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Short Title: Nitisinone causes acquired tyrosinosis in alkaptonuria

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Abstract

Background: For over two decades, Nitisinone (NTBC) has been successfully used to manipulate the tyrosine degradation pathway and save the lives of many children with hereditary tyrosinaemia type1 (HT1). More recently, NTBC has been used to halt homogentisic acid accumulation in alkaptonuria (AKU) with evidence suggesting its efficacy as a disease modifying agent. NTBC induced hypertyrosinemia has been associated with cognitive impairment and potentially sight-threatening keratopathy. In the context of a non-lethal condition (i.e. AKU), these serious risks call for an evaluation of the wider impact of NTBC on the tyrosine pathway. We hypothesized that NTBC increases the tyrosine pool size and concentrations in tissues.

Methods: In AKU mice tyrosine concentrations of tissue homogenates were measured before and after treatment with NTBC. In Humans, pulse injection with L-[^{13}C$_9$]tyrosine and L-[d$_8$]phenylalanine was used along with compartmental modelling to estimate the size of tyrosine pools before and after treatment with NTBC.

Results: NTBC increased tyrosine concentrations in murine tissues by five to nine folds. It also significantly increased the tyrosine pool size in humans ($p<0.001$), suggesting that NTBC increases tyrosine not just in serum but also in tissues (i.e. acquired tyrosinosis).

Conclusions: This study provides, for the first time, the experimental proof for the magnitude of NTBC-related acquired tyrosinosis which should be overcome to ensure the safe use of NTBC in AKU.

Take-home message: This study provides, for the first time, the experimental proof for the magnitude of tyrosine increase following nitisinone therapy.

Compliance with Ethics Guidelines:

Conflict of Interest: Milad Khedr, Maggie S Cooper, Andrew T Hughes, Anna M Milan, Andrew S Davison, Brendan P Norman, Hazel Sutherland, Jonathan C Jarvis, Richard Fitzgerald, Louise Markinson, Eftychia-Eirini Psarelli, Parisa Ghane, Nicolaas EP Deutz, James A Gallagher, Lakshminarayan R Ranganath declare that they have no conflict of interest.. □

Informed Consent: All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000 (5). Informed consent was obtained from all patients for being included in the study.

Animal Rights: All institutional and national guidelines for the care and use of laboratory animals were followed. All procedures in the mice study were performed in accordance with the Animals Scientific Procedures Act (1986) and licensed by the United Kingdom Home Office under the project license 40/3743.

Details of the contributions of individual authors: MK, MC, EEP, LRR and JAG contributed to the planning, conduct, and reporting of the analytical, animal and human studies. AMM, ATH and ASD contributed to the planning, conduct, and reporting of the stable isotopes measurements by LC-MSMS. JCJ and HS contributed to the planning, conduct, and reporting of the animal study. NEPD and PG contributed to the planning and reporting of the human study. BPN contributed to the reporting of the human study. LM and RF contributed to the planning and conduct of the human study. All authors gave final approval of the manuscript version to be published.

Guarantor: Milad Khedr

Data and materials availability: All data associated with this study are present in the paper or the supplementary material.

Introduction

Alkaptonuria (AKU) is a rare inborn error of tyrosine metabolism, (OMIM 203500), caused by a deficiency in the homogentisate dioxygenase enzyme (HGD, EC 1.13.11.5) (O'Brien et al., 1963) which leads to increased homogentisic acid in body fluids and tissues. This results in the formation of a melanin-like pigment (Figure S1) in a process called ochronosis (Zannoni et al., 1969).

Ochronosis is the main pathophysiological process in AKU. It changes the mechanical properties of tissues and gives rise to the various manifestations of AKU (Ranganath et al., 2019). These

include features such as stones (kidney, prostate, salivary and gall bladder), ruptures (tendons, muscle, ligaments), hearing impairment, external ocular and auricular ochronosis, cardiac (mainly aortic) valve disease, bone fractures and most significantly, arthritis (Ranganath et al., 2019).

The management of AKU has been mainly supportive without addressing the underlying pathophysiological mechanisms. However, NTBC, a competitive reversible inhibitor of the hydroxyphenylpyruvic acid dioxygenase enzyme (HPPD, E.C. 1.13.11.27), can decrease urinary excretion of homogentisic acid by 98.8% (Ranganath et al., 2016). NTBC is already licensed and has been used for over two decades for the treatment of HT1 in children (Ranganath et al., 2013). Since 2012, NTBC has been used off-label in the NHS England designated National Alkaptonuria Centre (NAC), at the Royal Liverpool University Hospital. All the known AKU patients from England and Scotland attend the NAC annually and receive NTBC 2 mg daily as part of their standard care. Animal studies have demonstrated that ochronosis can be arrested at any stage and completely prevented if the treatment with NTBC started early in life (Preston et al., 2014, Keenan et al., 2015). Data from the NAC cohort suggest that NTBC arrests ochronosis (Ranganath et al., 2018).

