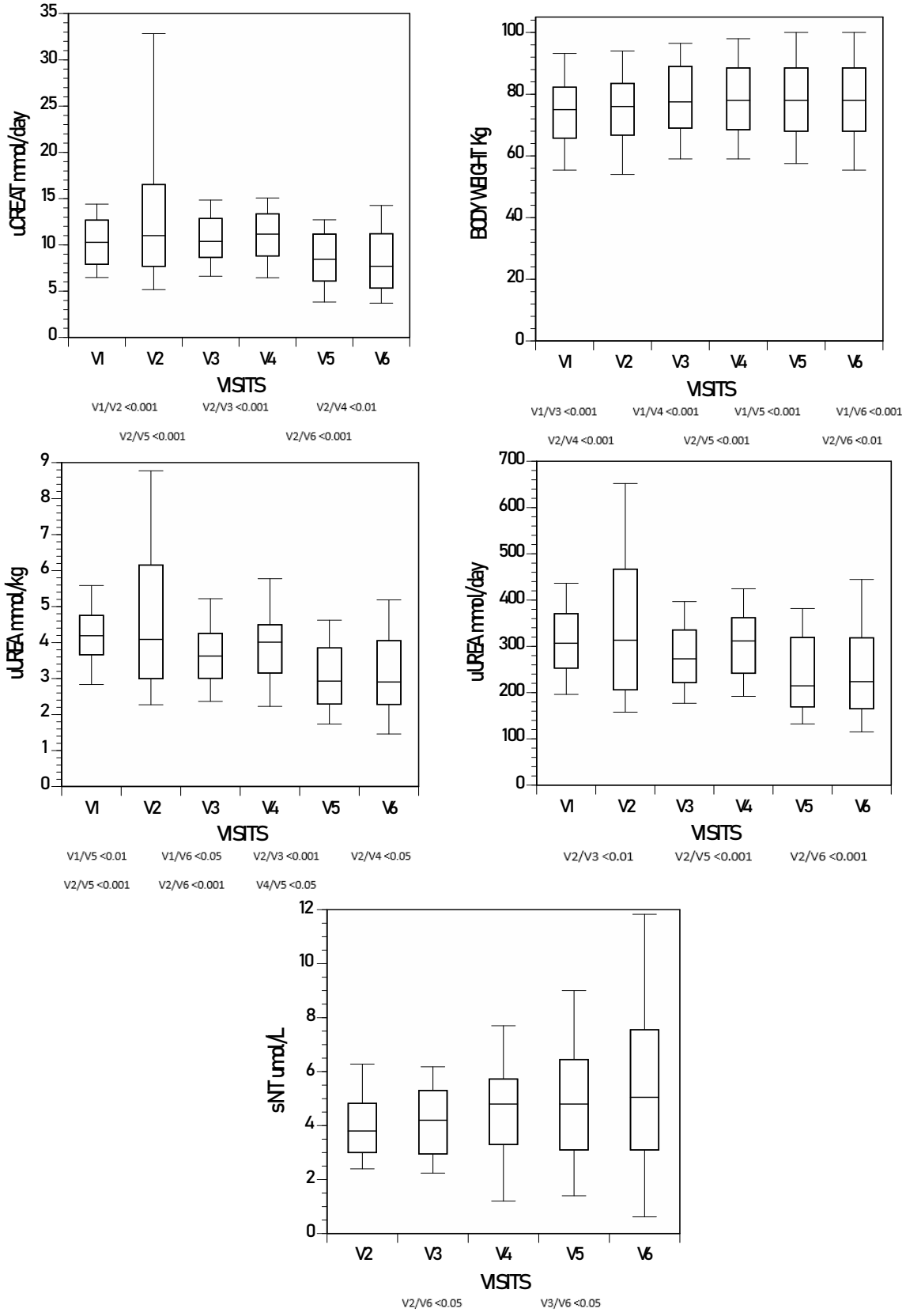
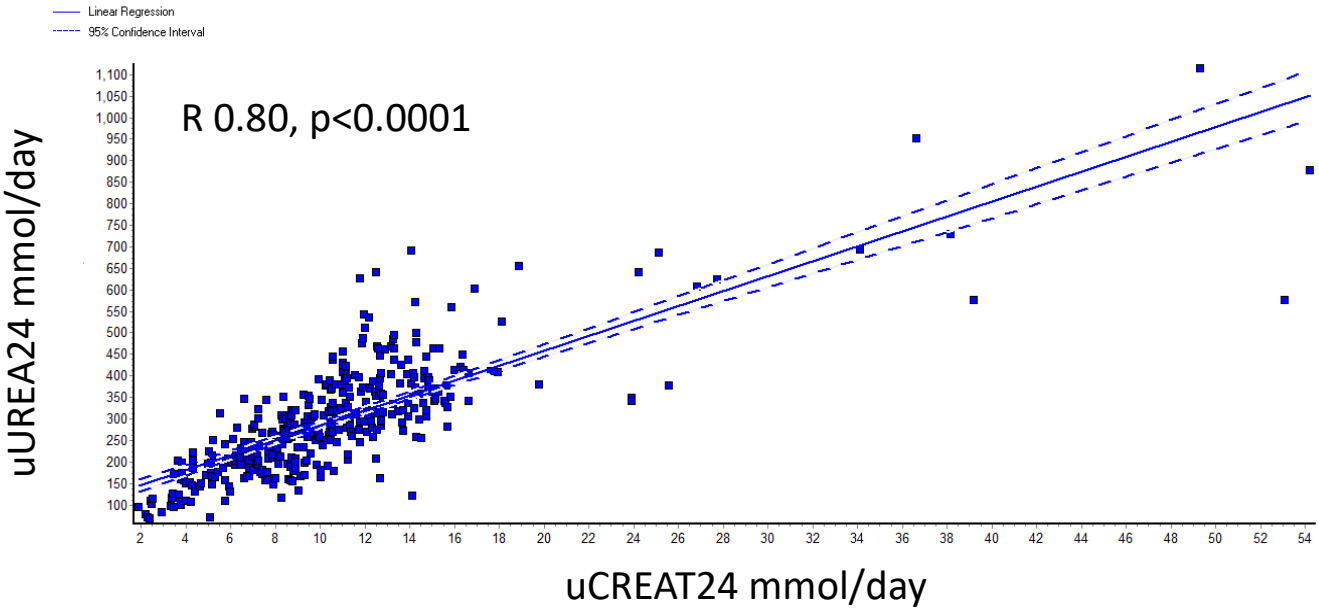

