Pathophysiological and therapeutic studies in Alkaptonuria

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Medicine

by

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This work is dedicated to Malack

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Abstract

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Dr Milad Khedr

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) which leads to increased homogentisic acid in body fluids and tissues. This results in the formation of a melanin-like pigment in a process called ochronosis. Ochronosis is the main pathophysiological process in AKU. It changes the mechanical properties of tissues and gives rise to the various manifestations of AKU. 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.

The management of AKU has been mainly supportive without addressing the underlying pathophysiological mechanisms. However, nitisinone (NTBC), a competitive reversible inhibitor of the hydroxyphenylpyruvic acid dioxygenase enzyme (HPPD, EC 1.13.11.27), can decrease urinary excretion of homogentisic acid by 98.8%. NTBC is already licensed and has been used for over two decades for the treatment of hereditary tyrosinaemia type 1 (HT-1) in children. Since 2012, NTBC has been used off-label in the NHS England designated National Alkaptonuria Centre (NAC), at the Royal Liverpool University Hospital. Data from the NAC cohort suggest that NTBC reverses ochronosis. In October 2020 and following the positive clinical outcomes of SONIA 2, NTBC has been licensed for the treatment of AKU in adults. 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 which has been associated with cognitive impairment in children, skin rash, vitiligo, cataract, and potentially sight-threatening keratopathy. These serious risks call for an evaluation of the wider impact of NTBC on the tyrosine pathway. It is hypothesised that NTBC increases the tyrosine pool size and concentrations in tissues.

An analytical method has been developed to measure tyrosine and phenylalanine tracers. 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 in AKU patients and also measure concentrations of tracers in AKU mice, healthy volunteers and AKU patients.

NTBC significantly increased the tyrosine pool size in humans. It also increased the tyrosine concentrations in murine tissues, suggesting that NTBC increases tyrosine not just in serum but also in tissues (i.e., acquired tyrosinosis). This study provides, for the first time, the experimental proof for the magnitude of NTBC-related acquired tyrosinosis that should be overcome to ensure the safe use of NTBC in AKU.

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ABBREVIATIONS

AKU	Alkaptonuria
AKUSSI	AKU Severity Score Index
ANOVA	Analysis of Variance
AUC	Area Under the Curve
BMI	Body Mass Index
BQA	Benzoquinone acetic acid
BW	Body Weight
EC	Extracellular
EMA	European Medicines Agency
FFM	Fat Free Mass
FFM%	Fat Free Mass as a percentage of body weight
HGA	homogentisic acid
HGD	Homoentisate 1,2-dioxygenase
HPLA	4-hydroxyphenyllactic acid
HPLC	high performance liquid chromatography
HPPA	4-hydroxyphenylpyruvic acid
HPPD	4-hydroxyphenylpyruvic acid dioxygenase
HT-1	Hereditary Tyrosinaemia type I
HT-2	Hereditary Tyrosinaemia type II
HT-3	Hereditary Tyrosinaemia type III
IC	Intracellular
LAT1	Large Neutral Amino Acid Transporter 1
LC-MS/MS	Liquid Chromatography- Tandem Mass Spectrometry
NAC	National Alkaptonuria Centre
NICU	Neonatal Intensive Care Unit
NTBC	2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione; nitisinone
PAL	Phenylalanine Ammonia Lyase
Phe	Phenylalanine
PKU	Phenylketonuria
SOFIA	Subclinical Ochronotic Features In Alkaptonuria
SONIA 1	Suitability Of Nitisinone In Alkaptonuria 1
SONIA 2	Suitability Of Nitisinone In Alkaptonuria 2
TAL	Tyrosine Ammonia Lyase
TBW	Total Body Water
TTR	Tracer to Tracee Ratio
Tyr	Tyrosine
WB-PB	Whole Body Protein Breakdown
WB-PS	Whole Body Protein synthesis
WT	Wild Type

1.0 GENERAL INTRODUCTION

Alkaptonuria (AKU) is a rare inborn error of tyrosine metabolism (OMIM 203500). It is inherited in an autosomal recessive fashion. It is characterised by high circulating homogentisic acid (HGA) due to a genetic defect in the enzyme homogentisate dioxygenase (HGD, EC 1.13.11.5) (Phornphutkul et al., 2002). Ochronosis is the main pathogenetic event in AKU and it results from the conversion of HGA to a melanin-like HGA-pigment (Figure 1), that has affinity for connective tissues, especially cartilage (Zannoni et al., 1969, Ranganath et al., 2019). Ochronosis leads to arthritis, valvular heart disease, nephrolithiasis, and tendon ruptures (O'Brien et al., 1963, Ranganath et al., 2019).

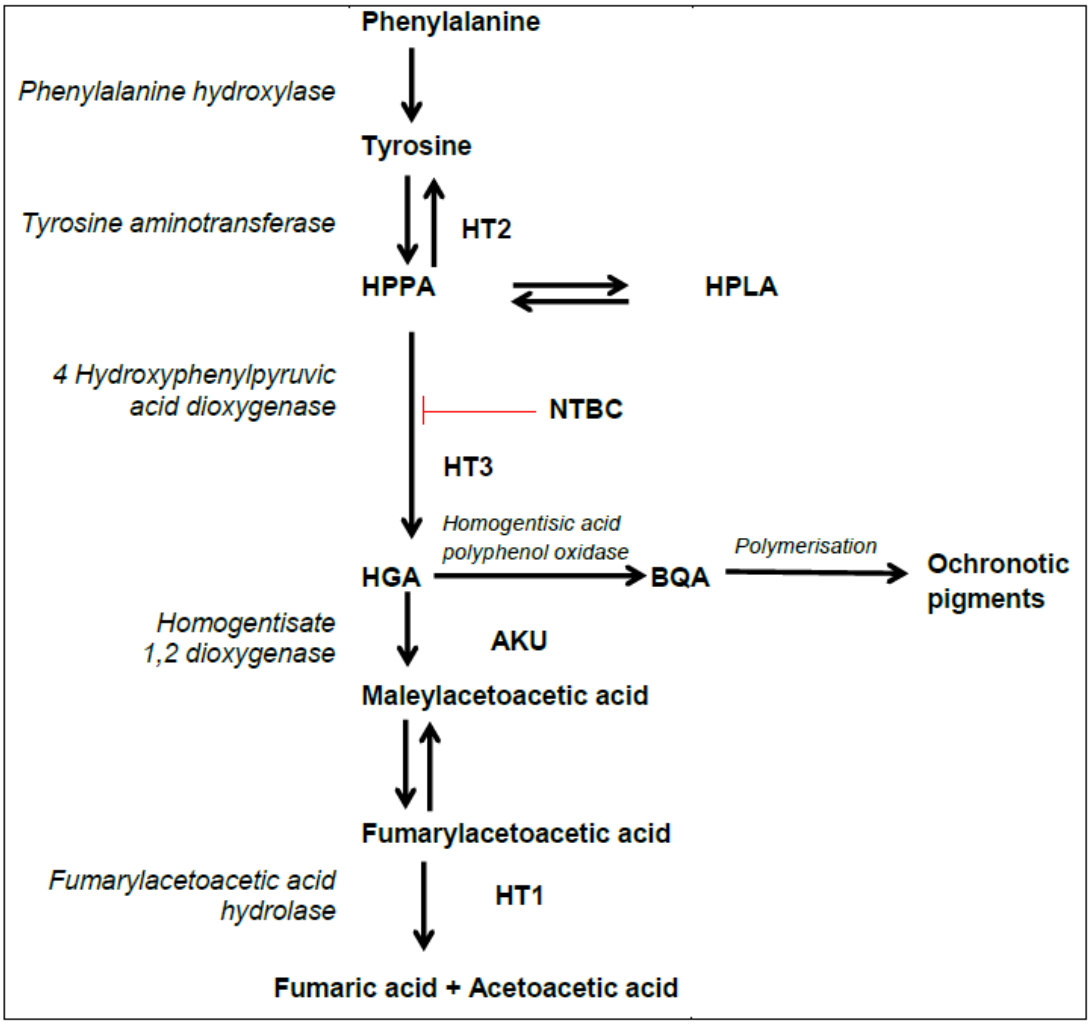

Figure 1: Tyrosine degradation pathway.
HPPA: hydroxyphenylpyruvic acid, HPLA: hydroxyphenyllactic acid, HGA: homogentisic acid, NTBC: nitisinone, AKU: alkaptonuria, HT1: hereditary tyrosinaemia type 1, HT2: hereditary tyrosinaemia type 2, HT3: hereditary tyrosinaemia type 3. BQA: Benzoquinone acetic acid.

1.1 History

The earliest evidence of ochronosis was identified in Harwa, an Egyptian mummy who worked as a granary custodian who lived around 1500 B.C (Stenn et al., 1977). The term ‘Alcapton’ was first used to describe a reducing substance found in the urine of a female patient (Boedeker, 1859) . This was later confirmed to be homogentisic acid (Wolkow and Baumann, 1891). Sir Archibald Garrod discovered that AKU follows Mendelian inheritance (Garrod, 1908).

1.2 Genetics of alkaptonuria

The HGD gene maps to chromosome 3q, which encodes for the HGD enzyme (Pollak et al., 1993). Homozygous or compound heterozygous mutations cause decreased function or complete inactivation. AKU results from homozygous or compound heterozygous loss-of-function mutation of the HGO gene (Fernández-Cañón et al., 1996). Nearly two thirds of these are missense mutations (Davison and Norman, 2023).

As of January 2023, there are 254 known HGD gene variants (Zatkova et al., 2020). These are curated on an online HGD mutation database (http://hgddatabase.cvtisr.sk/) (Zatkova et al., 2012).

1.3 Epidemiology

AKU has an estimated prevalence of 1 in 250,000 to 1 million (Phornphutkul et al., 2002). A six year screening programme in Slovakia which tested over half a million new-born, demonstrated the highest prevalence of 1 in 19000(Sršeň and Varga, 1978, Sršeň et al., 1978). High prevalence has also been reported in the Dominican republic(Milch, 1960), in Jordan (Al-Sbou and Mwafi, 2012, Al-Sbou et al., 2012) and in India (Sakthivel et al., 2014). Notably,

the frequency of AKU in Wales has been reported to be 1 in 44,800 over seven year period between 1970 and 1977(Harper and Bradley, 1978).

1.4 Clinical manifestations

Although the biochemical deficit is present since birth, AKU patients are largely asymptomatic until the third decade of life, except for constant dark urine (Zatkova et al., 2020). The Subclinical Ochronotic Features in Alkaptonuria study (Cox et al., 2019) has demonstrated eye pigmentation in a 22-year-old patient and biopsy evidence of ear pigmentation in a 20-year-old patient. This supports the view that ochronosis starts earlier in life than the major clinical signs.

AKU is a multi-system disease (Helliwell et al., 2008). To capture disease severity, progression and response to therapy, a scoring system was developed (Ranganath and Cox, 2011, Cox and Ranganath, 2011), the AKU Severity Score Index (AKUSSI). AKUSSI was used to report on the outcomes of NTBC use in the NAC (Ranganath et al., 2018). Features of the AKUSSI are summarised in Table 1. A more comprehensive version of this scoring system was used in Suitability of Nitisinone in Alkaptonuria 2 study (SONIA 2) to assess disease progression and response to NTBC (Ranganath et al., 2020d). This was further modified to produce the “flex-AKUSSI” which would be valuable in resources-limited settings (Cant et al., 2022).

AKU can be very variable phenotypically. Zatkova et al. (2022) have found significant variation in AKU phenotype in 24 paired siblings /groups in SONIA 2. Furthermore, accumulation of HGA in AKU patients appears to be more related to protein intake rather than the genotype (Ascher et al., 2019).

While AKU does not usually affect lifespan (Srsen et al., 1985), the quality of life worsens with age (Phornphutkul et al., 2002), mainly due to progressive arthropathy (Zatkova et al., 2020).

Table 1: Summary of Alkaptonuria Severity Score Index (AKUSSI).

CT-BMD: Computed tomography bone densitometry, 18FPET-CT: Positron emission tomography–computed tomography scan. Eye pigmentation: 1, 2 and 3 points for slight, moderate, and marked conjunctival pigmentation and 4, 6 and 8 points for scleral pigmentation. Ear pigmentation: 2 and 4 points for slight and marked pigmentation.

Feature	Test	Feature	Test
Clinical AKUSSI			
Eye ochronosis			
Right eye nasal	Photograph	Left eye nasal	Photograph
Right eye temporal	Photograph	Left eye temporal	Photograph
Ear ochronosis:			
Right ear	Photograph	Left ear	Photograph
Prostate stones: (4 per episode)	Ultrasound/history	Kidney stones (4 per episode)	Ultrasound/history
Osteopenia: (4)	CT-BMD	Hearing impairment	History
Aortic sclerosis: (6); aortic stenosis: mild, moderate, severe (8, 10, 12)	Echocardiography		
Joint AKUSSI			
Fracture (8 per fracture)	History	Muscle rupture (8 per rupture)	History
Ligament rupture (8 per rupture)	History	Tendon rupture (8 per rupture)	History
Joint pain score: (1 for each large joint area; 14 large joint areas) History			History
Scintigraphic scan joint score: (2 for each large joint; 14 large joint areas)			18FPET-CT
Number of arthroscopies: (2 each) History			History
Number of joint replacements: (4 each) History			History
Spine AKUSSI			
Spinal pain score: (2 each for cervical, thoracic, lumbar, sacroiliac)			History
Scintigraphic scan spine score: (6 areas; 4 points for each area; pubic symphysis,costochondral, lumbar, thoracic, cervical, sacroiliac)			18FPET-CT
Kyphosis (4), Scoliosis (4)			X-ray
All AKUSSI (Clinical+ Joint+ Spine)			

1.4.1 Ocular manifestations

Ocular signs were present in two thirds of the patients in one review (Lindner and Bertelmann, 2014). Conjunctival (Chévez Barrios and Font, 2004) and scleral (Ranganath et al., 2020c) pigmentations were the most common ocular features. Cataract was not widely recognised as a common feature in AKU until recent review of NAC cohort revealed that 76% of patients had cataract as baseline. This is thought to be due to lifelong exposure to HGA which mediates oxidative stress process which eventually leads to cataract formation. Accumulation of ochronotic pigment in the lens has been implicated but not proved. Prevalence of cataract appears to increase further following NTBC therapy, presumably due to NTBC-induced tyrosinaemia (Ahmad et al., 2022). Other ocular problems that have been reported include glaucoma, acute recurrent anterior uveitis, central vein occlusion and progressive astigmatism (Lindner and Bertelmann, 2014).

1.4.2 ENT manifestations

ENT signs or symptoms were present in 90% of AKU patients. The most common ENT findings are discolouration of the pinna and cerumen. Pigmentations of the tympanic membrane, the middle ear and the nasal septum have also been reported. In nearly a third of the cases reported, there was mild hearing loss. This was predominantly, high frequency sensorineural loss (Steven et al., 2015, Al-Shagahin et al., 2019). Pigmentation of the laryngeal cartilage has also been reported (Helliwell et al., 2008).

1.4.3 Other connective tissues

Findings include thickened Achilles tendons, rupture of tendons, ligaments, and muscles; joint effusions and synovitis have been reported too

(Phornphutkul et al., 2002, Manoj Kumar and Rajasekaran, 2003, Alajoulin et al., 2015, Jiang et al., 2019).

1.4.4 Spondyloarthropathy

Low back pain is very common and has been reported in 94% of AKU patient before the age of 40 in a US cohort (Phornphutkul et al., 2002). In the NAC cohort, there was evidence of uptake on positron emission tomography computerised tomography (PET-CT) in all patients. However, pain was reported in only 85.7% suggesting that spondylo-arthropathic disease process takes place before symptoms appear (Ranganath et al., 2021d). In the latter cohort, pain affected joints in the following descending order: knees, shoulders, hips, ankle, feet, elbows, and wrists. In the spine, pain was mostly reported on the lumbar region (nearly 80%) followed by cervical (60%), thoracic (~40%), and sacroiliac (~20%) regions.

Arthroplasty in the NAC cohort was reported in 36.8% of patients, with nearly a third of the patients having multiple arthroplasties. Joint replacements were commonly reported in the knees, followed by the hips and the shoulders. (Ranganath et al., 2021b).

1.4.5 Metabolic bone disease

Bone turnover is increased in AKU and osteoporosis was reported in 24.7% in the NAC cohort, while fractures were prevalent at 44.8% (Ranganath et al., 2020a).

1.4.6 Cardiovascular manifestations

Prevalence of cardiovascular manifestations was found to be around 40% (Phornphutkul et al., 2002, Pettit et al., 2011). Features include aortic dilation and cardiac valves involvement with aortic valve disease being more

predominant. Phornphutkul et al. (2002) have reported CT- evidence of coronary artery calcifications in 50 % of AKU patients by the age of 59 years, although this had no correlation to elevated serum cholesterol concentrations. Within the NAC cohort, the prevalence of aortic stenosis was 22.2% while 6.2% of patients have undergone aortic valve replacement. Data suggests that NTBC slows progression of aortic stenosis (Ranganath et al., 2021c).

1.4.7 Urolithiasis

Prostate stones were found in a nearly third of the male participants in one case series (Phornphutkul et al., 2002).They can be quite extensive compared to non-AKU related cases (Masoud et al., 2017). Half of the AKU patients have history of renal stones by the age of 64 years (Introne WJ, 2003).

1.4.8 Thyroid disorders

Primary hypothyroidism was found to be over 4 times as prevalent compared to the general population. It is postulated that HGA accumulation interferes with thyroid hormones production. Notably, prevalence of thyroid nodules and cancers was comparable to the general population (Avadhanula et al., 2020).

1.4.9 Acute haemolysis and methaemoglobinaemia

Kidneys are essential in eliminating HGA, predominantly through tubular secretion, complemented by glomerular filtration (Ranganath et al., 2020b).

To date, twelve cases of haemolysis and methaemoglobinaemia have been reported. It is postulated that the worsening of renal function along with HGA elevation and oxidative stress lead to this lethal complication (Davison et al., 2016, Davison et al., 2020).

1.4.10 Neurological manifestations

Parkinson disease was reported to be nearly 20 times as prevalent in AKU patients compared to general population (Ranganath et al., 2023). This is thought to be related to life-time exposure to HGA. Dura mater pigmentations have been reported (Liu and Prayson, 2001, Helliwell et al., 2008).

1.4.11 Secondary amyloidosis

Millucci et al. (2012) have reported the presence of serum amyloid A (SAA) in several AKU tissues. It is postulated that HGA related oxidative stress response leads to chronic inflammation which results in secondary amyloidosis in AKU (Millucci et al., 2015).

1.5 Diagnosis

AKU is usually diagnosed by measuring HGA in urine (Ranganath et al., 2013). The latter is mostly done utilising chromatographic techniques (Davison et al., 2019a). Genetic testing has role in cascade testing in family members and pre-conception counselling.

1.6 Management

1.6.1 Supportive management

Pain relief, acupuncture and physiotherapy have been used as supportive management in AKU. Vitamin C, chondroitin and glucosamine are of unproven benefit in disease modification.

1.6.2 Disease modifying therapy

NTBC is a competitive reversible inhibitor of the hydroxyphenylpyruvic acid dioxygenase enzyme (HPPD, EC 1.13.11.27) (Lock et al., 1998), and can decrease urinary excretion of homogentisic acid in a dose-dependent fashion-up to 99.7% in SONIA 2 (Ranganath et al., 2016, Ranganath et al., 2020d).It

has been shown to completely prevent ochronosis in a mouse model of AKU (Preston et al., 2014, Keenan et al., 2015). NTBC is already licensed and has been used for over two decades for the treatment of hereditary tyrosinaemia type 1 (HT-1) in children (Lindstedt et al., 1992). Since 2012, NTBC has been used off-label in the NHS England designated National Alkaptonuria Centre (NAC), at the Royal Liverpool University Hospital. Data from the NAC cohort suggest that NTBC reverses ochronosis (Ranganath et al., 2018, Ranganath et al., 2020c). More recently, the SONIA 2 study have showed that NTBC decreases ochronosis and slows disease progression in AKU (Ranganath et al., 2020d). This has led to the approval of NTBC as the first disease modifying therapy for AKU in adults by the European Medicine Agency (EMA, 2020).

Subclinical Ochronotic Features In Alkaptonuria (SOFIA) study has demonstrated evidence of ochronosis in ear biopsies from the age of 20 (Cox et al., 2019). Since biochemical deficit is present since birth, treatment with NTBC to modify the course of AKU seems logical. A paediatric SOFIA study is planned and will seek to establish the earliest age for detecting ochronosis.

1.6.2.1 *NTBC-induced hypertyrosinaemia*

The tyrosinaemia that occurs during NTBC treatment resembles hereditary tyrosinaemia type 3. Adverse effects known to be associated with tyrosinaemia include dermal toxicity (Meissner et al., 2008, Stewart et al., 2014), vitiligo (Ranganath et al., 2021a), and cataract (Ahmad et al., 2022). In Children with HT-1 who have received NTBC, impairment of neurocognitive function has been reported (Macsai et al., 2001, Thimm et al., 2012, Bendadi et al., 2014, van Ginkel et al., 2016b, García et al., 2017, van Vliet et al., 2019). In AKU, recent studies have demonstrated that the use of NTBC 2 mg was not

associated with impairment in cognitive function or worsening of depression (Davison et al., 2018a, Davison et al., 2022a)

Tyrosinaemia related corneal lesions are reported to be less than 9% in children with hereditary tyrosinaemia type 1 (HT-1) who are treated with NTBC (Holme and Lindstedt 1998; Gissen et al. 2003). Schauwvlieghe et al. (2013) have reported tyrosine keratopathy in a 16-year-old male who received NTBC for HT-1. Although corneal symptoms resolved after stopping NTBC, tyrosine crystals were still detectable in the corneal epithelium using confocal microscopy and slit lamp examination.

In SONIA2, NTBC 10 mg dose was used and 14% of the treated patients developed keratopathy. Affected individuals were predominantly males (Ranganath et al., 2020d, Ranganath et al., 2022c). Keratopathy has also been reported in AKU patients treated with 2 mg dose (Introne et al., 2011, Stewart et al., 2014, Khedr et al., 2018).