The Subclinical Ochronotic Features in Alkaptonuria (SOFIA) study has demonstrated eye pigmentation in a 22-year-old patient and biopsy evidence of ear pigmentation in a 20-year-old patient. This suggests that ochronosis starts earlier in life than the major clinical signs (Cox et al., 2019). Since the biochemical deficit is present since birth, treatment with NTBC to modify the course of AKU seems logical. However, NTBC causes a significant rise in serum tyrosine (Phornphutkul et al., 2002, Introne et al., 2011, Ranganath et al., 2016, Milan et al., 2017). This can result in ocular tyrosine keratopathy (Introne et al., 2011, Stewart et al., 2014, Khedr et al.,

2018, White and C Tchan, 2018) which spontaneously resolves upon discontinuation of NTBC. Furthermore, there are concerns regarding effects on neurocognitive function as a result of NTBC-induced hypertyrosinemia in children with HT1 (McKiernan, 2013, van Ginkel et al., 2016, García et al., 2017). Although low phenylalanine levels, NTBC and the natural course in an older HT1 population may be responsible for neurocognitive impairment as well, not only tyrosine levels. These concerns are underscored by observations of significant dose-dependent increases in tyrosine metabolites following treatment with NTBC (Milan et al., 2019). This calls for further assessment of the extent of the NTBC-induced hypertyrosinemia.

In this study, we have assessed the magnitude of NTBC-induced hypertyrosinemia by measuring tyrosine concentrations in tissues harvested from NTBC treated AKU mice. Assessing tissue tyrosine concentrations in humans would be impractical. Therefore, we have used stable isotope methodology with compartmental modelling to estimate the intracellular and extracellular pool size of tyrosine before and after NTBC.

Materials and Methods

Biochemical analysis:

Subjects were asked to collect urine over the two hours of the blood sampling period. Collection bottles were stored away from light and in cool conditions. Urine was acidified using 5 N sulphuric acid to a pH of less than 2.5. Serum samples were collected in plain serum tubes (Sarstedt, Germany). At the end of the experiment, samples were centrifuged (10 min, 1500 xg, 4 °C). One serum aliquot was acidified (to stabilize HGA) by addition of a volume of 5.8 M perchloric acid (approx. 60% w/v) equivalent to 10% of the serum volume (Hughes et al., 2015). Acidified serum was centrifuged (10 min, 1500 xg, 4 °C) and supernatant was taken. All samples were frozen and stored at -80 °C until analysis. Tracer measurements were done in one batch for all samples. Measurement of native tyrosine, phenylalanine, HGA, HPPA and HPLA were done on all samples in a separate batch.

Native tyrosine, phenylalanine, HGA, HPPA and HPLA concentrations were measured in human serum and urine using Liquid chromatography–mass spectrometry (LC-MS/MS) (Hughes et al., 2015, Hughes et al., 2014). Tyrosine concentrations were also measured in murine plasma and tissue homogenates using LC-MS/MS (Hughes et al., 2015).

L-[^{13}C$_9$]tyrosine (95%) was obtained from Sigma- Aldrich (Dorset, UK). L-[*ring*-^{13}C$_6$]tyrosine (99%), L-[*ring*-^{13}C$_6$]phenylalanine (99%), L-[d$_7$]tyrosine (95%) and L-[d$_8$]phenylalanine (98%) were obtained from Cambridge Isotopes Laboratories (Andover, MA, USA). Using matrix matched calibrators, we have modified an existing LC-MS/MS method (Hughes et al., 2015) to enable measurements of serum L-[^{13}C$_9$]tyrosine, L-[d$_8$]phenylalanine and L-[d$_7$]tyrosine. This

was achieved following a 1 in 10 dilution with deionized water containing L-[*ring*-$^{13}C_6$]tyrosine, L-[*ring*-$^{13}C_6$] phenylalanine internal standards. The prepared sample was injected onto an Atlantis dC18 column (3 mm x 100 mm, 3 µm) using an Agilent 6490 triple quadrupole LC-MS/MS with Jet-Stream electrospray ionization (ESI-MS/MS) equipped with an Agilent 1290 infinity pump and autosampler. Assay was validated in accordance with international guidelines . Intra-batch accuracy (n=6) was 98 ± 5% for L-[$^{13}C_9$]tyrosine, 101 ± 4% for L-[d_8]phenylalanine and 101 ± 7% for L-[d_7]tyrosine; inter-batch accuracy (n=6) was 98 ± 2% for L-[d_7]tyrosine and 93 ± 13% for both L-[$^{13}C_9$]tyrosine and L-[d_8]phenylalanine. Precision, both intra- and inter-batch (n=6 for each), was <10% for L-[$^{13}C_9$]tyrosine, L-[d_8]phenylalanine and L-[d_7]tyrosine. Hughes et al (Hughes et al., 2015) demonstrated that the internal standard normalized matrix factor had <10% coefficient of variation in acidified serum tyrosine calibrators. Analyses of this study samples were carried out using the same LC-MS/MS analyzer as well as the same mass spectrometric parameters and chromatographic conditions. Therefore, matrix effects were not assessed here. L-[$^{13}C_9$]tyrosine, L-[d_8]phenylalanine and L-[d_7]tyrosine were stable after 24h storage at 20 °C, 4 °C and -20 °C. The assay was linear to 10 µM L-[$^{13}C_9$]tyrosine, 9.37 µM L-[d_8]phenylalanine and 1.2 µM L-[d_7]tyrosine. This range was sufficient to analyze all the study samples. The lower limit of quantitation was 50 nM, 47 nM and 21 nM for L-[$^{13}C_9$]tyrosine, L-[d_8]phenylalanine and L-[d_7]tyrosine, respectively. No carry over was observed.