uCREAT mmol/day
VISITS
V1 V2 V3 V4 V5 V6
V1/V2 <0.001
V2/V3 <0.001
V2/V4 <0.01
V2/V5 <0.001
V2/V6 <0.001
BODY WEIGHT Kg
V1/V3 <0.001
V1/V4 <0.001
V1/V5 <0.001
V1/V6 <0.001
V2/V4 <0.001
V2/V5 <0.001
V2/V6 <0.01
uUREA mmol/kg
V1/V5 <0.01
V1/V6 <0.05
V2/V3 <0.001
V2/V4 <0.05
V2/V5 <0.001
V2/V6 <0.001
V4/V5 <0.05
uUREA mmol/day
V2/V3 <0.01
V2/V5 <0.001
V2/V6 <0.001
sNT umol/L
V2/V6 <0.05
V3/V6 <0.05

Figure 5


Linear Regression
95% Confidence Interval
R 0.80, p<0.0001
uUREA24 mmol/day
uCREAT24 mmol/day

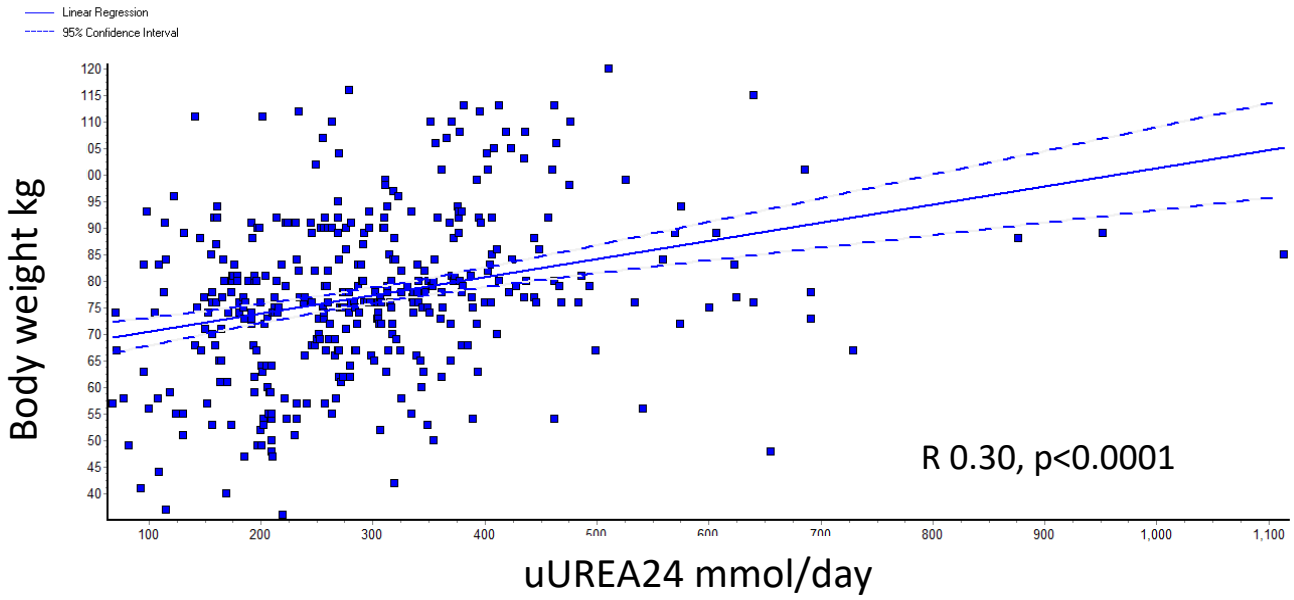

Linear Regression
95% Confidence Interval
R 0.30, p<0.0001
Body weight kg
uUREA24 mmol/day

Figure 6.