1.7 Use of stable isotopes to study the impact of NTBC on tyrosine degradation:

For over than 75 years, stable isotopes have been instrumental in unlocking the dynamics of metabolism in animals and humans (Beysen et al., 2019). This undoubtedly has provided invaluable insights into the metabolism of amino acids. Stable isotopes have replaced radioactive isotopes in metabolic research because they are safe, specific and behave metabolically in similar ways to native amino acids.

The use of stable isotopes in metabolic research has predominantly adopted a primed constant infusion approach (Wilkinson, 2018) which allows for reaching a ‘steady state’. Recently, there has been a shift towards a bolus

injection approach as it is more convenient for patients, less labour intensive and cheaper. Studies have looked at the bolus injection approach in animals (Zhang et al., 2002) and humans (Tang et al., 2007, Tuvdendorj et al., 2014, Mason et al., 2017). For the determination of the pool size, it is possible to use a single bolus injection study design (Wolfe and Chinkes, 2004). The tracer infusion protocol in this study is based on the work of Mason et al (Mason et al., 2017).

Phenylalanine hydroxylation to tyrosine is reduced in NTBC-treated patients. This could be due to negative feedback from increased tyrosine and would limit the flux of tyrosine down its degradation pathway. This has implications in terms of dietary manipulation as restricting phenylalanine alone in NTBC treated patients will not help in reducing the burden of hypertyrosinaemia. In NTBC treated AKU mice, restriction of phenylalanine alone did not lower serum tyrosine significantly; whereas restriction of both phenylalanine and tyrosine led to a significant does- dependent reduction of serum tyrosine (Hughes et al., 2020). A review of the literature, as regards phenylalanine hydroxylation, reveals a heterogeneity in the studies performed in terms of participants demographics, stable isotopes used and infusion protocols (Clarke and Bier, 1982, Thompson et al., 1989, Cortiella et al., 1992, Marchini et al., 1993, Mason et al., 2017) (summarised in Table 2). This work has not been done before in AKU.

Protein turnover: Data from the NAC suggest that AKU patients are in a state of protein energy malnutrition at presentation before starting NTBC (Judd et al., 2020). Following NTBC therapy, low protein diet results in loss of muscle mass (Ranganath et al., 2022b). Since managing NTBC-induced

hypertyrosinaemia involves a degree of dietary restriction of protein, it is of interest to assess protein synthesis and breakdown in this group of patients. Although this study did not intend to assess protein synthesis and breakdown, its design and use of stable isotopes has enabled the estimation of both. Historically continuous infusions of tracers were required. However, recent studies showed that pulse methods can be used reliably to study protein metabolism (Engelen et al., 2019). This is discussed further later in this thesis (fourth chapter).

1.8 Study aims and objectives

1.8.1 Aims

• To characterise and quantify the effects of nitisinone on the tyrosine pathway in AKU mice.

• To characterise and quantify the effects of nitisinone on the tyrosine pathway in AKU patients and compare them to healthy volunteers who did not receive nitisinone.

1.8.2 Objectives

1.8.2.1 *Primary objectives*

- To develop and validate a simple LC-MSM method for measurements of tyrosine and phenylalanine stable isotopes.
- To measure tissue homogenates concentrations of the tyrosine pathway compounds in AKU mice before and after nitisinone.
- To estimate the tyrosine pool size in AKU patients before and after nitisinone treatment.
- To estimate the tyrosine pool size for tyrosine in healthy volunteers.

1.8.2.2 *Secondary objectives*

- To assess protein turnover in AKU mice before and after nitisinone treatment.
- To estimate the extent of phenylalanine hydroxylation to tyrosine in healthy volunteers.
- To estimate the extent of phenylalanine hydroxylation to tyrosine in AKU patients.
- To assess protein turnover in AKU patients and healthy volunteers.

Table 2: A comparison of the total bolus/ prime doses given in previous studies with the current study.
*A fixed dose was given. For illustration purpose only, the equivalent weight adjusted dose calculated here from the fixed dose for a presumed 50 kg subject.

Study	**Tyrosine tracer Bolus Dose** (µmol/kg)	**Total tyrosine bolus dose for a 50 Kg participant** (µmol)	**Phenylalanine tracer Bolus Dose** (µmol/kg)	**Total Phenylalanine bolus dose for a 50 Kg participant** (µmol)
Clarke and Bier 1982	2.7 µmol/kg	135	No bolus dose given	-
Thompson et al 1989	1.8 µmol/kg	90	2.9 µmol/kg	145
Cortiella et al 1992	5.4 µmol/kg	270	3.1 µmol/kg	155
Marchini et al 1993	4.3 µmol/kg	215	5.8 µmol/kg	290
Castillo et al 1994	6.1 µmol/kg	305	5.4 µmol/kg	270
Sanchez et al 1995	5.5 µmol/kg	275	4.5 µmol/kg	225
Clark et al 1996	2.5 µmol/kg	125	4 µmol/kg	200
Roberts et al 1998	3.6 µmol/kg	180	15.6 µmol/kg	780
Roberts et al 2001	3.6 µmol/kg	180	15.6 µmol/kg	780
De-Betue et al 2011	2.5 µmol/kg	125	4.4 µmol/kg	220
Meesters et al 2009	1.47 µmol/kg	73.5	3.65 µmol/kg	182.5
Moran et al 2001	2.8* µmol/kg	140	3.6 µmol/kg	180
This study	2.1* µmol/kg	105	3.8* µmol/kg	289 µmol

2.0 DEVELOPMENT AND VALIDATION OF LC-MS/MS METHOD FOR THE MEASUREMENT OF TYROSINE AND PHENYLALANINE STABLE ISOTOPES

2.1 Abstract

Background: AKU is an ultra-rare disease that is inherited in an autosomal recessive fashion. It is caused by deficiency of homogentisate 1,2 dioxygenase (HGD) enzyme activity which results in HGA accumulation in tissues and ochronosis related morbidity. NTBC decreases serum and urine HGA but causes tyrosinaemia. In this study, stable isotopes methodology was deployed to further examine the tyrosine degradation pathway in AKU patients and mice before and after NTBC, as well as in healthy volunteers. To enable this, it was necessary to develop a robust and reliable analytical method to measure the concentrations of tyrosine and phenylalanine stable isotopes used in the study.

Methods: Using matrix matched calibrators, an existing LC-MS/MS method was modified to enable measurements of L-[$^{13}C_9$]tyrosine, L-[d_7]tyrosine and L-[d_8]phenylalanine in serum, urine. This was achieved following 1 in 10 dilution with deionised 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 × 100 mm, 3 µm) using an Agilent 6490 triple quadrupole LC-MS/MS with Jet-Stream electrospray ionisation equipped with an Agilent 1290 infinity pump and autosampler. Assay was validated in accordance with international guidelines across serum, urine, and aqueous matrices. The analytical range was sufficient to analyse all the study samples across the three matrices. The assay had good accuracy and

precision. L-[$^{13}C_9$]tyrosine, L-[d_7]tyrosine and L-[d_8]phenylalanine were stable after 24 hours storage at 20°C, 4°C and −20°C. No carry over was observed.

Conclusion: a simple and reliable LC-MSMS method was validated for measurement of L-[$^{13}C_9$]tyrosine, L-[d_7]tyrosine and L-[d_8]phenylalanine in matrices obtained from humans and mice. The method has a short cycle time, requires a small sample volume and no derivatisation. The assay meets the international validation criteria and the requirements for the study.

2.2 Introduction

Stable isotopes have been used for over three decades in studying human metabolism. Because phenylalanine is an essential amino acid (Womack and Rose, 1934), that is disposed of by conversion to tyrosine (Thompson et al., 1989) phenylalanine and tyrosine stable isotopes have been used extensively to study whole body protein synthesis and breakdown in humans.

Historically, gas chromatography mass spectrometry (GC-MS) has been used to measure the enrichment of tyrosine and phenylalanine stable isotopes (Clarke and Bier, 1982, Matalon et al., 1982, Thompson et al., 1989, Krempf et al., 1990, Marchini et al., 1993, Castillo et al., 1994, Kilani et al., 1995, Tessari et al., 1999, Møller et al., 2000, Boirie et al., 2004). Recently the application of liquid chromatography-tandem mass spectrometry (LC-MS/MS) for the analysis of tyrosine and phenylalanine stable isotope enrichments has become popular (Engelen et al., 2000, Engelen et al., 2003, van Eijk et al., 2007, Meesters et al., 2009, Mason et al., 2017).

LC-MS/MS methods for the measurements of native tyrosine and phenylalanine in serum and urine have already been published by the Liverpool group (Hughes et al., 2014, Hughes et al., 2015). This study aimed to modify the existing methods to enable measurement L-[$^{13}C_9$]tyrosine, L-[d_7]tyrosine and L-[d_8]phenylalanine in both serum and urine samples. This enabled further examination of the tyrosine degradation pathway in alkaptonuria patients.

Rationale for measurements of the above three tracers: estimation of tyrosine pools utilises stable isotopes techniques that are used for studying

protein turnover. Phenylalanine is an indispensable amino acid which is exclusively disposed of by conversion to tyrosine (Thompson et al., 1989). In this study, L-[d_8]phenylalanine is hydroxylated to L-[d_7]tyrosine; and measurement of both is necessary to estimate the whole body production of phenylalanine. The measurement of L-[$^{13}C_9$]tyrosine is necessary for calculating the whole body production of tyrosine. The inclusion of L-[$^{13}C_9$]tyrosine strengthen the robustness of calculations used to estimate protein turnover and tyrosine pool sizes (Engelen et al., 2019).

2.3 Materials and methods

2.3.1 Chemicals and materials

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). Formic acid was obtained from Biosolve. LC–MS grade methanol was obtained from Sigma Aldrich (Dorset, UK). Water was purified in-house by DIRECT-Q 3UV Millipore water purification system. Oxygen free nitrogen was supplied by a Peak nitrogen generator. All dilutions and sample preparation were performed in glass.

2.3.2 Instrumentation and operating conditions

The prepared samples were analysed using an Agilent 6490 Triple Quadrupole mass spectrometer with Jet-Stream® electrospray ionisation (ESI-MS/MS) equipped with an Agilent 1290 infinity UHPLC pump and 1290 multi-sampler. Mass Hunter software package (version B.06.00) was used for qualitative and quantitative analysis of data. The optimal operating conditions are summarised in Table 3.

Table 3: A summary of the optimum operating ESI conditions.

ESI Parameter	
Gas Temp (°C)	150
Gas Flow (L/min)	17
Nebulizer (psi)	40
Sheath Gas Heater (°C)	320
Sheath Gas Flow (L/min)	12
Capillary (V)	3500
Ion Funnel Parameters	
High Pressure RF (V)	110
Low Pressure RF (V)	60

Chromatographic conditions: An Atlantis dC18 column (100 mm × 3.0 mm, 3 µm, Waters) was used to achieve chromatographic separation. The column temperature was maintained at 35°C. The mobile phase gradient was optimised by changing the percentage composition of mobile phase A and B over a five-minute time window. The gradient used is outlined in Table 4. Mobile phase (A) was composed of deionised water plus 2.0mmol/L ammonium acetate and 0.1% formic acid, and (B) methanol plus 2.0mmol/L ammonium acetate and 0.1% formic acid. Buffer A is water (1L and 1mL formic acid), Buffer B is MeOH (1L and 1mL formic acid), Wash is 50:50 of A&B. Injected sample volume was 2 µL and the total run time was 7 minutes.

Table 4: Mobile phase gradient used for optimisation chromatography. Buffer A is water, Buffer B is methanol, Wash is 50:50 of A&B.

	Time	**A**	**B**	**Flow**
1	0.50 min	80.00 %	20.00 %	0.4 mL/min
2	2.50 min	10.00 %	90.00 %	0.4 mL/min
3	3.50 min	10.00 %	90.00 %	0.4 mL/min
4	3.60 min	0.00 %	100.00 %	0.4 mL/min
5	4.90 min	0.00 %	100.00 %	0.4 mL/min
6	5.00 min	80.00 %	20.00 %	0.4 mL/min

2.3.3 Preparation of standard solutions

Super-stock standard solutions of L-[d_7]tyrosine, L-[$^{13}C_9$]tyrosine and L-[d_8]phenylalanine were prepared: L-[d_7]tyrosine and L-[$^{13}C_9$]tyrosine in 0.5N sulphuric acid (H2SO4) and L-[d_8]phenylalanine in deionised water at concentrations of 10 mmol/L. These were stored at -80°C.

A superstock of L-[ring-$^{13}C_6$]tyrosine and L-[ring-$^{13}C_6$]phenylalanine internal standards at concentrations of 10 mmol/L was prepared in 0.5N sulphuric acid and deionised water. These were stored at -80°C.

2.3.4 Preparation of calibrators and controls

2.3.4.1 *Preparation of calibrators and controls- urine assay*

Because all urine samples from AKU patients were acidified (5N sulphuric acid), acidified urine pools for dilution were used. Intermediate stocks were prepared at 10 times the final required concentrations. These were diluted, at 1 in 10 using acidified urine, to create combined calibrators solution with concentrations ranging from 200 to 800 nmol/L for L-[d_7]tyrosine, 40 to 3000 nmol/L for L-[$^{13}C_9$]tyrosine and 50 to 3000 nmol/L for L-[d_8]phenylalanine.

Internal quality controls (IQC) were prepared from separate pools in a similar fashion and their concentrations were chosen carefully to cover the analytical range of the assay. L-[d_7]tyrosine IQC values were 250-650 nmol/L, L-[$^{13}C_9$]tyrosine and L-[d_8]phenylalanine IQC values were 75-1000 nmol/L.

The sample pre-diluent consisted of a combined internal standard solution combining final concentrations of 50nmol/L L-[ring-$^{13}C_6$]tyrosine, 40 nmol/L L-[ring-$^{13}C_6$]phenylalanine, and 0.1% formic acid (v/v) in deionised water. This

was used, at 1 in 10 dilution, to dilute samples, IQC and calibrators as a preparation for the assay.

2.3.4.2 *Preparation of calibrators and controls- serum assay*

Steroid depleted serum (BBI Solutions, SF236-7) was used ensure matrix matched calibration. Intermediate stocks were prepared at 10 times the final required concentrations. These were diluted, at 1 in 10 using steroid depleted serum, to create combined calibrators solution with concentrations ranging from 20 to 1200 nmol/L for L-[d_7]tyrosine, 20 to 10,000 nmol/L for L-[$^{13}C_9$]tyrosine and L-[d_8]phenylalanine.

Internal quality controls (IQC) were prepared from separate pools in a similar fashion and their concentrations were chosen carefully to cover the analytical range of the assay. L-[d_7]tyrosine IQC values were 35-900 nmol/L, L-[$^{13}C_9$]tyrosine and L-[d_8]phenylalanine IQC values were 35-7000 nmol/L.

To ensure stabilisation of HGA in study samples, all serum samples, calibrators and IQC were subjected to acidification and deproteinization using 60% perchloric acid (5.8 N) (ratio 1:11) (Hughes et al., 2015). Supernatant was diluted, at 1 in 10 dilution, using sample pre-diluent which consisted of a combined internal standard solution combining final concentrations of 20nmol/L L-[ring-$^{13}C_6$]tyrosine, 10 nmol/L L-[ring-$^{13}C_6$]phenylalanine, and 0.1% formic acid (v/v) in deionised water.

2.3.4.3 *Preparation of calibrators and controls- aqueous assay*

To ensure matrix matched calibration, 0.9% sodium chloride (referred to as normal saline in this document) was used. Intermediate stocks were prepared

at 10 times the final required concentrations. These were diluted, at 1 in 10 using normal saline, to create combined calibrators solution with concentrations ranging from 25 to 10,000 nmol/L for L-[$^{13}C_9$]tyrosine, and from 25 to 3000 nmol/L for L-[d_8]phenylalanine.

Internal quality controls (IQC) were prepared from separate pools in a similar fashion and their concentrations were chosen carefully to cover the analytical range of the assay. L-[$^{13}C_9$]tyrosine IQC values were 25-15,000 nmol/L, while L-[d_8]phenylalanine IQC values were 25- 2000 nmol/L.

The sample pre-diluent consisted of a combined internal standard solution combining final concentrations of 20 nmol/L L-[ring-$^{13}C_6$]tyrosine, 10 nmol/L L-[ring-$^{13}C_6$]phenylalanine, and 0.1% formic acid (v/v) in deionised water. This was used, at 1 in 10 dilution, to dilute samples, IQC and calibrators as a preparation for the assay.

2.3.5 Assay validation

The LC-MS/MS assay was developed for the purpose of measuring the concentrations of L-[$^{13}C_9$]tyrosine, L- [d_7]tyrosine and L-[d_8]phenylalanine in samples obtained from humans and mice including murine tissue homogenates. As part of quality assurance, the aqueous matrix assay was required to measure the concentrations of L-[$^{13}C_9$]tyrosine and L-[d_8]phenylalanine in the tracers infusate given to the study participants and to mice.

The assay validation was guided by in-house standard operating procedures which were based on international guidelines (CHMP, 2011, Honour, 2011, FDA, 2018a).

2.3.5.1 *Linearity*

Standard curves were fitted using linear regression with a 1/x weighting factor (which helps to improve fit and minimise assay errors at the lower end of the analytical range) and a minimum of six calibration points plus a blank sample (urine for the urine assay, steroid depleted serum (SDS) for the serum assay and normal saline for the aqueous assay) and curve fitting parameters excluded zero. Performance of fitted curves is presented as the coefficient of determination (R^2).

2.3.5.2 *Accuracy*

This describes the closeness of a measured value compared to the nominal concentration of the analyte. This was calculated as follows:

Accuracy= [measured concentration - nominal concentration]/

[nominal concentration] * 100%.

Accuracy was assessed for both intra- assay (n= 8) and inter assay (n=6).

2.3.5.3 *Precision*

Three Internal Quality Control (IQC) concentrations, covering the calibration curve range, were used. Intra-assay precision was determined by analysing, in a single run, five replicate samples per IQC concentration. Intra-assay precision was determined by analysing, in a single run, eight replicate samples per IQC concentration.

Precision was expressed as the coefficient of variation (CV). For both intra- and inter- assay precision, CV should not exceed 15% except for LLOQ which should not exceed 20%.

2.3.5.4 *Lower Limit of Quantification (LLOQ):*

This is considered being the lowest calibrator with CV is less than 20% and the signal to noise ratio is at least 5:1. Bias should not exceed 20% (Honour, 2011, CHMP, 2011, FDA, 2018a).

2.3.5.5 *Dilution integrity*

It is important to demonstrate that dilution of samples that contain the analyte with a concentration above the upper limit of quantification (ULOQ) does not compromise accuracy or precision.

Serum samples were assessed at x3, x5, and x10 dilution using steroid depleted serum (n=5). Whereas 1 in 10 dilutions with deionised water was

used for urine samples (n=5). For the aqueous matrix samples, normal saline was used to obtain the following dilutions: x2, x5, x10, x20, x500, and x2000 (n=5).

2.3.5.6 *Carryover*

Carryover experiments identify whether the high concentration of analytes from one sample influences the measurement of subsequent samples. This was assessed, in this study, by five separate water injections following the injection of the top calibrator in each of the serum, urine, and aqueous matrix assay. Peak areas were assessed in each instance for any evidence of carryover.

2.3.5.7 *Interferences study*

Due to the structural similarities between native tyrosine and phenylalanine as well as their stable isotopes, this study assessed whether there is cross talk/ interference between the native compounds and their counterparts of stable isotopes.

A combined calibrators solution, containing L-[$^{13}C_9$]tyrosine, L-[d_7]tyrosine and L-[d_8]phenylalanine, was diluted with IS and water (separately). This was assayed, in triplicates, using AKU serum markers assay (Hughes et al., 2015).

2.3.5.8 *Stability*

Stability was assessed using three levels of IQC materials. These were subjected to three freeze-thaw cycles (at −80°C). Additionally, they were used to assess stability after storing samples for 24 hr in the following condition:

a. room temperature at 20°C
b. at 4°C temperature (equivalent to the temperature of the sample manager attached to the Agilent 6490)
c. at −20°C

The on-board samples were stored in glass vials. Results are expressed as a percentage of nominal values determined against a fresh calibration curve.

2.4 Results

2.4.1 Method validation

Multiple Reaction monitoring (MRM) was used. All compounds were measured in positive ionisation mode which achieved optimal sensitivity. MS parameters are summarised in Table 5. Figure 2 displays chromatograms of each of the tyrosine and phenylalanine stable isotopes used in the study (including the internal standards: L-[ring-$^{13}C_6$]tyrosine and L-[ring-$^{13}C_6$]phenylalanine). Chromatograms are identical for the serum, urine, and aqueous matrix assays. L-[d_7]tyrosine was not measured in the aqueous matrix assay

Table 5: Parameters for MS Detection of L-[d_7]tyrosine, L-[$^{13}C_9$]tyrosine, and L-[d_8]phenylalanine.
CE: collision energy. L-[ring-$^{13}C_6$]tyrosine and L-[ring-$^{13}C_6$]phenylalanine are the internal standards.