Mice study
Mice were bred, housed and maintained within the Liverpool John Moores University Life Science Support Unit in accordance with the Home Office UK guidelines. A total of 18 BALB/c mice were split into 3 groups:

- Wild type (WT) group: (n=6; median age= 24.8 weeks; individual age values: 17.4, 17.4,27.6, 27.6, 24.7, 24.9 weeks; all males)

- AKU treated group (*HGD*-/-) :(n=6; median age= 8.5 weeks; individual age values: 6.6, 8.6, 8.4, 8.4, 24.4, 24.4 weeks; 4males and 2 females)

- AKU control group (*HGD*-/-): (n=6; median age= 21.1 weeks; individual age values:6.7,6.7, 23.6, 23.7, 23.7, 18.6 weeks; 3 males and 3 females)

Tissues homogenates were prepared as 30 mg/mL solution. Tyrosine concentrations were all normalized and expressed as µmol/gram tissue.

Human study

Study participants

This was a non-randomized study involving a group of AKU patients aged 24 to 66 years; and a group of healthy volunteers aged 24 to 41 years. Healthy volunteers were recruited from the Royal Liverpool University Hospital Consent for Consent database. AKU patients were recruited from the National AKU center. Recruitment took place between July and December 2017. Seven AKU patients and seven healthy volunteers were enrolled in the study. Height was measured by stadiometer. Weight, whole body fat free mass, total body water and lean body mass were obtained using bioelectric impedance analysis (BIA,TANITA body composition analyzer DC-430MA). The flow of participants in this study is shown in Figure S2. All participants completed the study. The characteristics of the study subjects are shown in Table S1.

Study design

The healthy volunteers received a single infusion of stable isotopes during the study. The AKU patients received two: one isotopes infusion was given while they were on NTBC and the other while off NTBC. Six of the AKU patients were already receiving NTBC 2 mg daily each and were asked to stop NTBC for at least four weeks. Only one AKU patient was not on NTBC when enrolled in the study. He was started on NTBC 2 mg daily after receiving the first isotopes infusion.

Stable isotopes infusion Protocol

Microbiological and Pyrogen Tested L-[^{13}C$_9$]tyrosine (99%) and L-[d$_8$]phenylalanine (98%) were obtained from Cambridge Isotopes Laboratories (Andover, MA, USA). These were prepared into sterile infusions (20 mg of L-[^{13}C$_9$]tyrosine and 50 mg of L-[d$_8$]phenylalanine in a 50 mL of 0.9% saline), under good manufacturing practice conditions, in the radiopharmacy department at the Royal Liverpool University Hospital.

All subjects were studied at the NIHR Royal Liverpool University Hospital Clinical Research Facility. All subjects were instructed to attend the facility in the morning after an overnight fast. One peripheral venous line was placed in each arm. One was used for blood sampling and the other for infusing stable isotopes. The sampling line was placed in the dorsum of the hand or as close to it as possible. Arterialized blood samples were obtained using a thermostatically controlled heated- hand box (air temperature 55°C). Two patients could not tolerate the heated hand box and had a heating pad applied instead. Baseline samples were taken before the infusion. Subsequent samples were taken at the following time points: t=5,10,15,20,25,30,40,50,60,90,120 min.

Statistics

AKU is an ultra-rare metabolic disease with an estimated prevalence of 1 in 250,000 to 1,000,000 people (11). Prevalence may be up to 1:120.000 in central Europe and much higher in other areas of the world. In the UK, at the time of planning this study, there were 58 AKU patients who attended the NAC. The sample size was chosen on what can be realistically achieved in term of subject recruitment.

Continuous variables are presented with mean and standard deviation whereas categorical variables (such as gender and race/ethnicity of participants) are presented as counts. Shapiro-Wilk test was used to assess normality of the data set in each group. Where data were normally distributed a paired *t*-test was used to conduct comparisons within the AKU groups; and unpaired *t*-test was used to compare data between HV and AKU groups. Depending on normality of the variable of interest, Mann-Whitney test was used to compare tyrosine concentrations in murine brain homogenates in the AKU on and off NTBC groups, one way ANOVA was used to compare L-[d_8]phenylalanine doses given to human subjects in the three groups while a Kruskal-Wallis test was used to compare L-[$^{13}C_9$]tyrosine doses given to human subjects as well as area under the curve (AUC) for L [$^{13}C_9$]tyrosine in the three groups. Analyses were conducted in GraphPad Prism version 8.1.0 (GraphPad software), using two-sided significance tests at the 5% statistical significance level.