Compound	Ionisation	Product ion (Quantifier) [CE]	Product ion (Qualifier) [CE]
L-[$^{13}C_9$]tyrosine	Positive	191.0 > 144.0 [12]	191.0 > 98.0 [34]
L-[d_7]tyrosine	Positive	189.0 > 143.0 [12]	189.0 > 96.0 [36]
L-[d_8]phenylalanine	Positive	174.0 > 128.0 [14]	174.0 > 108.0 [36]
L-[ring-$^{13}C_6$]tyrosine	Positive	188.0 > 97.0 [32]	188.0 > 125.0 [20]
L-[ring-$^{13}C_6$]phenylalanine	Positive	172.0 > 126.0 [12]	172.0 > 109.0 [32]

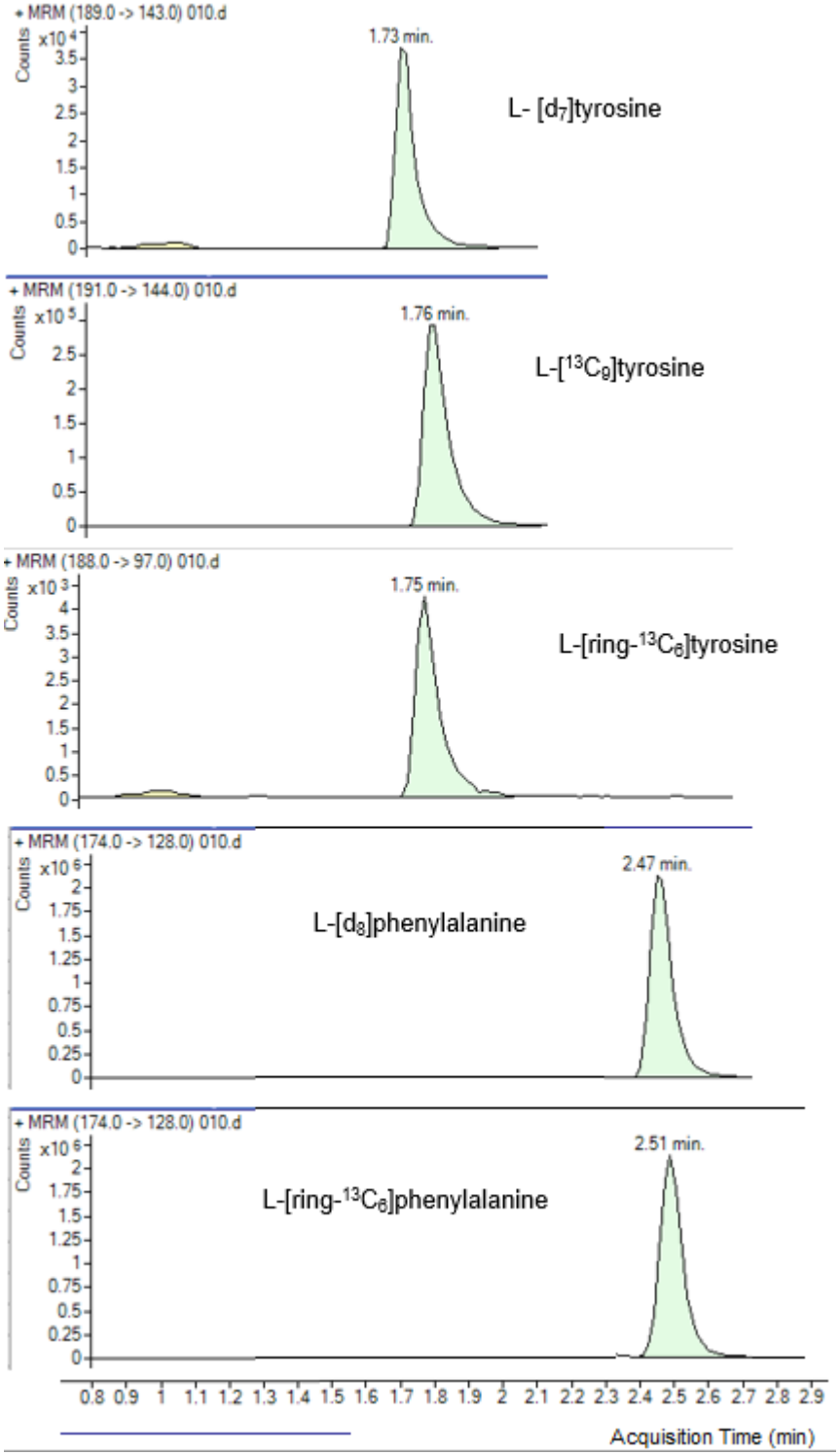

Figure 2: Chromatograms showing all quantifiable compounds in a serum sample matrix assay.
L-[ring-$^{13}C_6$]tyrosine and L-[ring-$^{13}C_6$]phenylalanine are the internal standards.

2.4.1.1 *Linearity*

2.4.1.1.1 Linearity in the serum assay

The serum assay used six calibrators plus serum blank. All calibration curves were constructed using linear regression with 1/x weighting factor and demonstrated a good fit with minimal inter-assay variability: L-[d_7]tyrosine R^2= 0.996 (n=6), L-[$^{13}C_9$]tyrosine R^2 =0.998 (n=6) and L-[d_8]phenylalanine R^2= 0.999 (n=6). The serum assay was linear to 10 µmol/L for L-[$^{13}C_9$]tyrosine, 1.2 µmol/L for L-[d_7]tyrosine and 9.37 µmol/L for L-[d_8]phenylalanine.

2.4.1.1.2 Linearity in the urine assay

The urine assay used six calibrators plus urine blank. All calibrations curves were constructed using linear regression with 1/x weighting factor (which exclude zero) and demonstrated a good fit with minimal inter-assay variability: L-[d_7]tyrosine R^2 = 0.980 (n=6), L-[$^{13}C_9$]tyrosine R^2 = 0.998 (n=6) and L-[d_8]phenylalanine R^2= 0.996 (n=6). The urine assay was linear to 3 µmol/L for L-[$^{13}C_9$]tyrosine, 800 nmol/L for L-[d_7]tyrosine and 3 µmol/L for L-[d_8]phenylalanine.

2.4.1.1.3 Linearity in the aqueous matrix assay

The aqueous matrix used six calibrators plus normal saline blank. All calibrations curves were constructed using linear regression with 1/x weighting factor (which excludes zero to detect any contamination or baseline drift) and demonstrated a good fit with minimal inter-assay variability with R^2 = 0.981 (n=6) for both L-[$^{13}C_9$]tyrosine (over a concentration range of 25 to

25,000 nmol/L) and L-[d_8]phenylalanine (over a concentration range of 25 to 3000 nmol/L).

The aqueous matrix assay was linear to 25 µmol/L for L-[$^{13}C_9$]tyrosine, and L-[d_7]tyrosine and 3 µmol/L for L-[d_8]phenylalanine.

2.4.1.2 *Accuracy*

2.4.1.2.1 Accuracy in the serum assay

Intra-batch accuracy (n=8) was 98 ± 5% for L-[$^{13}C_9$]tyrosine, 101 ± 4% for L-[d_8]phenylalanine and 101 ± 7% for L-[d7]tyrosine. Inter-batch accuracy (n=6) was 98 ± 2% for L-[d7]tyrosine and 93 ± 13% for both L-[$^{13}C_9$]tyrosine and L-[d_8]phenylalanine.

2.4.1.2.2 Accuracy in the urine assay

In the urine matrix assay, intra (n=8) and inter-assay (n=6) accuracy for L-[d_7]tyrosine, L-[$^{13}C_9$]tyrosine, and L-[d_8]phenylalanine was within ±12% of nominal values. This is summarised in Table 6. Results are represented as percentage recovery of a nominal amount of the stable isotope spiked into urine.

Table 6: Intra- and inter-batch accuracy in urine.
Results are expressed as mean ± SD.

	Urine matrix	
Expected concentration (nmol/L)	Intra-assay	Inter-assay
L-[d_7]tyrosine		
250	98.8 ± 9.2	98.9 ± 4.8
450	97.4 ± 8.7	98.4 ± 3.2
650	98.5 ± 8.7	100.3 ± 10.2
L-[$^{13}C_9$]tyrosine		
75	101.3 ± 6.6	102.8 ± 4.7
500	96.6 ± 10.7	95.0 ± 5.1
1000	98.9 ±8.6	100.4 ± 5.0
L-[d_8]phenylalanine		
75	100.3 ± 6.4	100.4 ± 5.0
500	101.1 ± 11.1	96.5 ± 7.8
1000	101.0 ± 10.0	99.7 ± 11.8

2.4.1.2.3 Accuracy in the aqueous matrix assay

Intra (n=8) and inter-assay (n=6) accuracy for L-[$^{13}C_9$]tyrosine in the aqueous matrix assay was within ±15% of nominal values. At 25 nmol/L (i.e. LLOQ), inter- assay accuracy was within ±21% of the nominal value for L-[d_8]phenylalanine in the aqueous matrix. At other concentrations of L-[d_8]phenylalanine, inter- and intra- assay accuracy was within ±15% of nominal values. This is summarised in Table 7.

Table 7: Intra- and inter-assay accuracy for L-[$^{13}C_9$]tyrosine, L-[d_8]phenylalanine in the aqueous matrix assays.
Results are represented as percentage recovery of a nominal amount of L-[$^{13}C_9$]tyrosine and L-[d_8]phenylalanine spiked into normal saline (mean ± SD).

	Aqueous matrix	
Expected concentration (nmol/L)	Intra-assay	Inter-assay
L [$^{13}C_9$]tyrosine		
25	109.0 ± 0.6	91.2 ± 13.7
1000	92.4 ± 2.5	93.0 ± 5.3
15000	99.6 ± 1.9	98.0 ± 15.6
L-[d_8]phenylalanine		
25	107.6 ± 0.7	79.3 ± 10.8
300	98.0 ± 1.6	98.3 ± 4.3
2000	98.3 ± 1.9	97.0 ± 13.5

2.4.1.3 *Precision*

2.4.1.3.1 Precision - serum assay:

In the serum matrix assay, intra- (n=8) and inter-assay (n=6) precision was less than 10% for L-[d_7]tyrosine (35- 900 nmol/L), L-[$^{13}C_9$]tyrosine (35- 7000 nmol/L), and L-[d_8]phenylalanine (35- 7000 nmol/L).

2.4.1.3.2 Precision - urine assay:

In the urine matrix assay, intra- (n=8) and inter-assay (n=6) precision was less than 12% for L-[d_7]tyrosine (250- 650 nmol/L), L-[$^{13}C_9$]tyrosine (75- 1000 nmol/L), and L-[d_8]phenylalanine (75- 1000 nmol/L).

2.4.1.3.3 Precision - aqueous matrix assay:

Intra-assay precision, in the aqueous matrix, was <5% for L-[$^{13}C_9$]tyrosine (25- 15000 nmol/L), and for L-[d_8]phenylalanine (25- 2000 nmol/L); while Inter-assay precision was <16% for L-[$^{13}C_9$]tyrosine (25- 15000 nmol/L), and <14% for L-[d_8]phenylalanine (25- 2000 nmol/L).

2.4.1.4 *LLOQ*

2.4.1.4.1 LLOQ- serum assay

LLOQ in the serum assay was 21, 50, and 47 nmol/L for L-[d_7]tyrosine, L-[$^{13}C_9$]tyrosine and L-[d_8]phenylalanine, respectively (CV < 5%, Bias < 10%).

2.4.1.4.2 LLOQ- urine assay

LLOQ in the urine assay was 203, 45, and 53 nmol/L for L-[d_7]tyrosine, L-[$^{13}C_9$]tyrosine and L-[d_8]phenylalanine, respectively (CV < 5%, Bias < 5%).

2.4.1.4.3 LLOQ- aqueous matrix assay

LLOQ in the aqueous matrix assay was 25, and 25 nmol/L for L-[$^{13}C_9$]tyrosine and L-[d_8]phenylalanine, respectively (CV < 11%, Bias < 20%).

2.4.1.5 *Carryover:*

The top serum and urine calibrators contained L-[d_7]tyrosine, L-[$^{13}C_9$]tyrosine and L-[d_8]phenylalanine, while the aqueous matrix top calibrator contained only L-[$^{13}C_9$]tyrosine and L-[d_8]phenylalanine.

2.4.1.5.1 Carryover- serum assay:

Transition windows of L-[d_7]tyrosine, L-[$^{13}C_9$]tyrosine and L-[d_8]phenylalanine were examined for the presence of discernible peaks. There were no detectable peaks (areas) in the five blank water samples injected following the injection of the serum top calibrator (L-[d_7]tyrosine 1.2 µmol/L, L-[$^{13}C_9$]tyrosine 10 µmol/L and L-[d_8]phenylalanine 10 µmol/L). Similarly, there were no peaks in the L-[ring-$^{13}C_6$]tyrosine and L-[ring-$^{3}C_6$]phenylalanine (i.e. internal standards) transition windows.

2.4.1.5.2 Carryover- urine assay:

Transition windows of L-[d_7]tyrosine, L-[$^{13}C_9$]tyrosine and L-[d_8]phenylalanine were examined for the presence of discernible peaks. There were no detectable peaks (areas) in the five blank water samples injected following the injection of the urine top calibrator (L-[d_7]tyrosine 0.8 µmol/L, L-[$^{13}C_9$]tyrosine 3 µmol/L and L-[d_8]phenylalanine 3 µmol/L). Similarly, there were no peaks in the L-[ring-$^{13}C_6$]tyrosine and L-[ring-$^{3}C_6$]phenylalanine (i.e. internal standards) transition windows.

2.4.1.5.3 Carryover- aqueous matrix assay:

Transition windows of L-[$^{13}C_9$]tyrosine and L-[d_8]phenylalanine were examined for the presence of discernible peaks. There were no detectable peaks (areas) in the five blank water samples injected following the injection of the combined aqueous matrix top calibrator (L-[$^{13}C_9$]tyrosine 25 µmol/L and L-[d_8]phenylalanine 3 µmol/L). Similarly, there were no peaks in the L-[ring-$^{13}C_6$]tyrosine and L-[ring-$^{3}C_6$]phenylalanine (i.e. internal standards) transition windows.

2.4.1.6 *Dilution integrity*

2.4.1.6.1 Dilution integrity- serum assay:

Recoveries of L-[d_7]tyrosine, L-[$^{13}C_9$]tyrosine and L-[d_8]phenylalanine were assessed post-dilution. Overall, these were within ±15% of expected values. This summarised in Table 8.

2.4.1.6.2 Dilution integrity- urine assay:

Recoveries of L-[$^{13}C_9$]tyrosine and L-[d_8]phenylalanine were assessed post-dilution. Despite a trend for over-recovery, overall, these were within ±15% of expected values. For L-[d_7]tyrosine, there was under-recovery at 1 in 3 dilution and over-recovery at 1in 5 and 1 in 10 dilution. This summarised in Table 9.

Table 8: Post-dilution recovery in the serum matrix

	Post-dilution recovery in the serum matrix		
	L-[d_7]tyrosine	L-[$^{13}C_9$]tyrosine	L-[d_8]phenylalanine
1 in 2 dilution	88.4 ± 2.3	91.1 ± 4.0	95.7 ± 2.6
1 in 5 dilution	100.4 ± 5.2	95.8 ± 2.6	102.7 ± 3.0
1 in 10 dilution	88.2 ± 7.5	91.2 ± 2.1	96.6 ± 2.4
1 in 20 dilution	90.9 ± 9.0	96.4 ± 4.7	103.6 ± 1.0

Table 9: Post-dilution recovery in the urine matrix

	Post-dilution recovery in the urine matrix		
	L-[d_7]tyrosine	L-[$^{13}C_9$]tyrosine	L-[d_8]phenylalanine
1 in 3 dilution	77.4 ± 3.2	105.4 ± 2.4	112.0 ± 11.8
1 in 5 dilution	128.1 ± 12.6	106.8 ± 2.0	106.2 ± 1.1
1 in 10 dilution	107.6 ± 18.9	101.4 ± 4.7	103.9 ± 1.3

2.4.1.6.3 Dilution integrity- aqueous matrix assay

Recoveries of L-[$^{13}C_9$]tyrosine and L-[d_8]phenylalanine were assessed post-dilution. Dilutions of 1 in 500 and 1 in 2000 were examined as these were needed as part of quality assurance for the tracers infusates given to study participants. Overall, recoveries were within ±15% of expected values. This summarised in Table 10.

Table 10: Post-dilution recovery in the aqueous matrix.
L-[d_7]tyrosine was not included in the tracers infusate. Therefore, it was not assessed.

	Post-dilution recovery in the aqueous matrix	
	L-[$^{13}C_9$]tyrosine	L-[d_8]phenylalanine
1 in 2 dilution	95.2 ± 1.9	96.8 ± 2.1
1 in 5 dilution	90.6 ± 1.7	94.2 ± 2.6
1 in 10 dilution	91.6 ± 5.1	96.9 ± 2.5
1 in 20 dilution	87.0 ± 2.1	100.4 ± 4.0
1 in 500 dilution	93.6 ± 2.2	85.1 ± 1.8
1 in 2000 dilution	87.8 ± 3.4	91.3 ± 1.5

2.4.1.7 *Stability of L-[d_7]tyrosine, L-[$^{13}C_9$]tyrosine and L-[d_8]phenylalanine.*

2.4.1.7.1 Freeze- thaw stability

Stability of serum samples after three freeze-thaw cycles demonstrated an average recovery of 96.9 ±4.4 for serum L-[d_7]tyrosine, 98.4 ±2.3 for serum L-[$^{13}C_9$]tyrosine and 98.9 ±2.3 for serum L-[d_8]phenylalanine. The average recovery for urine samples was 102.9 ±10.5 for urine L-[d_7]tyrosine, 102.0 ±7.5 for urine L-[$^{13}C_9$]tyrosine and 102.2 ±8.4 for urine L-[d_8]phenylalanine. In the aqueous matrix, the average recovery was 102.6 ±5.2 for L-[$^{13}C_9$]tyrosine and 101.5 ±5.0 for L-[d_8]phenylalanine.

2.4.1.7.2 Stability at room temperature (20°C), 4°C, and −20°C

In the serum matrix, stability studies demonstrated average recoveries 101 ±1.2, for L-[d_7]tyrosine, L-[$^{13}C_9$]tyrosine and L-[d_8]phenylalanine, at 20°C, 4°C, and −20°C as summarised in Table 11.

In the urine matrix, there was a trend of “over- recovery” in the L-[d_7]tyrosine most notably at room temperature. This is summarised in Table 11.

As normal saline infusates were stored at 4°C, stability in the aqueous matrix was only assessed after 48hr storage at 4°C. The long-term stability was tested for a duration of 5 and 6 months at 4°C by analysing in triplicates two separate infusates. One left for 5 months and the other left for 6 months. L-[d_7]tyrosine was not included in the infusate. Therefore, it was not assessed.

Table 11: Summary of 24 hr stability experiments at 20°C, 4°C and -20°C.
* Recoveries are expressed as percentages of nominal values, NA: not assessed.

Matrix	Storage Temperature	Average Recoveries*		
		L-[d_7]tyrosine	L-[$^{13}C_9$]tyrosine	L-[d_8]phenylalanine
Serum	20°C	100.0 ± 3.3	99.0 ± 2.3	102.7± 4.0
	4°C	100.6 ± 5.9	103.5 ± 3.6	100.8 ± 4.1
	-20°C	100.2 ± 4.1	101.4 ± 3.4	102.5 ± 5.2
Urine	20°C	110.0 ± 25.1	106.1 ± 2.4	106.1± 2.0
	4°C	111.4 ± 9.0	105.7 ± 2.1	105.4 ± 1.5
	-20°C	118.6 ± 4.7	104.8 ± 2.8	106.5 ± 2.7
Aqueous	4°C (for 48 hr)	NA	101.3 ± 5.4	100.1 ± 1.8
	4°C (for 5 mths)	NA	93.9 ± 4.0	94.5 ± 2.4
	4°C (for 6 mths)	NA	95.2 ± 0.5	106.2 ± 2.5

2.4.1.8 *Interferences study*

Due to the structural similarities between native tyrosine and phenylalanine as well as their stable isotopes, this study has assessed whether there is cross talk/ interference between the native compounds and their counterparts of stable isotopes.

A combined calibrators solution, covering the analytical range for the tracers' serum assay and containing L-[$^{13}C_9$]tyrosine, L-[d_7]tyrosine and L-[d_8]phenylalanine, was diluted with internal standard solution (containing internal standards (L-[$^{13}C_6$]homogentisic acid, L-[d_2]-tyrosine) in the first experiment. For the second experiment, water was used for dilution. In both experiments, samples were assayed, in triplicates, using AKU serum markers assay (Hughes et al., 2015). None of the native compounds (namely, phenylalanine, tyrosine, HGA, HPLA and HPPA) were detected in the combined calibrators solution.

2.5 Discussion

This chapter has described the development and validation of an LC-MS/MS method for the measurement of phenylalanine and tyrosine stable isotopes in human and murine urine and serum samples, as well as tissues homogenates obtained from mice.

NTBC is now an approved therapy for AKU adults (EMA, 2020). It decreases serum and urine HGA but causes tyrosinaemia. In this study, stable isotopes methodology was deployed to further examine the tyrosine degradation pathway in AKU patients and mice before and after NTBC, as well as in healthy volunteers. To enable this, it was necessary to develop a robust and reliable analytical method to measure the concentrations of tyrosine and phenylalanine stable isotopes used in the study.

Although this was a research assay, the method validation still followed the international guidelines used for validating mainstream clinical assays deployed in patient care. This is to ensure that high quality data are obtained. As the Liverpool group have already validated a method for the measurements of native tyrosine and phenylalanine in serum and urine, it made sense to modify the existing analytical method and LC-MS/MS analyser.

This method required an injection volume of 2 µL and a simple dilution step in contrast to other methods which required a relatively a larger injection volume of 10uL as well as derivatisation (Meesters et al., 2009). The samples obtained from mice in this study (plasma, urine, and tissue homogenates) were very small in volume, so it was advantageous that the developed assay required a very small injection volume and no derivatisation or extraction. Furthermore,

the run time was 7 minutes much shorter compared to 20 minutes(Meesters et al., 2009). A previously published method for measuring amino acid enrichment required 5 µL of deproteinised plasma but had a cycle run time of 45 minutes and required derivatisation with 9-fluorenylmethylchloroformate (van Eijk et al., 2007). An earlier method has used o-phthaldialdehyde derivatisation and had a similar run time of 45 minutes (van Eijk et al., 1999).

The sample volume meant that the assay can be conveniently used for precious samples, such as the ones obtained from mice. The simple preparation which consisted of a simple dilution step made it straightforward to process samples and very easy to perform the assay.

Accuracy and precision: Both were good across three matrices.

LLOQ: Across all matrices, the assay was sensitive at very low concentrations of the stable isotopes of interest. This is a strong point of the assay to be able to reliably measure lower concentrations of stable isotopes, if extended serial sampling periods are used in future studies. In this study, it was not necessary to use LLOQ as sampling was done over 2- hour periods and concentrations were higher than LLOQ.

Matrix effects: Hughes et al. (2015) demonstrated that there was no ion suppression in either in acidified serum tyrosine calibrators or internal standards. The internal standard normalised matrix factor was <10%. There was no evidence of matrix effect in acidified urine as far as tyrosine measurements (Hughes et al., 2014). There was minimal ion suppression for

phenylalanine in both serum and urine matrices with a CV<10% satisfying the validation criteria (Hughes et al., 2022).

L-[d_8]phenylalanine has an elution time (2.47min) that is very similar to native phenylalanine (2.4 min) (Hughes et al., 2022); while both L-[$^{13}C_9$]tyrosine and L- [d_7]tyrosine have retention times (1.76 min and 1.73 min, respectively) that are almost identical to native tyrosine (1.8 min) (Hughes et al., 2014). Furthermore, this method was developed using the same column, analytical platform, and buffers. Analyses of this study samples were carried out using the same LC-MS/MS analyser as well as the same mass spectrometric parameters and chromatographic conditions. Therefore, matrix effects were not assessed here.

Dilution: L- [d_7]tyrosine showed under-recovery at 1 in 3 dilution and over-recovery at 1:5 and 1 in dilutions. It is possible that the diluting the matrix (using deionised water) may have had some effect. This can further be examined by repeating the experiment using urine rather than deionised water. In practical terms, validation process used samples obtained from the earlier experiments in the study to optimise the analytical range for the assay. This has enabled the analysis of all the study samples with no /minimal dilution.

In the aqueous matrix, dilution examined up to 1 in 2000 while maintaining matrix integrity by using normal saline for dilution.

Stability: There was a trend to increasing urine L- [d_7]tyrosine concentrations after 24 hr storage especially in room temperature. All samples were analysed

in one batch and were stored at -80C°. Therefore, all samples would have been equally subjected to the same degree of positive bias/ over-recovery.

The stability of tissue homogenates samples was not tested. It is reasonable to assume that they have similar stability to serum samples. Furthermore, they were stored at -80C° until time of analysis and were all processed in one batch.

In the aqueous matrix, stability over 48hr was assessed as tracers' infusate were freshly prepared within that time of giving it to study participants.

There was no evidence of carryover. Furthermore, there was no interferences from native tyrosine and phenylalanine.

LC/MS/MS was found to be very precise and reliable particularly in small quantity samples when compared to other GC-based methods used in measuring phenylalanine tracer enrichment (Zabielski, *et al* (2013). The data on the assay presented here add further evidence to the reliability of the LC-MS/MS in measuring enrichment of stable isotopes in metabolic research studies.

2.6 Conclusion

In summary, this study validated a simple method for measurement of tyrosine and phenylalanine stable isotopes. The assay performed well to the standards expected in clinical assays used in direct patient care. This was made possible with the use of matrix matched calibrators to minimise matrix effects along with the assay favourable accuracy and precision, simple sample preparation, short running time as well as the lack of carry over. The small sample volume

meant that the assay could be utilised in measuring precious samples (e.g., murine tissue homogenates).

For this research project, the assay was instrumental in enabling further examination of the tyrosine degradation pathway. On a wider scale, this simple, reliable, and robust assay would be of interest to research groups deploying tyrosine and phenylalanine stable isotopes in studying protein metabolism.

2.7 Declaration and acknowledgments

I am grateful to my colleagues Dr Anna Milan, Dr A T Hughes, and Dr A Davison for their guidance during method development and validation.

3.0 INVESTIGATING THE EFFECTS OF NTBC ON THE TYROSINE DEGRADATION PATHWAY IN MICE:

3.1 Introduction

Until recently, the management of AKU has been supportive without addressing the underlying pathophysiological mechanisms. However, NTBC, a competitive reversible inhibitor of the 4-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 HT-1 in children (Lindstedt et al., 1992, 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. Results from SONIA 2 study have shown positive outcomes in terms of clinical efficacy, reversal of ochronosis and safety of NTBC when used in AKU (Ranganath et al., 2020d).

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 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 hypertyrosinaemia in children with HT-1 (McKiernan, 2013, van Ginkel et al., 2016a, García et al., 2017). Although low phenylalanine levels and NTBC 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 hypertyrosinaemia.

This study assessed the consequences of NTBC-induced hypertyrosinaemia by measuring tyrosine concentrations in tissues harvested from AKU mice on NTBC. It also explored the wider impact of NTBC effect on the tyrosine pathway compounds in murine plasma, urine, and tissue homogenates. Furthermore, It evaluated the decay of tyrosine and phenylalanine following pulse injection of L-[$^{13}C_9$]tyrosine and L- [d_8]phenylalanine. Additionally, it examined the effects of NTBC on protein turnover and phenylalanine hydroxylation in mice following NTBC. Figure 3 summarises the overall study design.

The estimation of tyrosine pools utilises stable isotopes techniques that are used for studying protein turnover. Phenylalanine is an indispensable amino acid which is exclusively disposed of by conversion to tyrosine (Thompson et al., 1989). In this study, L-[d_8]phenylalanine is hydroxylated to L-[d_7]tyrosine; and measurement of both is necessary to estimate the whole body production of phenylalanine. The measurement of L-[$^{13}C_9$]tyrosine is necessary for calculating the whole body production of tyrosine. The inclusion of L-[$^{13}C_9$]tyrosine strengthen the robustness of calculations used to estimate protein turnover and tyrosine pool sizes (Engelen et al., 2019).

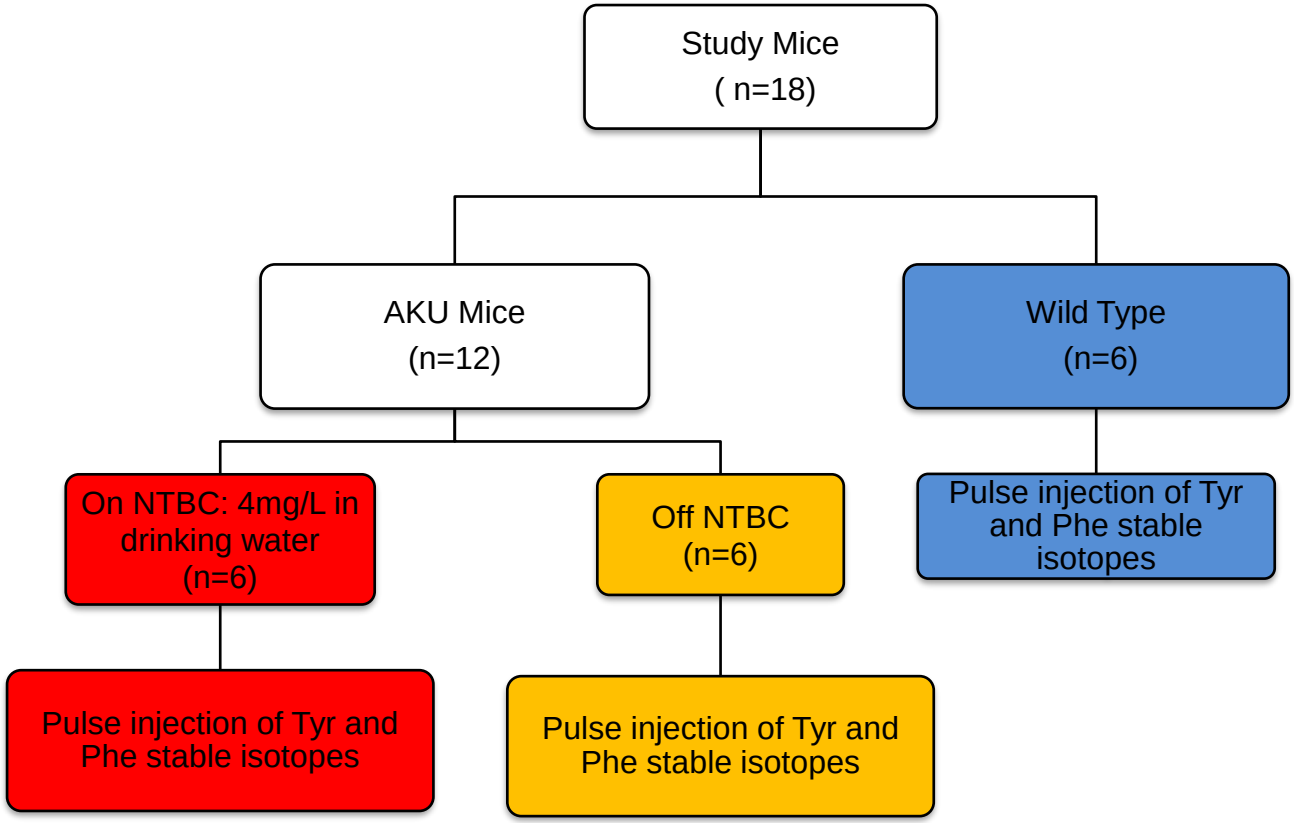

Figure 3: Summary of the overall study design in the mice experiment.
NTBC: nitisinone, Tyr and Phe stable isotopes are L-[$^{13}C_9$]tyrosine and L- [d_8]phenylalanine, respectively.

3.2 Material and methods

3.2.1 Chemicals

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).

3.2.2 Biochemical analysis

Native tyrosine, phenylalanine, homogentisic acid(HGA), hydroxyphenyllactic acid (HPLA) and hydroxyphenylpyruvic acid (HPPA) concentrations were measured in murine plasma, tissue homogenates and urine using Liquid chromatography–mass spectrometry (LC-MS/MS) (Hughes et al., 2014, Hughes et al., 2015, Hughes et al., 2022). Measurements of L-[$^{13}C_9$]tyrosine, L-[d_7]tyrosine and L-[d_8]phenylalanine were done in one batch for all samples. Measurements of native tyrosine, phenylalanine, HGA, HPLA and HPPA were done on all samples in a separate batch.

Using matrix matched calibrators, this study modified an existing LC-MS/MS method (Hughes et al., 2014, Hughes et al., 2015) to enable measurements of L-[$^{13}C_9$]tyrosine, L-[d_8]phenylalanine and L-[d_7]tyrosine in murine plasma, urine and tissue homogenates. 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. Method validation is reported in detail in chapter 2.

Urine and plasma samples from mice were all small volumes. Therefore, vial inserts (Agilent, UK) were used to overcome this and ensure optimal utilisation of the available samples.

3.2.3 Mice study

3.2.3.1 *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.

3.2.3.2 *Mice husbandry*

Mice were bred, housed, and maintained within the Liverpool John Moores University Life Science Support Unit in accordance with the Home Office UK guidelines. Mice fed on an ad libitum supply of vegetal diet A30 (SAFE, Augy, France) with a tyrosine and phenylalanine content of 0.72% and 1.10% respectively. It is estimated that the daily intake, per 30 gram of body weight, for BALB/cByJ mice is 5 gram of food and 6 mL of water (Bachmanov et al., 2002).

3.2.3.3 *NTBC administration*

The intervention group was treated with NTBC for a week. It was administered through drinking water (4 mg/L).

3.2.3.4 *Pulse injection of tyrosine and phenylalanine stable isotopes in mice*

This protocol is based on previously validated studies (Engelen et al., 2019, Granados et al., 2020). Mice were fasted for 5 hours prior to the experiment. A total of 18 BALB/c mice were included. Demographics are summarised in Table 12. The mice were split into three groups:

- Wild type (WT) group: (n=6; median age=24.8 weeks; all males)
- AKU treated group (*HGD*-/-) :(n=6; median age=8.5 weeks; 4 males and 2 females)
- AKU control group (*HGD*-/-): (n=6; median age=21.1 weeks; 3 males and 3 females)

A bolus of 0.5 mL of 0.9% saline containing 0.2 mg (1.05 µmol) of L-[$^{13}C_9$]tyrosine and 0.5 mg (2.70 µmol) of L-[d_8]phenylalanine was injected into the tail vein of in each mouse at baseline. Plasma samples were taken at the following time points: 20, 40- and 60-min. Additional samples were taken at either 0- or 5-min. Experiment were done in staggered fashion. Initially samples were taken at 0-min. This was changed from 0-min to 5-min later during the study and allowed estimation of tracers concentrations after giving the pulse injection. Therefore, the 0-min sampling point was dropped in favour of the 5-min one.

Table 12: Details of mice characteristics in this study.

Group	Genotype	Sex	Age (weeks)	Weight (gram)
Non-treated Wild type group	Wild type	Male	17.4	28.0
			17.4	28.0
			27.6	31.4
			27.6	30.8
			24.7	29.4
			24.9	30.7
NTBC- treated AKU group (4mg/L of NTBC in drinking water for one week)	*HGD*$^{-/-}$	Male	6.6	22.0
			8.6	21.8
			8.4	22.6
			8.4	23.4
		Female	24.4	25.0
			24.4	25.0
Non- treated AKU group	*HGD*$^{-/-}$	Male	6.7	22.0
			6.7	22.0
			18.6	26.6
		Female	23.6	25.0
			23.7	25.1
			23.7	23.9

3.2.3.5 *Sampling*

Venous tail blood was collected using Microvette tubes (Lithium Heparin CB 300, LH: Starstedt). Mice were then schedule 1 culled and the following tissues were removed and weighed: liver, kidney, heart, brain, quadriceps muscle and femur.

3.2.3.6 *Plasma collection*

All samples were stored on ice during each experiment. Samples were spun at 1500 xg for 10 mins at 4 ºC. Supernatant was removed into Eppendorf and 5.8 M perchloric added to 10% of volume. Samples were vortexed, centrifuged at 1500 xg 10 mins 4 ºC and supernatant removed into new Eppendorf and frozen at -20 ºC until analysis.

3.2.3.7 *Urine collection*

Mouse urine was collected in cling film at the end of the experiment. Then it was pipetted into sample tubes and frozen at -20ºC. A more robust approach would have been to catheterise the mice which too invasive and stressful for the mice. It was not possible to collect urine from all mice.

Due the small volume of the urine samples obtained; it was not possible to measure urinary creatinine. The volume of urine would not have been enough to help set up the stable isotopes assay and measure native compounds as well as tracers. Therefore, it was presumed that urine samples volumes were comparable and compounds concentrations were expressed in µmol/L.

3.2.3.8 *Homogenisation of tissues*

For kidney, brain, heart, and quadriceps muscle: weighed samples were added to the lysis matrix tubes (Matrix D: MP Biomedicals) and one mL of 70% HPLC grade methanol added. Samples were homogenized three times in the Roche Magnalyser (at 6000 cycles per minute, for 40 seconds) with a 5-minute interval on ice to cool the samples. Then samples were centrifuged at 2000 g for 5 minutes and supernatant removed.

For femur and liver, samples were ground to powder under liquid nitrogen in a mortar and pestle. For the liver, this was to obtain a homogenous sample of the whole liver. For the bone, it was to break up the bone. One mL of methanol (70%) was added to the weighed sample and homogenized as above.

Tissues homogenates were prepared as 30 mg/mL solution. Samples were diluted to 30 mg/mL wet weight with 70% methanol. Tyrosine concentrations were all normalized and expressed as µmol/gram wet weight of tissue.

3.2.4 Statistical methods

Data was expressed as Mean and SD unless indicated otherwise. Analyses were conducted in GraphPad Prism version 8.1.0 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com. To maintain consistency of statistical approach and ensure that all experiments were analysed in the same way, all samples were presumed to follow a normal distribution. Parametric testing was the preferred approach because non-parametric tests have little or no power to find a significant difference when the sample number is less than12 (Motulsky, n.d.-b, Motulsky, n.d.-a). One-way ANOVA test was carried out with Tukey's corrections. The two-tailed significance was set at the 5% statistical significance level.

3.2.4.1 *Urinary concentrations of stable isotopes and native compounds in the tyrosine pathway:*

Volumes of urine obtained were presumed to be comparable. It was not possible to measure urinary creatinine in the urine samples obtains due to the small sample size. Comparison of stable isotopes and native compounds concentrations in the three groups was by one-way ANOVA with Tukey's multiple comparisons test.

3.2.4.2 *Comparison of the tyrosine pathway compounds concentrations in plasma against tissue homogenates in mice*

In mice, water constitutes approximately 70% of body weight (Bailey et al., 1960, Durbin et al., 1992) with nearly 65% of the total body water is contained in the intracellular compartment while 35% exists in the extracellular space (Chapman et al., 2010). Average blood volume in the mouse is 7-8%. Of note, water content of tissues varies with the skin, bone and adipose tissues were reported as having the lowest water content; whilst the lungs, heart, muscle, brain, liver and kidney were reported as having the highest water content (Reinoso et al., 1997).

In the current study, compound concentration is plasma and tissue homogenates are in completely different units: µmol/L vs µmol/g of tissue. It was presumed that 1 Litre of plasma weighs approximately one kilogram, and plasma concentrations of compounds were converted from µmol/L to µmol/gram so comparison can be made with tissue homogenates concentrations.

3.2.4.3 *Curve fitting of tracer data in mice*

Since one of the assumptions for non-compartmental modelling is the steady state of tracee, the Tracer-Tracee Ratio (TTR) was calculated 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 mouse separately. Moreover, the TTR values for each subject were normalized by the amount of administered tracer and mouse weight. All TTR data were combined

for mice in each group and fitted in one curve. Tracer to tracee ratio (TTR) data for L-[$^{13}C_9$]tyrosine, L-[d_8]phenylalanine and L-[d_7]tyrosine was fitted using the two-exponential model. Calculations of the area under the curve (AUC) for each stable isotope and curve fitting of data from TTR and changes in tracer concentrations against time were performed using GraphPad Prism 8.0.2.

3.2.4.4 *Protein turnover calculations in mice*

AUCs were calculated using GraphPad Prism 8.0.2. Protein turnover parameters were calculated as follows (Mason et al., 2017)

- Whole body production of phenylalanine (WBP PHE)= 1/ Total Peak Area of L-[d_8]phenylalanine (PHE_8)
- SE- WBP PHE= (1/ total Peak Area of PHE_8) * SQRT (Std Error)^2+(Total Peak Area of PHE_8)^2
- Whole body production of tyrosine (WBP TYR)= 1/ Total Peak Area of L-[$^{13}C_9$]tyrosine (TYR_9)
- SE- WBP TYR= (1/ Total Peak Area of TYR_9) * SQRT (Std Error)^2+(Total Peak Area of TYR_9)^2
- Phenylalanine hydroxylation to tyrosine (PHE>TYR) (µmol/kg ffm/min) = WBP TYR*[(AUC-TYR7)/(AUC-PHE8)]
- In the post-absorptive state (Engelen et al., 2000, Wolfe and Chinkes, 2004):
    - Protein breakdown (PB) = Protein synthesis (PS)+ (PHE>TYR)
    - Whole-body Protein Breakdown (µmol/kg ffm/min) = WBP PHE

- Whole-body Protein Synthesis (µmol/kg ffm/min) = WBP PHE-[PHE>TYR (µmol/kg ffm/min)]

3.3 Results

3.3.1 Comparison of mice characteristics in the study groups

There is no statistically significant difference in age in mice amongst the three group. However, the weight in the WT mice is significantly larger than in the AKU mice on NTBC (P<0.001) and the AKU mice off NTBC (P<0.001). The difference in weight between AKU mice on NTBC and AKU mice off NTBC was not significant (P=0.658). This is summarised in Figure 4 and Table 13. In the current study, WT mice were older and heavier in weight compared to AKU mice. This is consistent with previous observations that weight is significantly affected by age in mice leading to increase weight in older age (Yanai and Endo, 2021)

Table 13: Comparison of mice characteristics in the study groups.

WT: wild type mice, AKU on NTBC: AKU mice treated with NTBC, AKU off NTBC: AKU mice that were not treated with NTBC. Ordinary one-way ANOVA with Tukey's multiple comparisons test was used to compare data in the three groups. †Comparison of WT and AKU on NTBC mice. ††Comparison of AKU on NTBC and AKU off NTBC mice. †††Comparison of WT and AKU off NTBC mice. All AKU mice on NTBC received NTBC for one week. In the AKU mice off NTBC: four mice did not receive NTBC at all, while two mice had NTBC for three days followed by 7 days withdrawal of NTBC prior to enrolling them in the AKU off NTBC group.