Data analysis

Since one of the assumptions for non-compartmental modelling is the steady state of tracee, we calculated the Tracer-Tracee Ratio (TTR) at each time point as the ratio of the tracer concentration and the pooled tracee concentration, when the pooled tracee concentration is the

median of the measured tracee in all blood draws and was calculated for each person separately. Moreover, the TTR values for each subject were normalized by the amount of administered tracer and fat free mass. We combined all TTR data for subjects in each group and fitted one curve on the combined dataset. The mean and standard deviation of parameters from the best fit were used for the calculation of tracee quantities by means of non-compartmental modelling. Curve fitting on the TTR data was performed using GraphPad Prism 8.0.2. Further details on compartmental modelling can be found in the supplementary material.

Results

Effect of NTBC on tyrosine concentrations in murine tissues

NTBC leads to five- to nine-fold increase in tyrosine concentrations in tissues in the AKU treated group compared to the AKU control group. This is summarized in Table S2 and Figure 1.

Effects of NTBC on serum compounds in the tyrosine pathway in humans

Serum phenylalanine (s-Phe), serum tyrosine (s-Tyr), serum hydroxyphenyllactic acid (s-HPLA), serum hydroxyphenylpyruvic acid (s-HPPA) and serum homogentisic acid (s-HGA) were measured. The concentrations of s-Tyr, s-Phe, s-HGA and s-HPLA were all significantly higher in the non-treated AKU group when compared to healthy volunteers (p <0.001). s-HPPA was not measurable in either group. The use of NTBC in AKU caused significant increase in the concentration of s-Tyr (p <0.001), s-HPLA (p <0.001) and s-HPPA. It also reduced the concentrations of s-HGA by seven folds (p <0.001). No effect was noted on s-Phe. This is summarized in Figure S3 and Table S3.

Effects of NTBC on urinary compounds in the tyrosine pathway in humans

We measured the two-hour urinary excretion of phenylalanine (u-Phe), urinary tyrosine (u-Tyr), urinary hydroxyphenyllactic acid (u-HPLA), urinary hydroxyphenylpyruvic acid (u-HPPA) and urinary homogentisic acid (u-HGA). The use of NTBC in AKU increased the concentration of u-Tyr by six folds (p =0.014), u- HPLA by 31-folds (p =0.004) and u-HPPA by 18-folds (p =0.007). In contrast, it reduced the concentration of u-HGA by six folds (p =0.002). No effect was noted on u-Phe. This is summarized in Figure S4 and Table S4.

Effects of NTBC on tyrosine decay curves in humans

Tracer to tracee ratio (TTR) data for L-[^{13}C$_9$]tyrosine, L-[d$_8$]phenylalanine and L-[d$_7$]tyrosine were fitted using the two-exponential model. A summary of average parameters is included in Table S5. The area under the curve (AUC) for L-[^{13}C$_9$]tyrosine was significantly lower in NTBC- treated AKU group compared to the control AKU group (0.19+0.02 vs 0.70+0.21 min; p = 0.026). However, in AKU patients who were not on NTBC, there was no statistically significant difference in L-[^{13}C$_9$]tyrosine- AUC when compared to that in healthy volunteers (0.70+ 0.21 vs 0.88+0.07 min; p =0.845). Tyrosine degradation in the AKU patients who are not on NTBC is comparable to that in healthy volunteers; whereas in AKU patients who received NTBC, tyrosine undergoes a minimal degree of degradation (Figure2, panels A and B). In contrast, phenylalanine degradation is not affected (Figure 2, panel C).

Effects of NTBC on Tyrosine pool size in humans

Comparison of the tyrosine estimated pool size across the three groups has demonstrated that the tyrosine extracellular pool size in NTBC treated AKU patients is nearly five- folds larger than that of AKU patients who did not receive NTBC ($p<0.001$). Furthermore, the tyrosine intracellular pool size in NTBC treated AKU patients is almost three- folds larger than that of

AKU patients who did not receive NTBC ($p<0.001$). Compared to healthy volunteers, the tyrosine extracellular pool size in AKU patients off NTBC is 2.7-folds larger ($p= 0.044$). Furthermore, the tyrosine extracellular pool size in AKU patients off NTBC is 1.4- folds larger when compared to healthy volunteers ($p= 0.027$). This is summarized in Figure 3 and Table S6.