Parameter	Mice study groups											
	WT				AKU on NTBC				AKU off NTBC			
	n	Mean ± SD		$p^{\dagger}$	n	Mean ± SD		$p^{\dagger\dagger}$	n	Mean ± SD		$p^{\dagger\dagger\dagger}$
Mouse Age (weeks)	6	23 ± 5		0.087	6	13 ± 9		0.669	6	17 ± 8		0.352
Mouse weight (gram)	6	30 ± 2		<0.001	6	23 ± 1		0.658	6	24 ± 2		<0.001

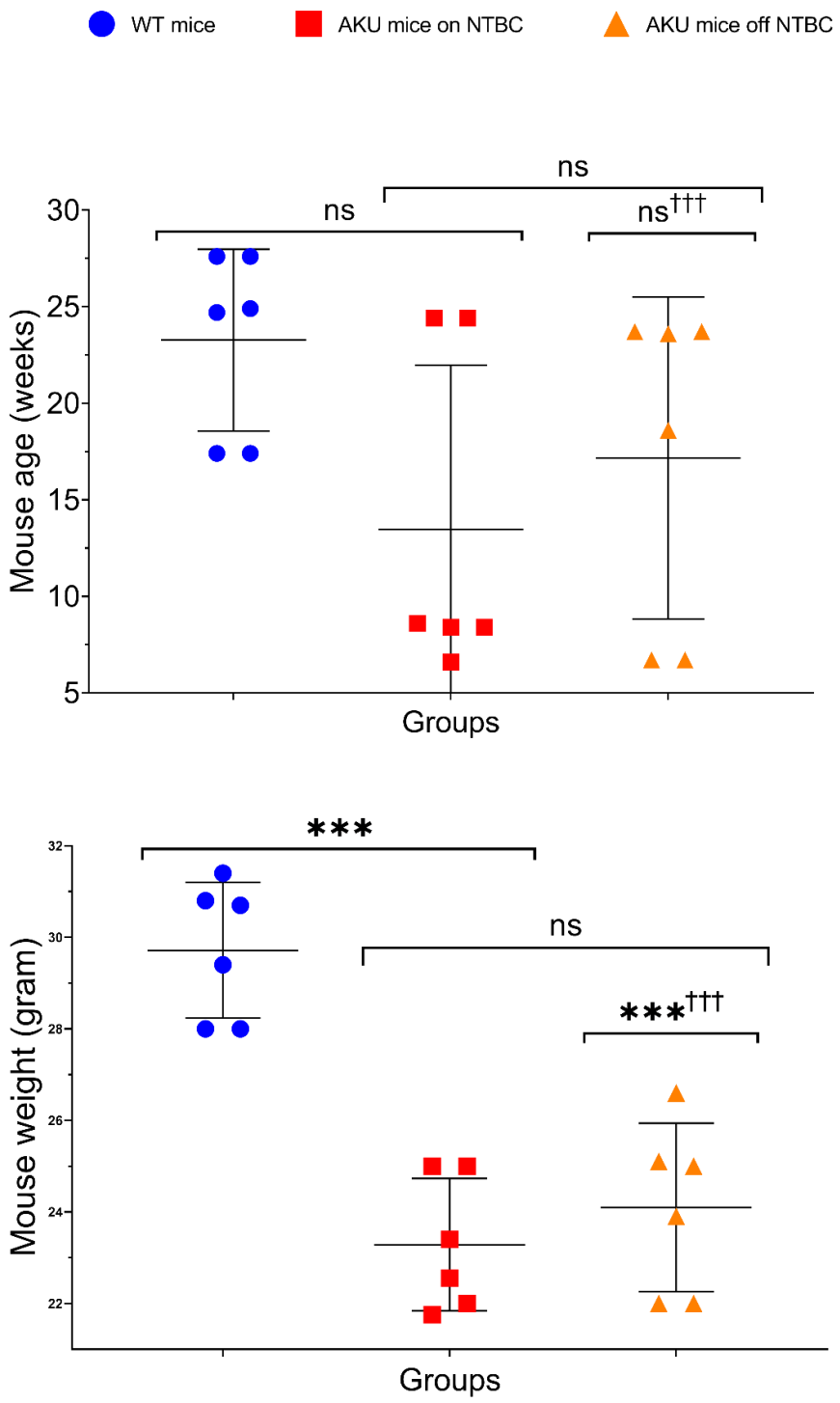

Figure 4: Comparison of mice characteristics in the study groups.
ns: *$P \geq 0.05$, ***$P < 0.001$.††† AKU off NTBC compared to WT mice. Line and error bars are mean ± SD. All AKU mice on NTBC received NTBC for one week. In the AKU mice off NTBC: four mice did not receive NTBC at all, while two mice had NTBC for three days followed by 7 days withdrawal of NTBC prior to enrolling them in the AKU off NTBC group.

3.3.2 Effects of NTBC on the plasma concentrations of native compounds in the tyrosine pathway in mice

Figure 5 and Table 14 provides an overall summary of the plasma concentrations of NTBC and the native compounds in the tyrosine pathway in mice across the three groups.

3.3.2.1 *Effects of NTBC on the plasma concentrations of phenylalanine in mice*

There was no significant difference in plasma concentrations of phenylalanine between WT and AKU mice off NTBC (*P*= 0.765). Furthermore, there was no significant difference in plasma concentrations of phenylalanine in the AKU mice on NTBC compared to WT mice (*P*= 0.839) or AKU mice off NTBC (*P*= 0.990).

3.3.2.2 *Effects of NTBC on the plasma concentrations of tyrosine in mice*

Plasma tyrosine concentrations were ten times larger in AKU mice on NTBC compared to WT mice (*P*< 0.001); and 8.4 times larger compared to AKU mice off NTBC (*P*< 0.001). There was no significant difference in plasma tyrosine concentrations between WT and AKU mice off NTBC (*P*= 0.986).

3.3.2.3 *Effects of NTBC on the plasma concentrations of HGA in mice*

Plasma HGA concentrations were 13 times larger in AKU mice off NTBC compared to WT mice (*P*= 0.003), and 7-fold larger compared to AKU mice on NTBC (*P*= 0.008). There was no significant difference in plasma HGA concentrations between WT and AKU mice on NTBC (*P*= 0.861).

3.3.2.4 *Effects of NTBC on the plasma concentrations of HPLA in mice*

Plasma HPLA concentrations were 179 times larger in AKU mice on NTBC compared to WT mice ($P< 0.001$), and 47 times larger compared to AKU mice off NTBC ($P< 0.001$). Plasma HGA concentrations in AKU mice off NTBC were four times larger compared to WT mice. But this difference was not significant ($P= 0.997$).

3.3.2.5 *Effects of NTBC on the plasma concentrations of HPPA in mice*

Plasma HPPA concentrations were 13 times larger in AKU mice on NTBC compared to AKU mice off NTBC ($P< 0.001$). No HPPA was detected in the plasma of WT mice.

Table 14: Plasma concentrations of NTBC and native compounds in the tyrosine pathway.
WT: wild type mice, AKU on NTBC: AKU mice treated with NTBC, AKU off NTBC: AKU mice that were not treated with NTBC. N: total number of samples per group. F1: the fold change in compound concentrations in the AKU on NTBC mice compared to WT mice, F2: the fold change in compound concentrations when comparing AKU on NTBC and AKU off NTBC mice, F3: fold change in compound concentrations in the AKU off NTBC mice compared to WT mice. ANOVA with Tukey's multiple comparisons test was used to compare data in the three groups. †Comparison of WT and AKU on NTBC mice. ††Comparison of AKU on NTBC and AKU off NTBC mice. †††Comparison of WT and AKU off NTBC mice. ND: not determined, NA: not available.

Compound	Plasma concentrations of compounds (µmol/L)															
	WT						AKU on NTBC						AKU off NTBC			
	N	Mean		SD	$p^{\dagger}$	***F1***	N	Mean		SD	$p^{\dagger\dagger}$	***F2***	N	Mean		SD	$p^{\dagger\dagger\dagger}$	***F3***
Phenylalanine	24	75	±	12	0.839	1.1	27	83	±	53	0.990	1.0	24	88	±	36	0.765	1.2
Tyrosine	24	75	±	25	<0.001	10	27	740	±	316	<0.001	8.4	24	90	±	46	0.986	1.2
HGA	24	2.5	±	1.4	0.861	13	27	34	±	14	0.008	0.14	24	240	±	202	0.003	96
HPLA	24		ND		NA	NA	27	72	±	49	<0.001	47	24	1.6	±	1.8	0.997	NA
HPPA	24		ND		NA	NA	27	114	±	75	<0.001	13	24	9	±	10	NA	NA
NTBC	24		ND		NA	NA	27	0.28	±	0.15	NA	NA	24		ND		NA	NA

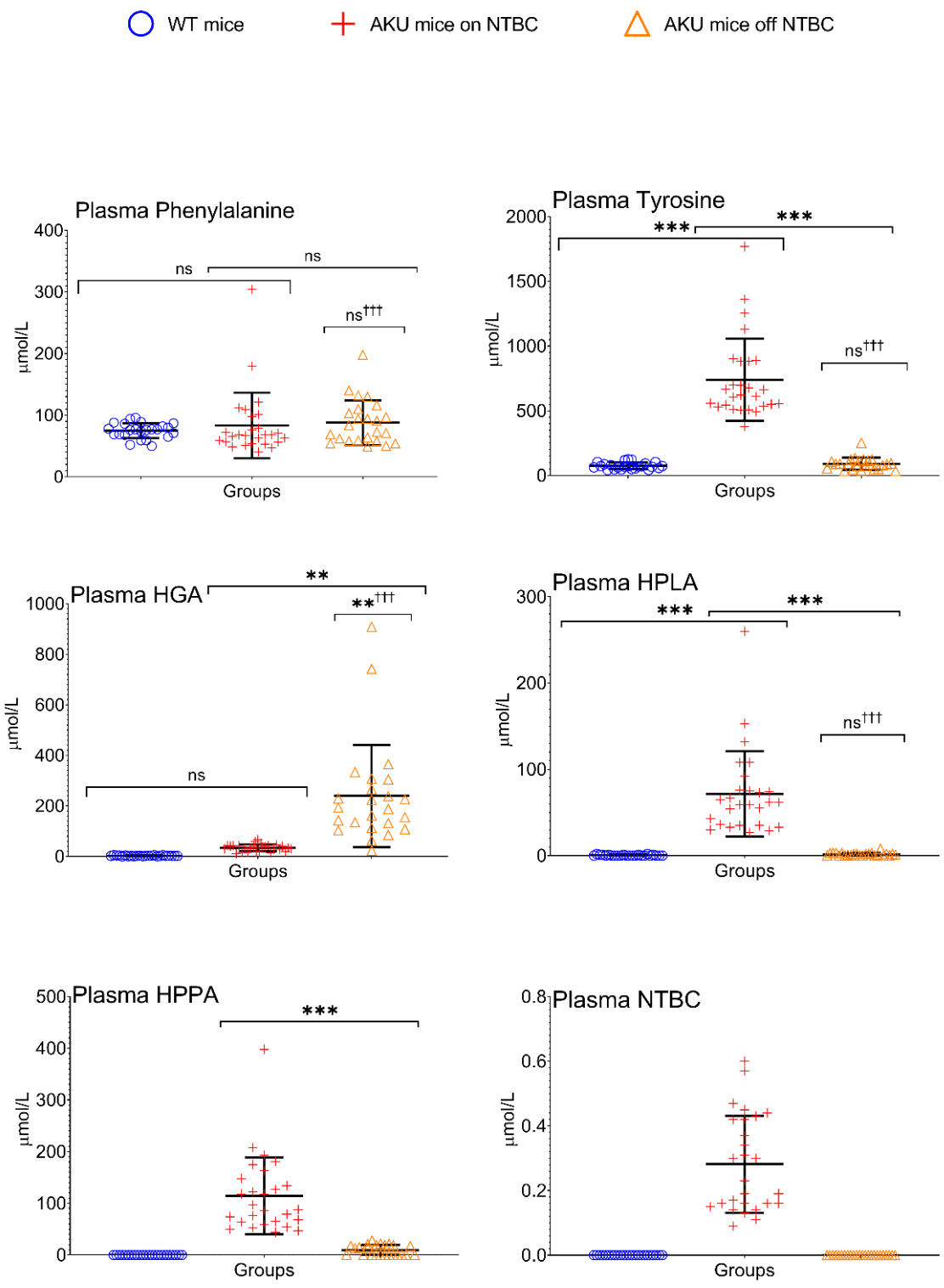

Figure 5:Summary of the plasma concentrations of NTBC and the native compounds in the tyrosine pathway in mice.
ANOVA with Tukey's multiple comparisons test was used to compare data in the three groups. ns: *$P \geq 0.05$, **$P < 0.01$, ***$P < 0.001$.††† AKU off NTBC compared to WT mice. Line and error bars are mean ± SD.

3.3.3 Effects of NTBC on tissue homogenates concentrations of native compounds in the tyrosine pathway in mice

3.3.3.1 *Effects of NTBC on tissue homogenates concentrations of phenylalanine*

The brain homogenate concentration of phenylalanine in the AKU mice on NTBC is 70% of that in the AKU mice off NTBC. There were no significant differences when comparing other tissues amongst the three groups. Figure 6 and Table 15 provides a summary of the tissue homogenates concentrations of phenylalanine in mice across the three groups.

3.3.3.1.1 Effects of NTBC on the femur bone homogenates concentrations of phenylalanine

There was no significant difference in phenylalanine concentrations of the bone homogenates between WT and AKU mice off NTBC (P> 0.999), WT and AKU mice on NTBC (P= 0.289) or NTBC-treated AKU and AKU mice off NTBC (P= 0.299).

3.3.3.1.2 Effects of NTBC on the brain homogenates concentrations of phenylalanine in

Mean phenylalanine concentration in the brain homogenates of AKU mice on NTBC is nearly 30% lower compared to AKU mice off NTBC (P= 0.006). There was no significant difference in brain homogenates concentrations of phenylalanine between WT and AKU mice off NTBC (P= 0.173). Similarly, there was no significant difference in phenylalanine concentrations in the brains between WT and AKU mice on NTBC (P= 0.167).

3.3.3.1.3 Effects of NTBC on heart homogenates concentrations of phenylalanine

There was no significant difference in phenylalanine concentrations of the heart homogenates between WT and AKU mice off NTBC (*P*= 0.727), WT and AKU mice on NTBC (*P*= 0.071), or NTBC-treated AKU and AKU mice off NTBC (*P*= 0.249).

3.3.3.1.4 Effects of NTBC on kidney homogenates concentrations of phenylalanine

There was no significant difference in phenylalanine concentrations of the kidney homogenates between WT and AKU mice off NTBC (*P*= 0.957), WT and AKU mice on NTBC (*P*= 0.679) or NTBC-treated AKU and AKU mice off NTBC (*P*= 0.845).

3.3.3.1.5 Effects of NTBC on liver homogenates concentrations of phenylalanine

There was no significant difference in phenylalanine concentrations of the liver homogenates between WT and AKU mice off NTBC (*P*= 0.876), WT and AKU mice on NTBC (*P*= 0.052), or NTBC-treated AKU and AKU mice off NTBC (*P*= 0.128).

3.3.3.1.6 Effects of NTBC on quadriceps muscle homogenates concentrations of phenylalanine

There was no significant difference in phenylalanine concentrations of the quadriceps muscle homogenates between WT and AKU mice off NTBC (*P*= 0.598), WT and AKU mice on NTBC (*P*= 0.870) or NTBC-treated AKU and AKU mice off NTBC (*P*= 0.882).

Table 15: Tissue homogenates concentrations of phenylalanine in mice.

WT: wild type mice, AKU on NTBC: AKU mice treated with NTBC, AKU off NTBC: AKU mice that were not treated with NTBC. Ordinary one-way ANOVA with Tukey's multiple comparisons test was used to compare data in the three groups. F1: the fold change in phenylalanine concentrations in the AKU on NTBC compared to WT mice, F2: the fold change in phenylalanine concentrations when comparing AKU on NTBC and AKU off NTBC mice, F3: the fold change in phenylalanine concentrations in the AKU off NTBC compared to WT mice. †Comparison of WT and AKU on NTBC mice. ††Comparison of AKU on NTBC and AKU off NTBC mice. †††Comparison of WT and AKU off NTBC mice.

Tissues	Phenylalanine concentrations (µmol/gram)															
	WT						AKU on NTBC						AKU off NTBC			
	n	Mean	±	SD	$p^{\dagger}$	F1	n	Mean	±	SD	$p^{\dagger\dagger}$	F2	n	Mean	±	SD	$p^{\dagger\dagger\dagger}$	F3
Femur bone	4	53	±	5.4	0.289	0.8	5	44	±	8.5	0.299	0.83	4	53	±	12	>0.999	1
Brain	4	42.2	±	5	0.167	0.8	5	34.6	±	5	0.006	0.69	4	50.2	±	7	0.173	1.2
Heart	4	92.1	±	11	0.071	0.7	5	64.6	±	14.7	0.249	0.78	4	83.2	±	22	0.727	0.9
Kidney	4	82.8	±	26	0.679	1.2	5	96.5	±	28.6	0.845	1.1	4	87.6	±	12	0.957	1.1
Liver	6	120	±	26	0.052	1.4	6	170	±	36.7	0.128	1.31	6	130	±	38	0.876	1.1
Quadriceps	6	78.4	±	17	0.870	1.1	6	89.1	±	47	0.882	0.9	6	99.3	±	40	0.598	1.3

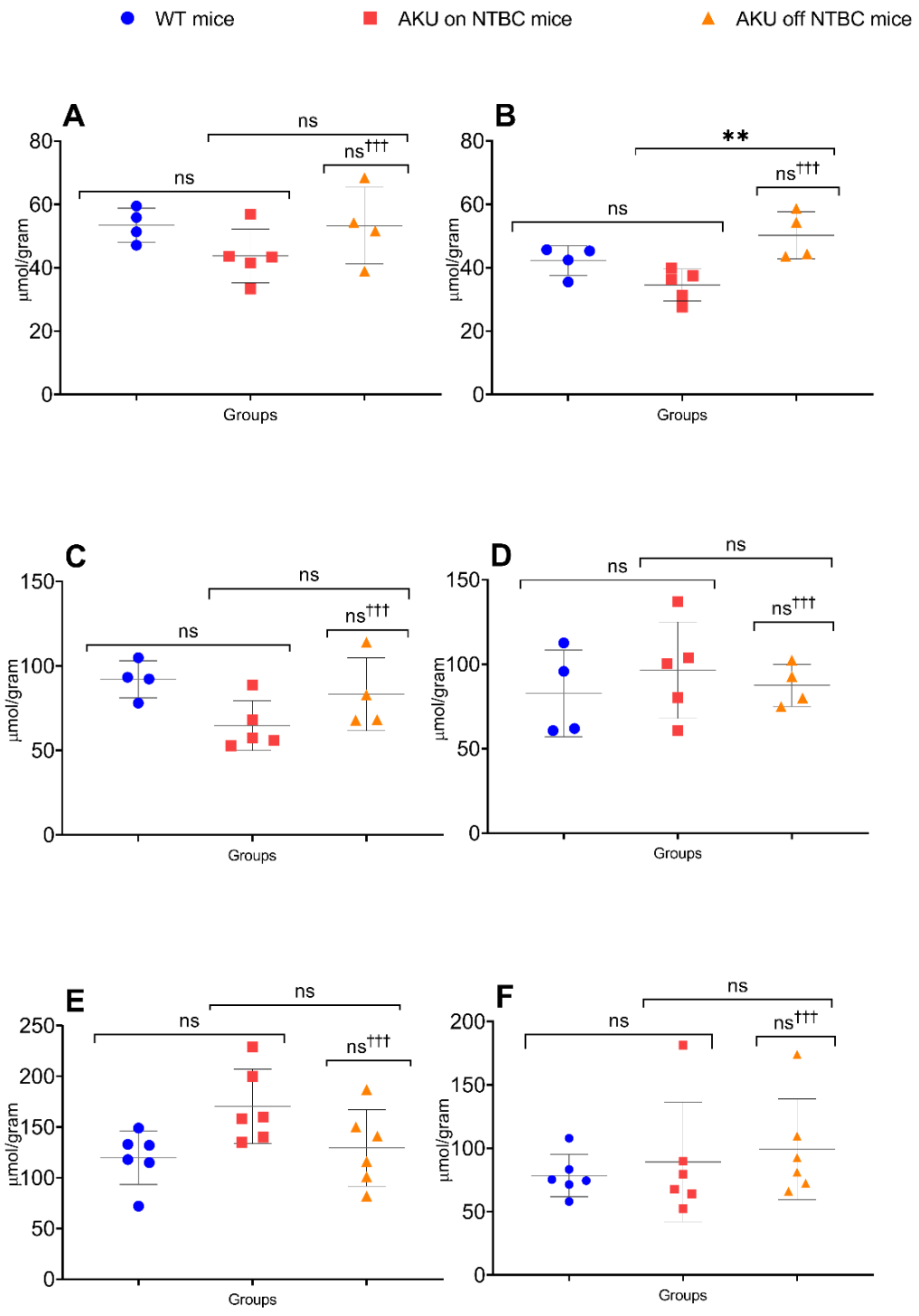

Figure 6: Tissue homogenates concentrations of phenylalanine in mice. (A) femur bone, (B) brain, (C) heart, (D) kidney, (E) liver, (F) quadriceps muscle. Line and error bars are mean ± SD. ns: *P ≥ 0.05, **P <0.01. Ordinary one-way ANOVA with Tukey's multiple comparisons test was used to compare data among the three groups. †††Comparison of WT and AKU off NTBC mice.

3.3.3.2 *Effects of NTBC on tissue homogenates concentrations of tyrosine in mice*

NTBC leads to five- to nine times increase in tyrosine concentrations in tissue homogenates in the AKU treated mice compared to the AKU mice off NTBC. There were no significant differences between the AKU mice off NTBC and WT mice. Notably, across the three groups, tyrosine concentrations are lower in the bone (due to a much lower water content compared to other tissues) and the brain (due to the presence of the blood brain barrier). Figure 7 and Table 16 provide a summary of the tissue homogenates concentrations of tyrosine in mice across the three groups.

3.3.3.2.1 Effects of NTBC on the femur bone homogenates concentrations of tyrosine in mice

Femur bone homogenates concentrations of tyrosine in mice were six times larger in AKU mice on NTBC compared to WT mice ($P< 0.001$), and 6.5 times larger compared to AKU mice off NTBC ($P< 0.001$). There was no significant difference in bone homogenates tyrosine concentrations between WT and AKU mice off NTBC ($P= 0.994$).

3.3.3.2.2 Effects of NTBC on the brain homogenates concentrations of tyrosine in mice

Brain homogenates concentrations of tyrosine in mice were nine times larger in AKU mice on NTBC compared to WT mice ($P< 0.001$), and nearly eight times larger compared to AKU mice off NTBC ($P< 0.001$). There was no significant difference in brain homogenates tyrosine concentrations between WT and AKU mice off NTBC ($P= 0.893$).

3.3.3.2.3 Effects of NTBC on the heart homogenates concentrations of tyrosine in mice

Heart homogenates concentrations of tyrosine in mice were nearly eight times larger in AKU mice on NTBC compared to WT mice ($P< 0.001$), and approximately nine times larger compared to AKU mice off NTBC ($P< 0.001$). There was no significant difference in heart homogenates concentrations of tyrosine between WT and AKU mice off NTBC ($P= 0.966$).

3.3.3.2.4 Effects of NTBC on the kidney homogenates concentrations of tyrosine in mice

Kidney homogenates concentrations of tyrosine in mice were seven times larger in AKU mice on NTBC compared to WT mice ($P< 0.001$) and, similarly, seven times larger compared to AKU mice off NTBC ($P< 0.001$). There was no significant difference in kidney homogenates concentrations of tyrosine between WT and AKU mice off NTBC ($P= 0.999$).

3.3.3.2.5 Effects of NTBC on the liver homogenates concentrations of tyrosine in mice

Liver homogenates concentrations of tyrosine in mice were almost six times larger in AKU mice on NTBC compared to WT mice ($P< 0.001$), and nearly five times larger compared to AKU mice off NTBC ($P< 0.001$). There was no significant difference in liver homogenates concentrations of tyrosine between WT and AKU mice off NTBC ($P= 0.940$).

3.3.3.2.6 Effects of NTBC on the quadriceps muscle homogenates concentrations of tyrosine in mice

Quadriceps muscle homogenates concentrations of tyrosine in mice were nearly nine times larger in AKU mice on NTBC compared to WT mice ($P< 0.001$), and six times larger compared to AKU mice off NTBC ($P< 0.001$). There was no significant difference in quadriceps muscle homogenates concentrations of tyrosine between WT and AKU mice off NTBC ($P= 0.883$).

Table 16: Tissue homogenates concentrations of tyrosine in mice.

WT: wild type mice, AKU on NTBC: AKU mice treated with NTBC, AKU off NTBC: AKU mice that were not treated with NTBC. Ordinary one-way ANOVA with Tukey's multiple comparisons test was used to compare data in the three groups. †Comparison of WT and AKU on NTBC mice. ††Comparison of AKU on NTBC and AKU off NTBC mice. †††Comparison of WT and AKU off NTBC mice. F1: the fold change in tyrosine concentrations in the AKU on NTBC mice compared to WT mice, F2: the fold change in tyrosine concentrations when comparing AKU on NTBC and AKU off NTBC mice, F3: the fold change in tyrosine concentrations in the AKU off NTBC compared to WT mice.

Tissues	Tyrosine concentrations (µmol/gram)															
	WT						AKU on NTBC						AKU off NTBC			
	n	Mean	±	SD	$p^{\dagger}$	F1	n	Mean	±	SD	$p^{\dagger\dagger}$	F2	n	Mean	±	SD	$p^{\dagger\dagger\dagger}$	F3
Femur bone	4	55	±	8	<0.001	6.3	5	347	±	44	<0.001	6.5	4	53	±	12	0.994	1.0
Brain	4	46	±	8	<0.001	9.2	5	421	±	37	<0.001	7.8	4	54	±	24	0.893	1.2
Heart	4	90	±	18	<0.001	7.7	5	689	±	100	<0.001	8.8	4	78	±	31	0.966	0.9
Kidney	4	109	±	31	<0.001	7.2	5	787	±	114	<0.001	7.4	4	106	±	20	0.999	1.0
Liver	6	153	±	36	<0.001	5.9	6	903	±	314	<0.001	4.8	6	189	±	59	0.94	1.2
Quadriceps	6	100	±	24	<0.001	8.9	6	887	±	271	<0.001	6.1	6	146	±	96	0.883	1.5

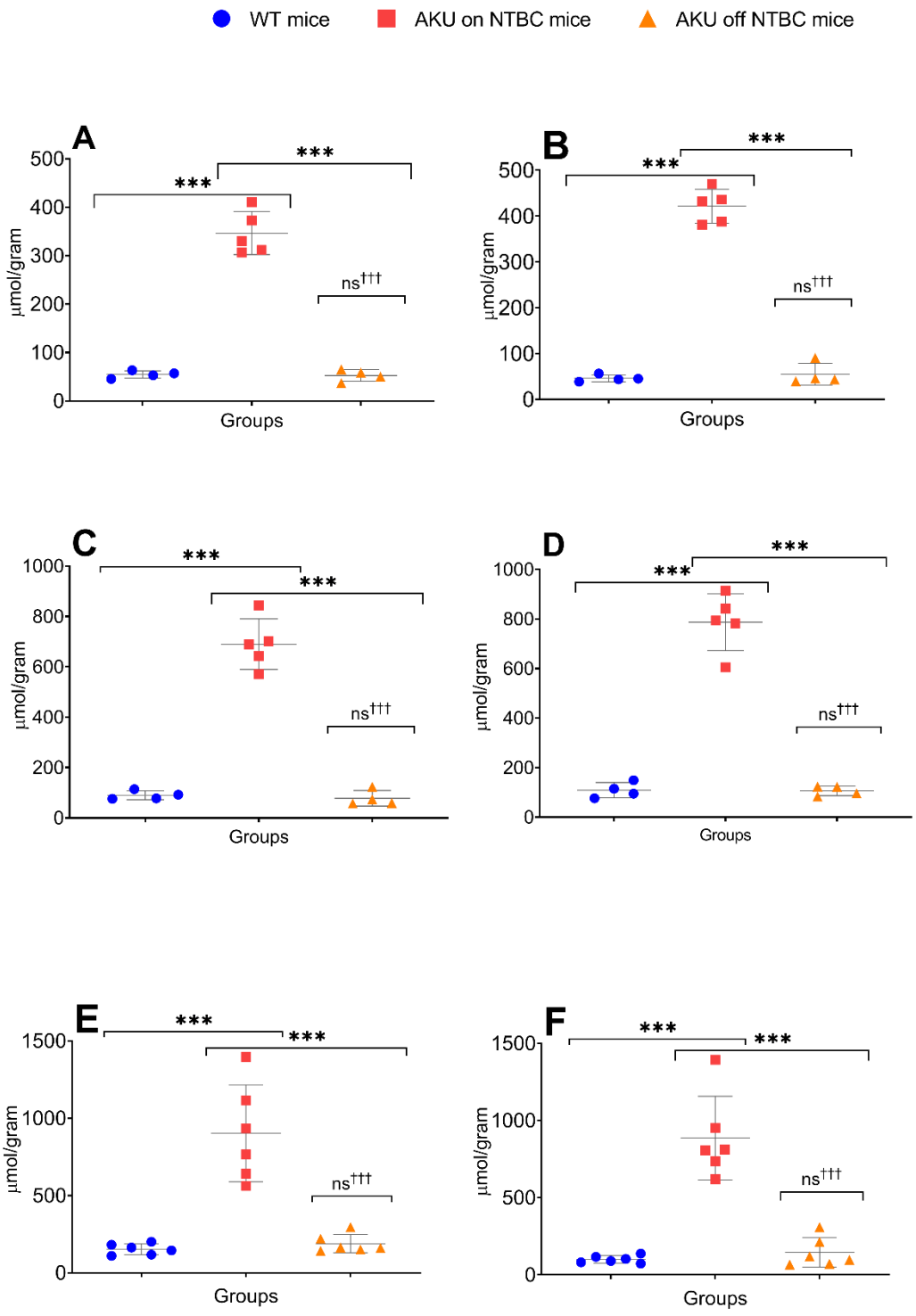

Figure 7: Tissue homogenates concentrations of tyrosine in mice.
(A) femur bone, (B) brain, (C) heart, (D) kidney, (E) liver, (F) quadriceps muscle. Line and error bars are mean ± SD. ns: *$P \geq 0.05$, ***$P < 0.001$. Ordinary one-way ANOVA with Tukey's multiple comparisons test was used to compare data among the three groups. †††Comparison of WT and AKU off NTBC mice.

3.3.3.3 *Effects of NTBC on the tissue homogenates concentrations of HGA in mice*

Tissue homogenates concentrations of HGA in the AKU mice off NTBC are two to fifty-three times larger compared to WT mice. There were no significant differences in tissue homogenates concentrations of HGA between the AKU mice on NTBC and WT mice. Figure 8 and Table 17 provide a summary of the tissue homogenates concentrations of HGA in mice across the three groups.

3.3.3.3.1 Effects of NTBC on the femur bone homogenates concentrations of HGA in mice

Femur bone homogenates concentrations of HGA in mice were seven times larger in AKU mice off NTBC compared to WT mice , and nearly six times larger compared to AKU mice on NTBC . There was no significant difference in femur bone homogenates concentrations of HGA between WT and AKU mice on NTBC .

3.3.3.3.2 Effects of NTBC on the brain homogenates concentrations of HGA in mice

Brain homogenates concentrations of HGA in mice were 2.3 times larger in AKU mice off NTBC compared to WT mice , and two times larger compared to AKU mice on NTBC . There was no significant difference in brain homogenates concentrations of HGA between WT and AKU mice on NTBC .

3.3.3.3.3 Effects of NTBC on the heart homogenates concentrations of HGA in mice

Heart homogenates concentrations of HGA were approximately five times larger in AKU mice off NTBC compared to WT mice , and four times larger

compared to AKU mice on NTBC . There was no significant difference in heart homogenates concentrations of HGA between WT and AKU mice on NTBC .

3.3.3.3.4 Effects of NTBC on the kidney homogenates concentrations of HGA in mice

Kidney homogenates concentrations of HGA were fifty-three times larger in AKU mice off NTBC compared to WT mice , and fifteen times larger compared to AKU mice on NTBC . The kidney homogenates concentrations of HGA in AKU mice on NTBC were 3.5 times larger compared to WT mice, though this difference was not statistically significant .

3.3.3.3.5 Effects of NTBC on the liver homogenates concentrations of HGA in mice

Liver homogenates concentrations of HGA were eighteen times larger in AKU mice off NTBC compared to WT mice , and seven times larger compared to AKU mice on NTBC . The liver homogenates concentrations of HGA in AKU mice on NTBC were 2.5 times larger compared to WT mice, though this difference was not statistically significant .

3.3.3.3.6 Effects of NTBC on the quadriceps muscle homogenates concentrations of HGA in mice

Quadriceps muscle homogenates concentrations of HGA were ten times larger in AKU mice off NTBC compared to WT mice ; and 6.5 times larger compared to AKU mice on NTBC. There was no significant difference in quadriceps muscle homogenates concentrations of HGA between WT and AKU mice on NTBC.

Table 17: Tissue homogenates concentrations of Homogentisic acid (HGA) in mice.WT: wild type mice, AKU on NTBC: AKU mice treated with NTBC, AKU off NTBC: AKU mice that were not treated with NTBC. Ordinary one-way ANOVA with Tukey's multiple comparisons test was used to compare data in the three groups. †Comparison of WT and AKU on NTBC mice. ††Comparison of AKU on NTBC and AKU off NTBC mice. †††Comparison of WT and AKU off NTBC mice. F1: the fold change in HGA concentrations in the AKU on NTBC mice compared to WT mice, F2: the fold change in HGA concentrations when comparing AKU on NTBC and AKU off NTBC mice, F3: the fold change in HGA concentrations in the AKU off NTBC compared to WT mice.

Tissues	HGA concentrations (µmol/gram)										
	WT			AKU on NTBC			AKU off NTBC				
	n	Mean	F1	n	Mean	F2	n	Mean		SD	F3
Femur bone	4	3	1.3	5	4	0.2	4	22	±	15	7.3
Brain	4	3	1.2	5	3	0.5	4	6	±	3	2.3
Heart	4	3	1.2	5	3	0.3	4	12	±	5	4.6
Kidney	4	3	3.5	5	9	0.1	4	138	±	122	53.2
Liver	6	2	2.5	6	5	0.1	6	36	±	10	18.0
Quadriceps	6	3	1.5	6	4	0.2	6	26	±	25	10.0

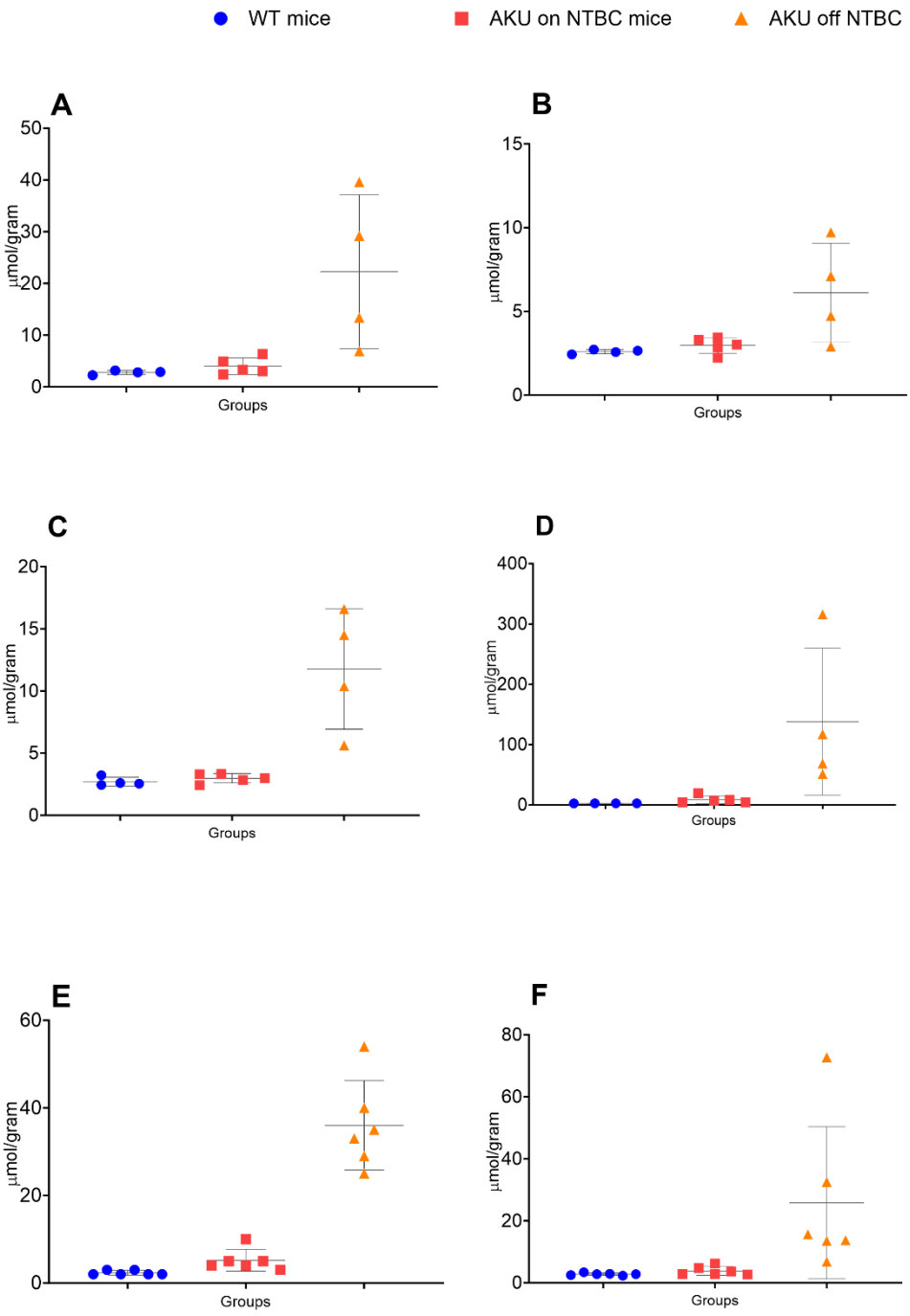

Figure 8: Tissue homogenates concentrations of Homogentisic acid (HGA) in mice.
(A) femur bone, (B) brain, (C) heart, (D) kidney, (E) liver, (F) quadriceps muscle. Line and error bars are mean ± SD.

3.3.3.4 *Effects of NTBC on tissue homogenates concentrations of HPLA in mice*

HPLA was not detectable in tissue homogenates of either WT mice or AKU mice off NTBC. In NTBC treated mice, kidney homogenates concentrations of HPLA were 4.4 times larger compared to liver homogenates and approximately five times larger compared to the quadriceps muscles homogenates. Figure 9 and Table 18 provide a summary of the tissue homogenates concentrations of HPLA in mice.

There is one outlier with a HPLA concentration of 25 µmol/gram in the heart homogenate of a mouse from the AKU off NTBC group. This was incongruous with HPLA data from the other heart homogenates and other tissues homogenates in the AKU off NTBC mice. On review of chromatography of samples from other mice in the AKU off NTBC group and the WT group, there were no discernible chromatographic peaks in the relevant transition windows. This suggests that this outlier is likely due to a pre- analytical error. To avoid skewing of results, it was discounted when calculating mean and SD of the group, but it was kept in the graphical representation to account for all data points.

Table 18: Tissue homogenates concentrations of hydroxyphenyllactic acid (HPLA) in mice.WT: wild type mice, AKU on NTBC: AKU mice treated with NTBC, AKU off NTBC: AKU mice that were not treated with NTBC. †One outlier removed (please see text for details). ND: not detectable.

Tissues	HPLA concentrations (µmol/gram)						
	WT		AKU on NTBC			AKU off NTBC	
	n	Mean	n	Mean	SD	n	Mean
Femur bone	4	ND	5	2	± 2	4	ND
Brain	4	ND	5	1	± 1	4	ND
Heart	4	ND	5	4	± 2	3†	ND
Kidney	6	ND	5	31	± 13	4	ND
Liver	6	ND	6	7	± 3	6	ND
Quadriceps	6	ND	6	6	± 4	6	ND

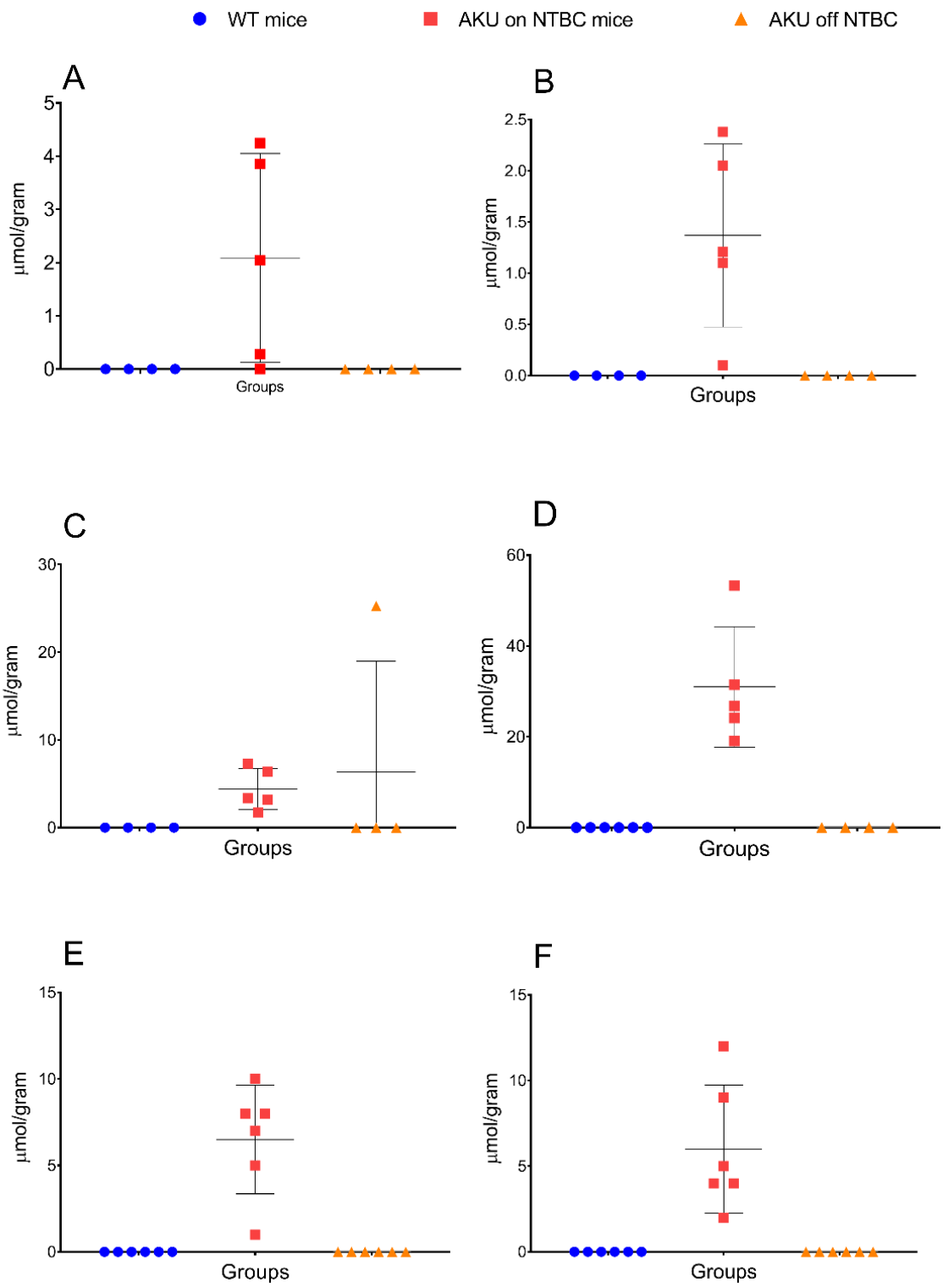

Figure 9: Tissue homogenates concentrations of hydroxyphenyllactic acid (HPLA) in mice. (A) femur bone, (B) brain, (C) heart, (D) kidney, (E) liver, (F) quadriceps muscle. Line and error bars are mean ± SD. One outlier in the AKU off NTBC group is included in panel C. There is one outlier in panel C: please see text for details.

3.3.3.5 *Effects of NTBC on the tissue homogenates concentrations of HPPA in mice*

Tissue homogenates concentrations of HPPA in the AKU mice on NTBC are nearly three times larger compared to WT mice and AKU mice off NTBC. There were no significant differences in tissue homogenates concentrations of HPPA between the AKU mice on NTBC and WT mice. Figure 10 and Table 19 provide a summary of the tissue homogenates concentrations of HPPA in mice across the three groups**.**

3.3.3.5.1 Effects of NTBC on the femur bone homogenates concentrations of HPPA in mice

There was no significant difference in the femur bone homogenates concentrations of HPPA between WT and AKU mice off NTBC (*P*= 0.839); WT and AKU mice on NTBC (*P*= 0.853); or non-treated AKU and AKU mice on NTBC (*P*= 0.511).

3.3.3.5.2 Effects of NTBC on the brain homogenates concentrations of HPPA in mice

There was no significant difference in the brain homogenates concentrations of HPPA between WT and AKU mice off NTBC (*P*=0.803); WT and AKU mice on NTBC (*P*= 0.473); or non-treated AKU and AKU mice on NTBC (*P*= 0.855).

3.3.3.5.3 Effects of NTBC on the heart homogenates concentrations of HPPA in mice

There was no significant difference in the heart homogenates concentrations of HPPA between WT and AKU mice off NTBC (*P*=0.997); WT and AKU mice on NTBC (*P*= 0.611); or non-treated AKU and AKU mice on NTBC (*P*= 0.660).