Discussion

In this study, we have assessed the extent of acquired tyrosinosis in NTBC treated AKU mice and humans. We have used stable isotopes in AKU for the first time to assess the magnitude of increased tyrosine pools in the context of NTBC therapy. Since 1932, the term 'tyrosinosis' has been used to described various metabolic disorders in which elevated blood tyrosine is a common feature (Halvorsen et al., 1966). After establishing the biochemical and genetic basis, the term was dropped in favor of tyrosinaemia. In this study we have used 'acquired tyrosinosis' in the context of NTBC therapy to emphasize the fact that increases in tyrosine are not restricted to the blood and that it extends to the tissues too.

Compared to healthy volunteers, the tyrosine extracellular pool size in AKU patients off NTBC is 2.7-folds larger ($p=0.044$). This is likely because the two groups are not matched for age or gender (control group participants were all Caucasian males aged 24-41 while the AKU group had 3 females and 4 males with an age range of 24-66).

We found that NTBC-induced HPPD inhibition causes increases in tyrosine and tyrosine metabolites upstream. This is in keeping with what others found (Phornphutkul et al., 2002, Ranganath et al., 2016, Milan et al., 2017). Tyrosine is a small molecule and distributes freely in the extracellular and intracellular compartments. Tyrosine crystals have been found in the cornea

in HT2 (Kocabeyoglu et al., 2014) and NTBC treated HT1 (Schauwvlieghe et al., 2013). Furthermore, tyrosine keratopathy has been reported in AKU patients treated with NTBC (Introne et al., 2011, Stewart et al., 2014, Khedr et al., 2018, White and C Tchan, 2018). But, the wider effects of increased tyrosine in other tissues (bone, heart, liver, muscles, brain and kidney) is largely unknown. It is not clear whether increased tyrosine in tissues leads to adverse impact on these tissues' structure or function.

Biochemically, NTBC produces a similar defect to what is seen in HT3 in which tyrosinosis is presumably present from the earliest embryonic stages. Neurological complications are common in HT3 despite reports of asymptomatic cases (Ellaway et al., 2001). In our study, NTBC caused an almost eight-fold increase in tyrosine concentration in the brains of AKU mice. Neural cells make up 70-80% of the total brain volume with the brain interstitial system taking up 10-20% of the total brain volume (Lei et al., 2017). It is reasonable to assume that the water in the brain is largely intracellular and that the measured tyrosine in brain tissue homogenates predominantly reflects the intracellular compartment. Studies in rats demonstrated that exposure to high levels of L-tyrosine were associated with impairment in energy metabolism in the brain (Ferreira et al., 2013). In the context of AKU, NTBC-induced hypertyrosinemia in mice was associated with increased concentrations of urinary 3-methoxytyramine indicating a change in the peripheral metabolism of catecholamines. However, it did not alter monoamine neurotransmitter metabolism in murine brain tissues (Davison et al., 2019). In humans, NTBC-induced hypertyrosinemia was not associated with altered mood (Davison et al., 2018).

The skin, also, can be affected by NTBC use with pruritus, erythematous rash and exfoliative dermatitis listed as side effects, but it is not clear if NTBC-induced hypertyrosinemia has any causative role in these NTBC related side effects. Notably, urticarial rash was reported in an AKU patient with NTBC-induced hypertyrosinemia. The rash resolved after discontinuation of NTBC (Stewart et al., 2014).

Notably, the concentrations of serum tyrosine, phenylalanine, HGA and HPLA are all significantly higher in the non-treated AKU group when compared to healthy volunteers. The AKU patients and healthy volunteers groups were not matched for age or gender. Furthermore, healthy volunteers were instructed not to make any changes to their diet whereas AKU patients were on protein- restricted diet to reduce the risks related to NTBC-induced hypertyrosinaemia. Fundamentally, the noted differences in these metabolites concentrations between the two groups are the likely result of HGD enzyme deficiency in AKU patients. It causes a block in the tyrosine pathway and an accumulation of HGA which could exert a negative feedback (product inhibition- like effect). This leads to an increase in the metabolites above the level of the block. It is unlikely that dietary factors could alone explain the differences between healthy volunteers and AKU groups. Of note, HPPA was not measurable in either group which may suggest that conversion of HPLA to HPPA is minimal in healthy and non-treated AKU subjects. This is supported by observations that HPPA and HPLA were undetected before starting NTBC in AKU (Milan et al., 2019).