3.3.3.5.4 Effects of NTBC on the kidney homogenates concentrations of HPPA in mice

In AKU mice on NTBC, kidney homogenates concentrations of HPPA were nearly three times larger compared to WT mice (*P*= 0.020); and to AKU mice off NTBC (*P*= 0.020). There was no significant difference in the kidney homogenates concentrations of HPPA between WT and AKU mice on NTBC (*P*= 0.944).

3.3.3.5.5 Effects of NTBC on the liver homogenates concentrations of HPPA in mice

There was no significant difference in the liver homogenates concentrations of HPPA between WT and AKU mice off NTBC (*P*= 0.974); WT and AKU mice on NTBC (*P*= 0.489); or non-treated AKU and AKU mice on NTBC (*P*= 0.619).

3.3.3.5.6 Effects of NTBC on the quadriceps muscle homogenates concentrations of HPPA in mice

There was no significant difference in the quadriceps muscle homogenates concentrations of HPPA between WT and AKU mice off NTBC (*P*=0.997); WT and AKU mice on NTBC (*P*= 0.099); or non-treated AKU and AKU mice on NTBC (*P*= 0.086).

Table 19: Tissue homogenates concentrations of hydroxyphenylpyruvic acid (HPPA) in mice.WT: wild type mice, AKU on NTBC: AKU mice treated with NTBC, AKU off NTBC: AKU mice that were not treated with NTBC. †Comparison of WT and AKU on NTBC mice. ††Comparison of AKU on NTBC and AKU off NTBC mice. †††Comparison of WT and AKU off NTBC mice. Ordinary one-way ANOVA with Tukey's multiple comparisons test was used to compare data in the three groups. F1: the fold change in HPPA concentrations in the AKU on NTBC mice compared to WT mice, F2: the fold change in HPPA concentrations when comparing AKU on NTBC and AKU off NTBC mice, F3: the fold change in HPPA concentrations in the AKU off NTBC compared to WT mice.

Tissues	HPPA concentrations (µmol/gram)																
	WT						AKU on NTBC						AKU off NTBC					
	n	Mean		SD	$p^{\dagger}$	F1	n	Mean		SD	$p^{\dagger\dagger}$	F2	n	Mean		SD	$p^{\dagger\dagger\dagger}$	F3
Femur bone	4	18	±	7	0.853	1.1	5	20	±	8	0.511	1.3	4	16	±	1	0.839	0.9
Brain	4	15	±	1	0.473	0.9	5	14	±	1	0.855	0.9	4	15	±	1	0.803	1.0
Heart	4	15	±	1	0.611	1.1	5	17	±	6	0.660	1.2	4	15	±	2	0.997	1.0
Kidney	4	14	±	1	0.020	2.9	5	41	±	19	0.020	2.9	4	14	±	1	0.944	1.0
Liver	6	16	±	3	0.489	1.2	6	18	±	5	0.619	1.1	6	16	±	3	0.974	1.0
Quadriceps	6	15	±	2	0.099	1.4	6	20	±	6	0.086	1.4	6	15	±	3	0.997	1.0

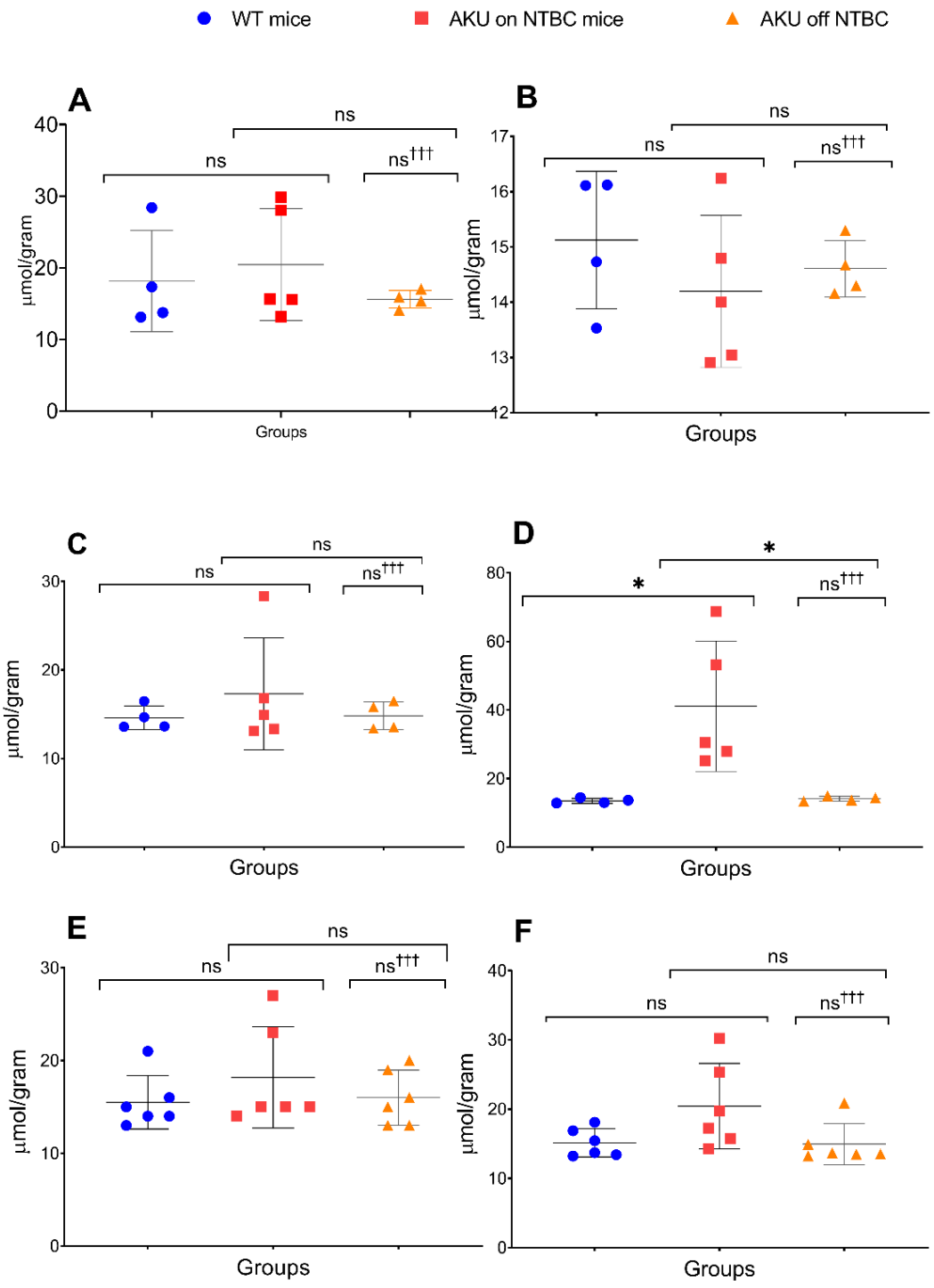

Figure 10: Tissue homogenates concentrations of hydroxyphenylpyruvic acid (HPPA) in mice.
(A) femur bone, (B) brain, (C) heart, (D) kidney, (E) liver, (F) quadriceps muscle. Line and error bars are mean ± SD. ns: $P \geq 0.05$, *$P < 0.05$. Ordinary one-way ANOVA with Tukey's multiple comparisons test was used to compare data among the three groups for all tissue except the kidney where ordinary one-way ANOVA with Holm-Sidak's multiple comparisons test. †††Comparison of WT and AKU off NTBC mice.

3.3.4 Comparison of the tyrosine pathway compounds concentrations in plasma against tissue homogenates in mice

The concentrations of compounds are expressed in µmol/L in plasma and in µmol/g in tissue homogenates. To aid comparison, plasma concentrations were converted from µmol/L to µmol/gram so comparison can be made with tissue homogenates concentrations. It was presumed that one litre of plasma weighs approximately one kilogram. It is important to bear in mind that water content of tissues varies with the skin, bone and adipose tissues were reported as having the lowest water content; whilst the lungs, heart, muscle, brain, liver and kidney were reported as having the highest water content (Reinoso et al., 1997). The difference in water content of tissues will be reflective in variable concentration of compounds across tissues.

3.3.4.1 *Comparison of phenylalanine concentrations in plasma against tissue homogenates in mice*

Phenylalanine concentration in tissue homogenates is markedly higher than plasma across all tissues and in all three study groups. To aid comparison, the Phenylalanine concentrations in tissue homogenates were divided by their plasma counterparts. Not surprisingly, the ratio of tissue homogenates to plasma concentrations of phenylalanine is ranges from 563 to 1600 in WT mice, 416 to 2048 in AKU mice on NTBC and 571 to 1477 in AKU mice off NTBC. This ratio tends to be the lowest on the bone homogenates and highest in liver homogenates across the three groups. This is summarised in Table 20 and Figure 11.

Table 20: Comparison of phenylalanine concentrations in the plasma and tissue homogenates in mice. WT: wild type mice, AKU on NTBC: AKU mice treated with NTBC, AKU off NTBC: AKU mice that were not treated with NTBC. $F1_{t\text{-}p}$: the fold change in tissue phenylalanine concentrations compared to plasma phenylalanine in the WT mice (obtained by dividing the tissue phenylalanine concentrations by plasma phenylalanine concentration in the WT mice), $F2_{t\text{-}p}$: the fold change in tissue phenylalanine concentrations compared to plasma phenylalanine in the AKU mice on NTBC (obtained by dividing the tissue phenylalanine concentrations by plasma phenylalanine concentration in the AKU mice on NTBC), $F3_{t\text{-}p}$: the fold change in tissue phenylalanine concentrations compared to plasma phenylalanine in the AKU mice off NTBC (obtained by dividing the tissue concentration of phenylalanine by plasma concentrations in the AKU mice off NTBC). ‡A total of twenty-four plasma samples were obtained from six mice in each of the AKU off NTBC group and the WT group. ‡‡A total of twenty-seven plasma samples were obtained from six mice in the AKU on NTBC group.

Tissues	Phenylalanine concentrations (µmol/gram)												
	WT					AKU on NTBC				AKU off NTBC			
	n	Mean	±	SD	$F1_{t\text{-}p}$	n	Mean	±	SD	$F2_{t\text{-}p}$	n	Mean	±	SD	$F3_{t\text{-}p}$
Femur bone	4	53	±	5	707	5	44	±	9	530	4	53	±	12	602
Brain	4	42	±	5	563	5	35	±	5	416	4	50	±	7	571
Heart	4	92	±	11	1,228	5	65	±	15	779	4	83	±	22	946
Kidney	4	83	±	26	1,104	5	97	±	29	1,163	4	88	±	12	995
Liver	6	120	±	26	1,600	6	170	±	37	2,048	6	130	±	38	1,477
Quadriceps	6	78	±	17	1,045	6	89	±	47	1,073	6	99	±	40	1,128
	Phenylalanine concentrations (µmol/gram)												
Plasma	6‡	0.075	±	0.012	NA	6‡‡	0.083	±	0.053	NA	6‡	0.088	±	0.036	NA

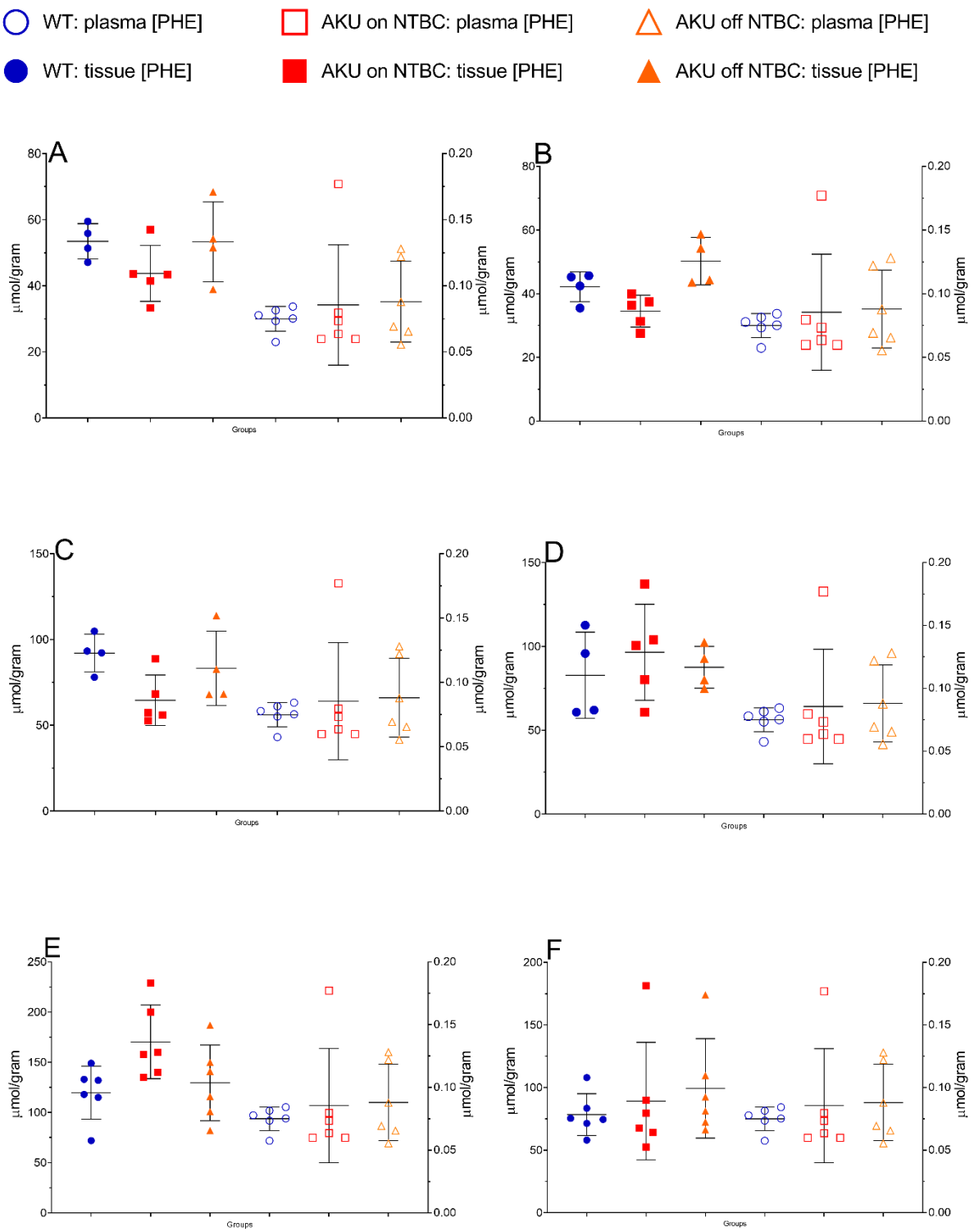

Figure 11: Comparison of phenylalanine (PHE) concentrations in plasma against tissue homogenates.
(A) plasma versus femur bone, (B) plasma versus brain, (C) plasma versus heart, (D) plasma versus kidney, (E) plasma versus liver, (F) plasma versus quadriceps muscle. Line and error bars are mean ± SD. Left Y axis is homogenates concentrations; right Y axis is plasma concentrations.

3.3.4.2 *Comparison of tyrosine concentrations in plasma against tissue homogenates in mice*

To aid comparison, the tyrosine concentration in tissue homogenates were divided by their plasma counterparts. The ratio of homogenate to plasma concentrations of tyrosine ranges from 613 (brain homogenates) to 2040 (Liver) in WT mice, 469 (bone homogenates) to 1220 (Liver homogenates) in AKU mice on NTBC and 589 (bone homogenates) to 2100 (Liver homogenates) in AKU mice off NTBC. This is summarised in Table 21 and Figure 12.

Table 21: Comparison of tyrosine concentrations in the plasma and tissue homogenates in mice.
WT: wild type mice, AKU on NTBC: AKU mice treated with NTBC, AKU off NTBC: AKU mice that were not treated with NTBC. (F1t-p): the fold change in tissue tyrosine concentrations compared to plasma in the WT mice (obtained by dividing the tissue tyrosine concentration by plasma tyrosine concentrations in WT mice), (F2t-p): the fold change in tissue tyrosine concentrations compared to plasma in the AKU mice on NTBC (obtained by dividing the tissue tyrosine concentration by plasma concentrations in the AKU mice on NTBC), (F3t-p): the fold change in tissue tyrosine concentrations compared to plasma tyrosine in the AKU mice off NTBC (obtained by dividing the tissue tyrosine concentration by plasma tyrosine concentrations in the AKU mice off NTBC). ‡A total of twenty-four plasma samples were obtained from six mice in each of the AKU off NTBC group and the WT group. ‡‡A total of twenty-seven plasma samples were obtained from six mice in the AKU on NTBC group.

	Tyrosine concentrations (µmol/gram)													
Tissues	WT					AKU on NTBC					AKU off NTBC				
	n	Mean		SD	$F1_{t\text{-}p}$	n	Mean		SD	$F2_{t\text{-}p}$	n	Mean		SD	$F3_{t\text{-}p}$
Femur bone	4	55	±	8	733	5	347	±	44	469	4	53	±	12	589
Brain	4	46	±	8	613	5	421	±	37	569	4	54	±	24	600
Heart	4	90	±	18	1,200	5	689	±	100	931	4	78	±	31	867
Kidney	4	109	±	31	1,453	5	787	±	114	1,064	4	106	±	20	1,178
Liver	6	153	±	36	2,040	6	903	±	314	1,220	6	189	±	59	2,100
Quadriceps	6	100	±	24	1,333	6	887	±	271	1,199	6	146	±	96	1,622
	Tyrosine concentrations (µmol/gram)														
Plasma	6‡	0.075	±	0.025	NA	6‡‡	0.740	±	0.316	NA	6‡	0.090	±	0.046	NA

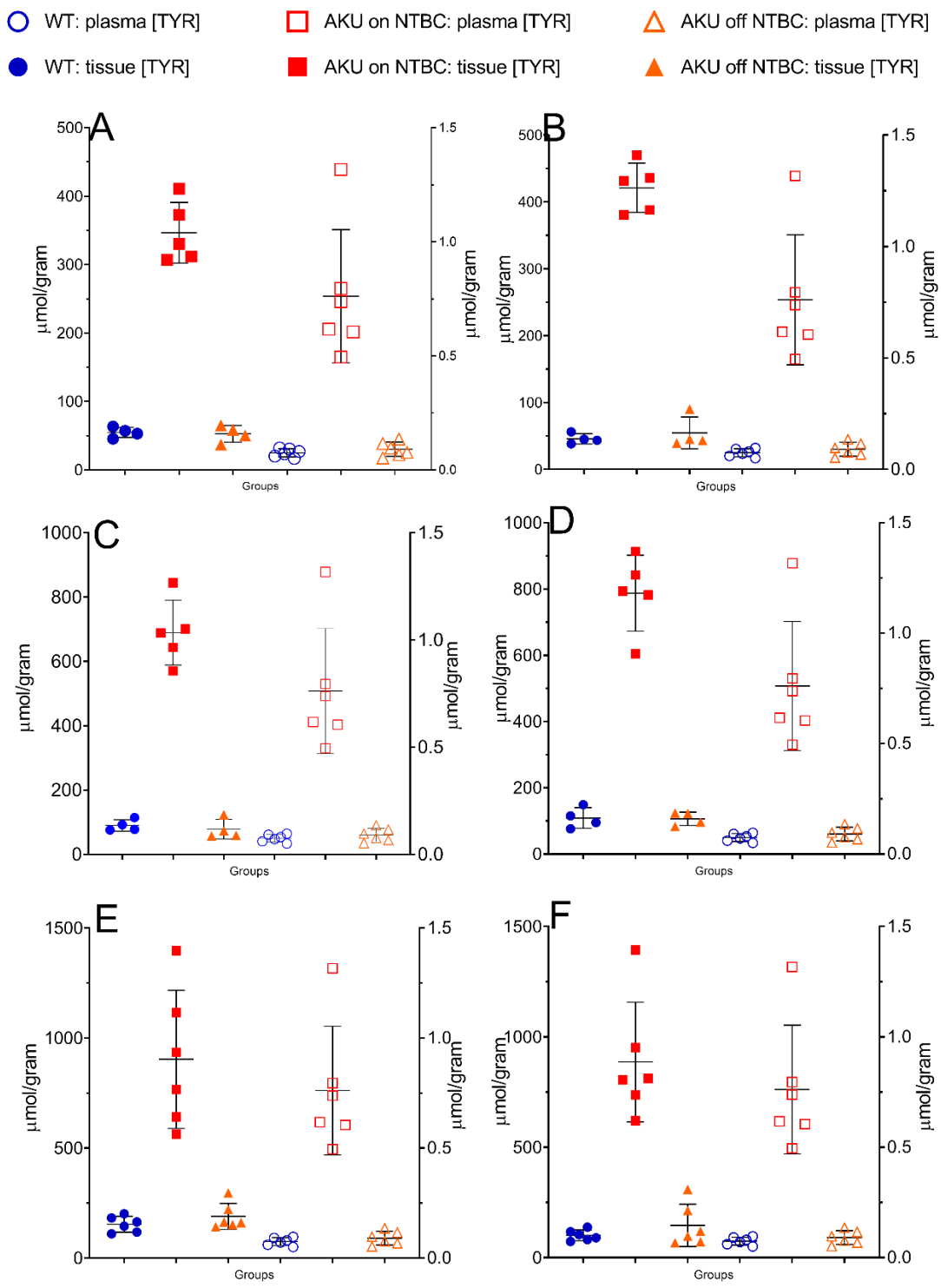

Figure 12: Comparison of tyrosine (TYR) concentrations in plasma against tissue homogenates.

(A) plasma versus femur bone, (B) plasma versus brain, (C) plasma versus heart, (D) plasma versus kidney, (E) plasma versus liver, (F) plasma versus quadriceps muscle. Line and error bars are mean ± SD. Left Y axis is homogenates concentrations, right Y axis is plasma concentrations. Plasma concentration in µmol/gram were obtained by dividing concentrations in µM by 1000. It was presumed that 1L of plasma weighs 1kg.