Our study has limitations. Firstly, the number of participants is relatively small making the statistical comparison not robust enough. Formal sample size calculation was not performed as

this would be impractical in the context of this rare disease. Secondly, the subjects in the control group were not matched with the AKU subjects. In the context of rare disease, there is a very limited pool of patients to recruit from. Our cohort of AKU subjects is quite heterogeneous. They were not matched with the control group participants who were all Caucasian males aged 24-41. The AKU group had 3 females and 4 males with age range of 24-66 years and BMI range of 19.80 to 36.10 kg/m^2. This heterogeneity could explain the wide distribution of data points observed in figure S4. Moreover, the SD values for the enrichment of tyrosine stable isotopes were quite high in the AKU off NTBC group (figure2). This is likely due to the use of fixed dose of stable isotopes in a heterogenous group of AKU patients. This source of variation would have been reduced by adjusting the stable isotope dose according to the participant's weight. Logistically, it was a challenge to match all AKU participants with healthy volunteers. Nonetheless, the homogeneity of the control group (all Caucasian males aged 24-41) ensured consistent data are produced from this group. A further limitation was the use of BIA in estimating fat free mass. BIA has large individual prediction errors compared to the reference standard of dual energy X-ray absorptiometry (DEXA) (Buckinx et al., 2018). Lastly, Use of L-[d$_8$]phenylalanine is reported to be associated with *in-vivo* conversion to L-[d$_7$]phenylalanine (Preston and Small, 2010) which could lead to underestimation of L-[d$_8$]phenylalanine. When setting up the stable isotopes analytical method, it was clear that ^{13}C based internal standards were more robust and stable compared to deuterated ones. Furthermore, the use of comparison, within the AKU group and against healthy volunteers, ensured that any error related to this *in vivo* conversion of L-[d$_8$]phenylalanine is replicated across groups without compromising the overall conclusion.

In summary, this study provides, for the first time, the experimental evidence for the extent of NTBC-related acquired tyrosinosis. We propose the use of the term 'acquired tyrosinosis' instead of 'hypertyrosinemia' to reflect the magnitude of tyrosine increases with NTBC use. Further research is required to assess its wider impact on organ function, if any, in AKU; to overcome this potentially serious side effect of NTBC and facilitate its safe use in modifying the natural history of AKU.

Supplementary Materials

1. Supplemental Methods

2. Supplemental Figures

3. Supplemental Tables

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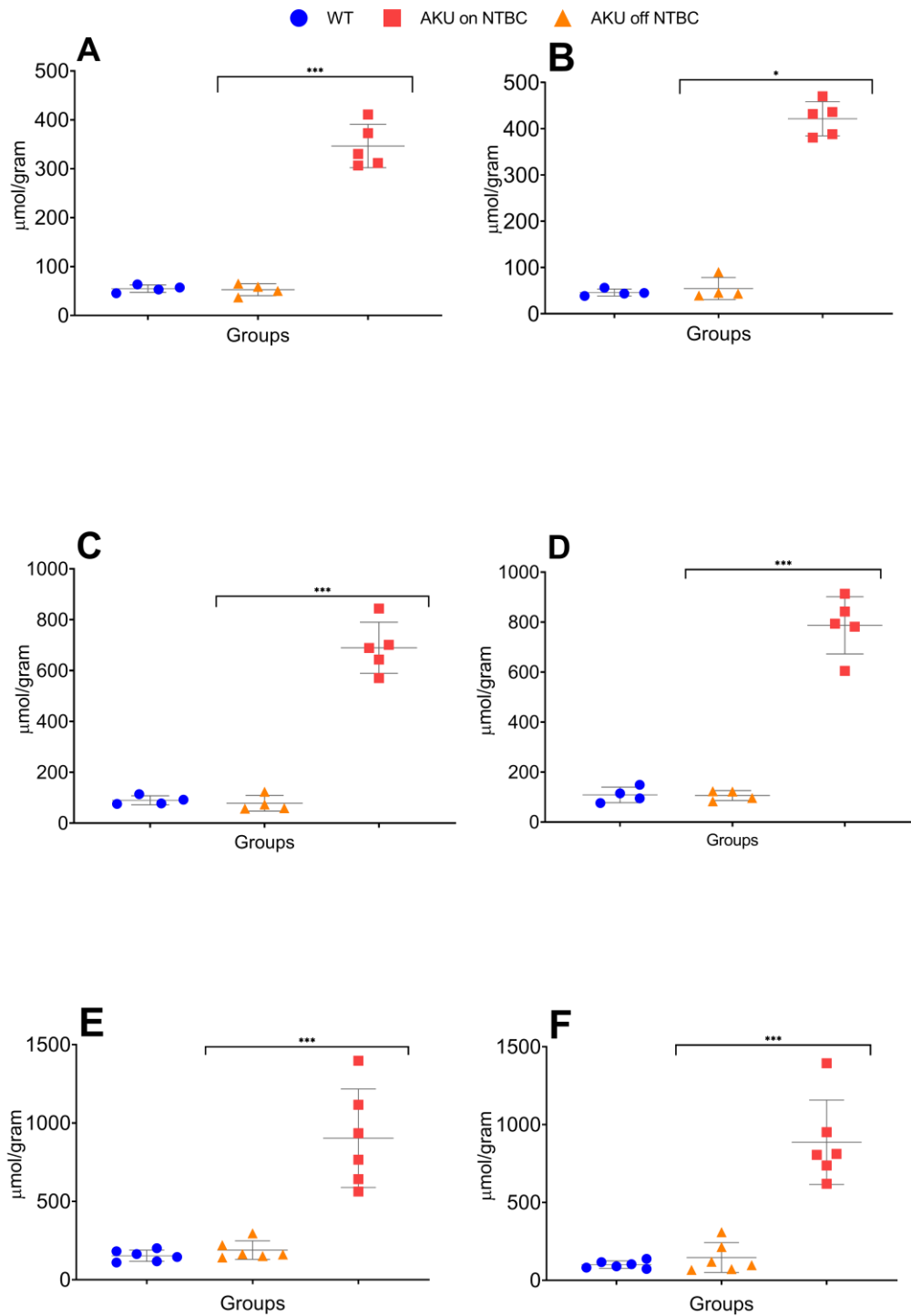
Figure 1: Tyrosine concentrations in murine tissue homogenates. (A) femur bone, (B) brain, (C) heart, (D) kidney, (E) liver, (F) quadriceps muscle. Line and error bars are mean ± SD.

*$P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Unpaired two-tailed t-test was used to compare data between the AKU on and off NTBC groups except for the brain homogenates where two-tailed Mann-Whitney test was used.
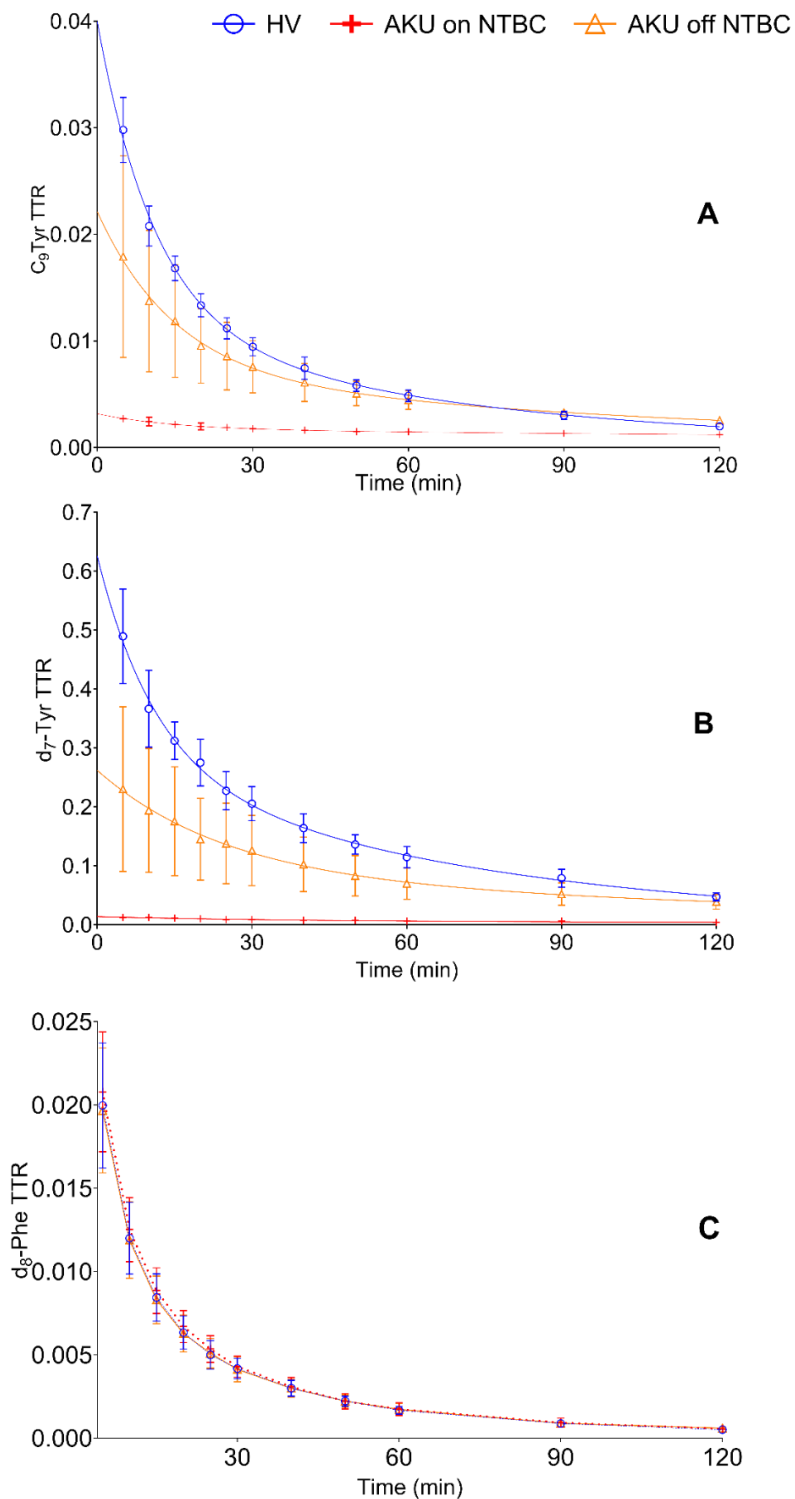
Figure 2: Comparison of tyrosine and phenylalanine enrichment versus time following a bolus injection of 105 µmol L-$[^{13}C_9]$tyrosine and 270 µmol of L-$[d_8]$phenylalanine in each of the three groups. A: comparison of L-$[^{13}C_9]$tyrosine enrichment versus time. C$_9$TyrTTR is

[L-[^{13}C$_9$]tyrosine]/[median of native tyrosine]; B: comparison of L-[d$_7$]tyrosine enrichment versus time, d$_7$TyrTTR is [L-[d$_7$]tyrosine]/[median of native tyrosine]; C: comparison of L-[d$_8$]phenylalanine enrichment versus time. d$_8$-PheTTR is [L-[d$_8$]phenylalanine]/ [median of native phenylalanine]. HV: healthy volunteers, AKU on NTBC: AKU patients on NTBC group, AKU off NTBC: AKU patients off NTBC group.
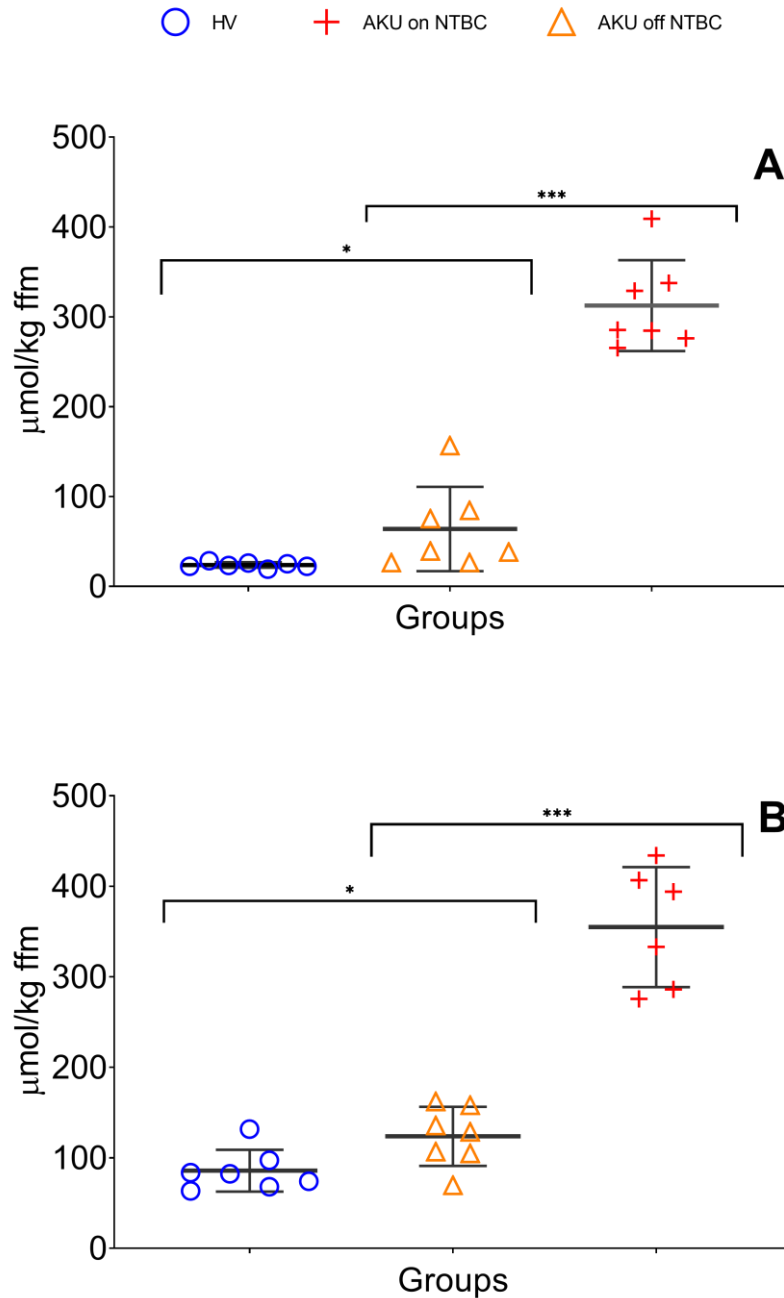
Figure 3: Estimated tyrosine pool size across the study groups. A: estimated pool size of the extracellular tyrosine, B: estimated pool size of the intracellular tyrosine HV: healthy volunteers group, AKU on NTBC: AKU patients on NTBC group, AKU off NTBC: AKU patients off NTBC group. Line and error bars are mean ± SD. Comparison of data in the AKU on NTBC group against AKU off NTBC group was by paired two-tailed *t*-test.

Comparison of data in the HV group against AKU off NTBC group was by unpaired two-tailed t-test. * $P < 0.05$, *** $P < 0.001$.

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