3.3.4.3 *Comparison of HGA concentrations in plasma against tissue homogenates in mice*

To aid comparison, the HGA concentrations in tissue homogenates were divided by their plasma counterparts. The ratio of homogenate to plasma concentrations of HGA ranges from 667 (liver homogenates) to 1000 (bone homogenates) in WT mice, 88 (brain and heart homogenates) to 265 (kidney homogenates) in AKU mice on NTBC and 25 (brain homogenates) to 575 (kidney homogenates) in AKU mice off NTBC. This is summarised in Table 22 and Figure 13.

The number of folds change between tissue homogenates and plasma concentrations of HGA is more evident in the WT groups and in the liver and kidney tissue homogenates of the AKU mice regardless of NTBC therapy. This reflects that both kidney and liver produce HGA. This is in addition to the larger intracellular compartment containing HGA, a small molecule that is distributing freely in water. Furthermore, it is possible that urine contaminating the kidney homogenates is contributing to high HGA concentrations. Both liver and kidney have rich blood supply and the possibility that HGA plasma, as a results, is also included when measuring kidney and liver HGA tissue homogenates.

Table 22: Comparison of HGA concentrations in the plasma and tissue homogenates in mice.

WT: wild type mice, AKU on NTBC: AKU mice treated with NTBC, AKU off NTBC: AKU mice that were not treated with NTBC. ($F1_{t\text{-}p}$): the fold change in tissue HGA concentrations compared to plasma in the WT mice (obtained by dividing the tissue HGA concentration by plasma HGA concentrations in WT mice), ($F2_{t\text{-}p}$): the fold change in tissue HGA concentrations compared to plasma HGA in the AKU mice on NTBC (obtained by dividing the tissue HGA concentration by plasma HGA concentrations in the AKU mice on NTBC), ($F3_{t\text{-}p}$): the fold change in tissue HGA concentrations compared to plasma HGA in the AKU mice off NTBC (obtained by dividing the tissue HGA concentration by plasma HGA concentrations in the AKU mice off NTBC). ‡A total of twenty-four plasma samples were obtained from six mice in each of the AKU off NTBC group and the WT group. ‡‡A total of twenty-seven plasma samples were obtained from six mice in the AKU on NTBC group.

Tissues	HGA concentrations (µmol/gram)											
	WT			AKU on NTBC			AKU off NTBC					
	n	Mean	$F1_{t\text{-}p}$	n	Mean	$F2_{t\text{-}p}$	n	Mean		SD	$F3_{t\text{-}p}$
Femur bone	4	3	1000	5	4	118	4	22	±	15	92
Brain	4	3	864	5	3	88	4	6	±	3	25
Heart	4	3	864	5	3	88	4	12	±	5	50
Kidney	4	3	864	5	9	265	4	138	±	122	575
Liver	6	2	667	6	5	147	6	36	±	10	150
Quadriceps	6	3	864	6	4	118	6	26	±	25	108
	HGA concentrations (µmol/gram)										
Plasma	6‡	0.003	± 0.001	NA	6‡‡	0.034	± 0.014	NA	6‡	0.240	± 0.202	NA

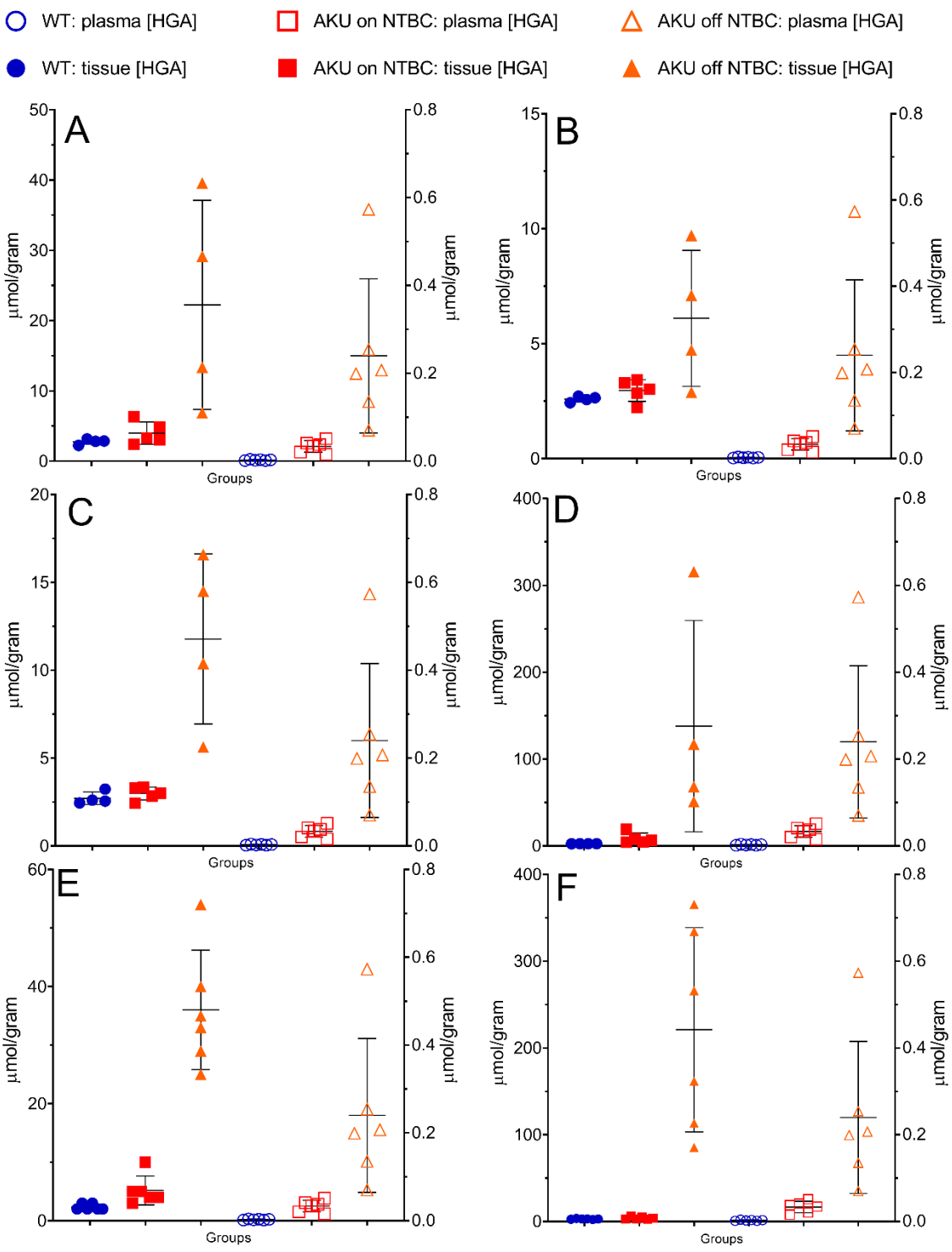

Figure 13: Comparison of HGA concentrations in plasma against tissue homogenates.

(A) plasma versus femur bone, (B) plasma versus brain, (C) plasma versus heart, (D) plasma versus kidney, (E) plasma versus liver, (F) plasma versus quadriceps muscle. Line and error bars are mean ± SD. Left Y axis is homogenates concentrations; right Y axis is plasma concentrations.

3.3.4.4 *Comparison of HPLA concentrations in plasma against tissue homogenates in mice*

To aid comparison, the HPLA concentrations in tissue homogenates were divided by their plasma counterparts. HPLA was not detectable in tissue homogenates of the WT mice or the AKU mice off NTBC. In the AKU mice on NTBC, the ratio of homogenate to plasma concentrations of HPLA ranges from 14 in the brain to 431 in the kidneys. This is summarised in Figure 14 and Table 14.

Table 23: Comparison of HPLA concentrations in the plasma and tissue homogenates in mice.
WT: wild type mice, AKU on NTBC: AKU mice treated with NTBC, AKU off NTBC: AKU mice that were not treated with NTBC. NA: not available ,($F1_{t\text{-}p}$): the fold change in tissue HPLA concentrations compared to plasma in the WT mice (obtained by dividing the tissue HPLA concentration by plasma HPLA concentrations in WT mice), ($F2_{t\text{-}p}$): the fold change in tissue HPLA concentrations compared to plasma HPLA in the AKU mice on NTBC, ($F3_{t\text{-}p}$): the fold change in tissue HPLA concentrations compared to plasma HPLA in the AKU mice off NTBC. ‡A total of twenty-four plasma samples were obtained from six mice in each of the AKU off NTBC group and the WT group. ‡‡A total of twenty-seven plasma samples were obtained from six mice in the AKU on NTBC group, ND: not detected.

	HPLA concentrations (µmol/gram)											
Tissues	WT			AKU on NTBC					AKU off NTBC			
	n	Mean	$F1_{t\text{-}p}$	n	Mean		SD	$F2_{t\text{-}p}$	n	Mean		$F3_{t\text{-}p}$
Femur bone	4	ND	NA	5	2	±	2	28	4	ND		NA
Brain	4	ND	NA	5	1	±	1	14	4	ND		NA
Heart	4	ND	NA	5	4	±	2	56	3	ND		NA
Kidney	6	ND	NA	5	31	±	13	431	4	ND		NA
Liver	6	ND	NA	6	7	±	3	97	6	ND		NA
Quadriceps	6	ND	NA	6	6	±	4	83	6	ND		NA
	HPLA concentrations (µmol/gram)											
Plasma	6‡	ND	NA	6‡‡	0.072	±	0.049	NA	6‡	0.002	± 0.002	NA

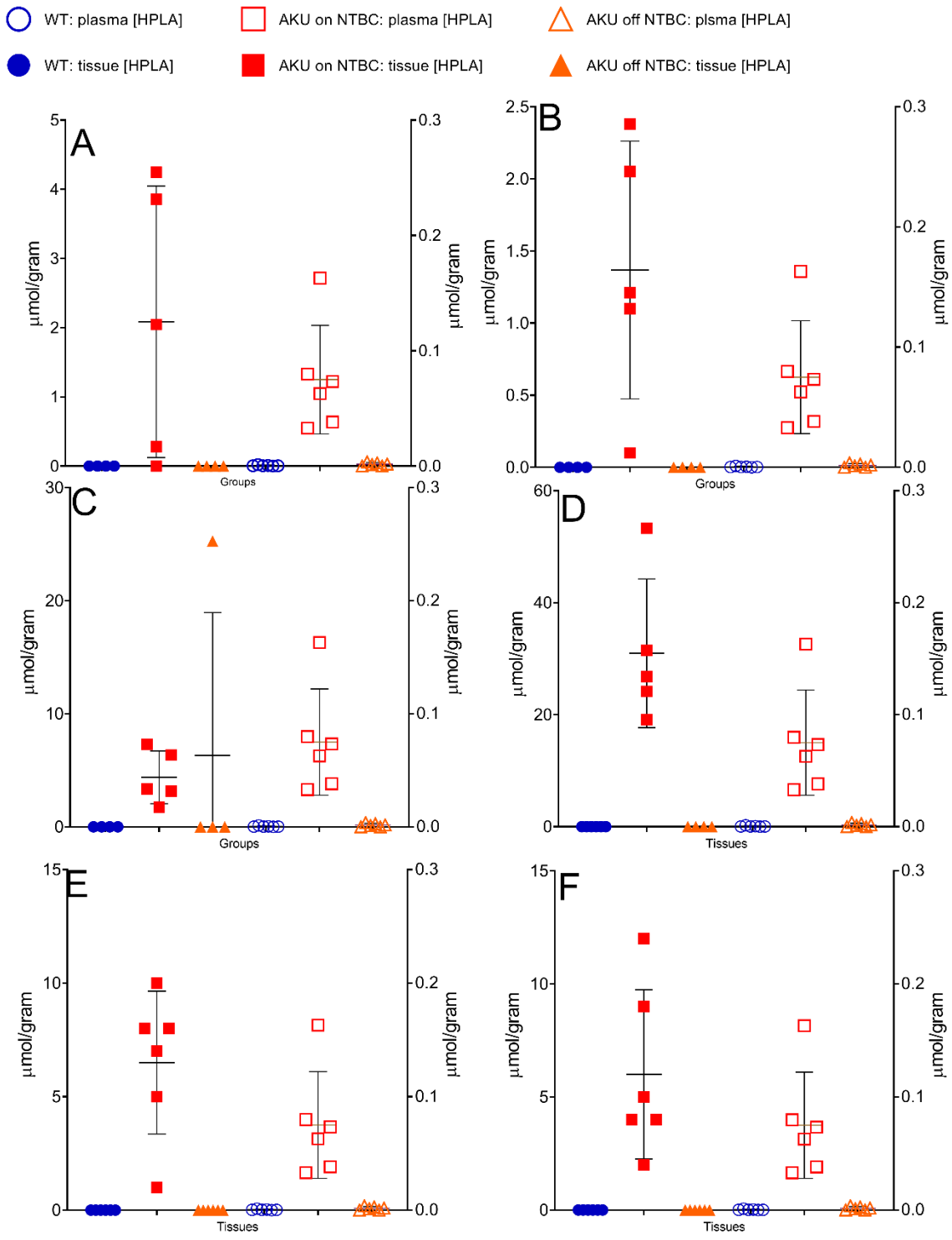

Figure 14: Comparison of HPLA concentrations in plasma against tissue homogenates.

(A) plasma versus femur bone, (B) plasma versus brain, (C) plasma versus heart, (D) plasma versus kidney, (E) plasma versus liver, (F) plasma versus quadriceps muscle. Line and error bars are mean ± SD. Left Y axis is homogenates concentrations; right Y axis is plasma concentrations. Plasma concentration in µmol/gram were obtained by dividing concentrations in µM by 1000. It was presumed that 1L of plasma weighs 1kg.

3.3.4.5 *Comparison of HPPA concentrations in plasma against tissue homogenates in mice*

To aid comparison, the HPPA concentrations in tissue homogenates were divided by their plasma counterparts. HPPA was not detectable in the plasma of the WT group. The ratio of homogenate to plasma concentrations of HPPA ranges from 123 to 361 in the AKU mice on NTBC and 1556 to 1778 in AKU mice off NTBC. This is summarised in Figure 15 and Table 24.

Table 24: Comparison of HPPA concentrations in the plasma and tissue homogenates in mice.
WT: wild type mice, AKU on NTBC: AKU mice treated with NTBC, AKU off NTBC: AKU mice that were not treated with NTBC. ($F1_{t\text{-}p}$): the fold change in tissue HPPA concentrations compared to plasma in the WT mice (NA: it was not calculated as plasma HPLA concentration was below detection limits), ($F2_{t\text{-}p}$): the fold change in tissue HPPA concentrations compared to plasma HPPA in the AKU mice on NTBC (obtained by dividing the tissue HPPA concentration by plasma HPPA concentrations in the AKU mice on NTBC), ($F3_{t\text{-}p}$): the fold change in tissue HPPA concentrations compared to plasma HPPA in the AKU mice off NTBC (obtained by dividing the tissue HPPA concentration by plasma HPPA concentrations in the AKU mice off NTBC). ‡A total of twenty-four plasma samples were obtained from six mice in each of the AKU off NTBC group and the WT group. ‡‡A total of twenty-seven plasma samples were obtained from six mice in the AKU on NTBC group. NA: not available, ND: not detected.

	HPPA concentrations (µmol/gram)													
Tissues	WT					AKU on NTBC					AKU off NTBC				
	n	Mean		SD	$F1_{t\text{-}p}$	n	Mean		SD	$F2_{t\text{-}p}$	n	Mean		SD	$F3_{t\text{-}p}$
Femur bone	4	18	±	7	NA	5	20	±	8	180	4	16	±	1	1736
Brain	4	15	±	1	NA	5	14	±	1	123	4	15	±	1	1680
Heart	4	15	±	1	NA	5	17	±	6	152	4	15	±	2	1667
Kidney	4	14	±	1	NA	5	41	±	19	361	4	14	±	1	1556
Liver	6	16	±	3	NA	6	18	±	5	160	6	16	±	3	1778
Quadriceps	6	15	±	2	NA	6	20	±	6	179	6	15	±	3	1667
	HPPA concentrations (µmol/gram)														
Plasma	6	ND			NA	6‡	0.114	±	0.075	NA	6‡	0.009	±	0.010	NA

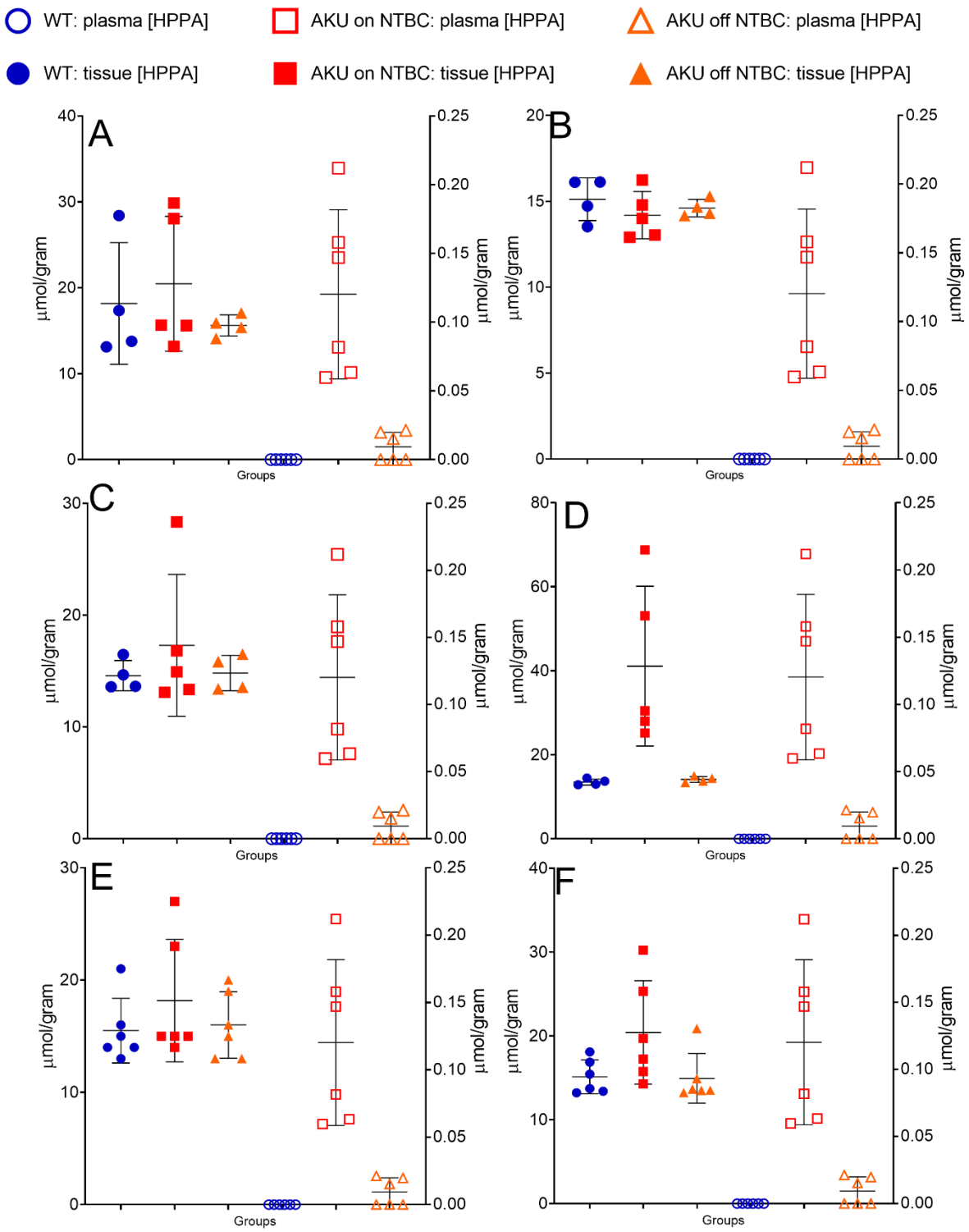

Figure 15: Comparison of HPPA concentrations in plasma against tissue homogenates.

(A) plasma versus femur bone, (B) plasma versus brain, (C) plasma versus heart, (D) plasma versus kidney, (E) plasma versus liver, (F) plasma versus quadriceps muscle. Line and error bars are mean ± SD. Left Y axis is homogenates concentrations; right Y axis is plasma concentrations.

3.3.5 Comparison of NTBC concentrations across plasma and tissue homogenates in mice

NTBC was present in all tissue of the AKU mice on NTBC but not in WT mice. In the AKU mice off NTBC, NTBC was only detected in the liver homogenates of two mice who, one week prior to the experiment, have received NTBC for only three days. The remaining four mice in the AKU mice off NTBC had no detectable NTBC in plasma or any of the examined tissues. This is summarised in Figure 16 and Table 25.

Table 25: NTBC concentrations across plasma and tissue homogenates in mice.

WT: wild type mice, AKU on NTBC: AKU mice treated with NTBC, AKU off NTBC: AKU mice that were not treated with NTBC. ($F_{t\text{-}p}$): the fold change in tissue NTBC concentrations compared to plasma in the AKU mice on NTBC (obtained by dividing the tissue NTBC concentration by plasma NTBC concentrations in the AKU mice on NTBC), ND: not detected. † Data taken from six mice with a total number of twenty-seven blood sampling points. √

	NTBC concentrations (µmol/gram)										
Tissues	WT		AKU on NTBC					AKU off NTBC			
	n	Mean	n	Mean		SD	$F_{t\text{-}p}$	n	Mean		SD
Femur bone	4	ND	5	0.6	±	0.1	1,867	4	ND		
Brain	4	ND	5	0.8	±	0.2	2,800	4	ND		
Heart	4	ND	5	2.2	±	0.4	7,333	4	ND		
Kidney	6	ND	5	7.1	±	2.8	23,667	4	ND		
Liver	6	ND	6	55.0	±	26.0	183,333	6	13.0	±	20.0
Quadriceps	6	ND	6	6.0	±	3.7	20,000	6	ND		
	NTBC concentrations (µmol/gram)										
Plasma	6	ND	6†	0.0003	±	0.0002	-	6	ND		

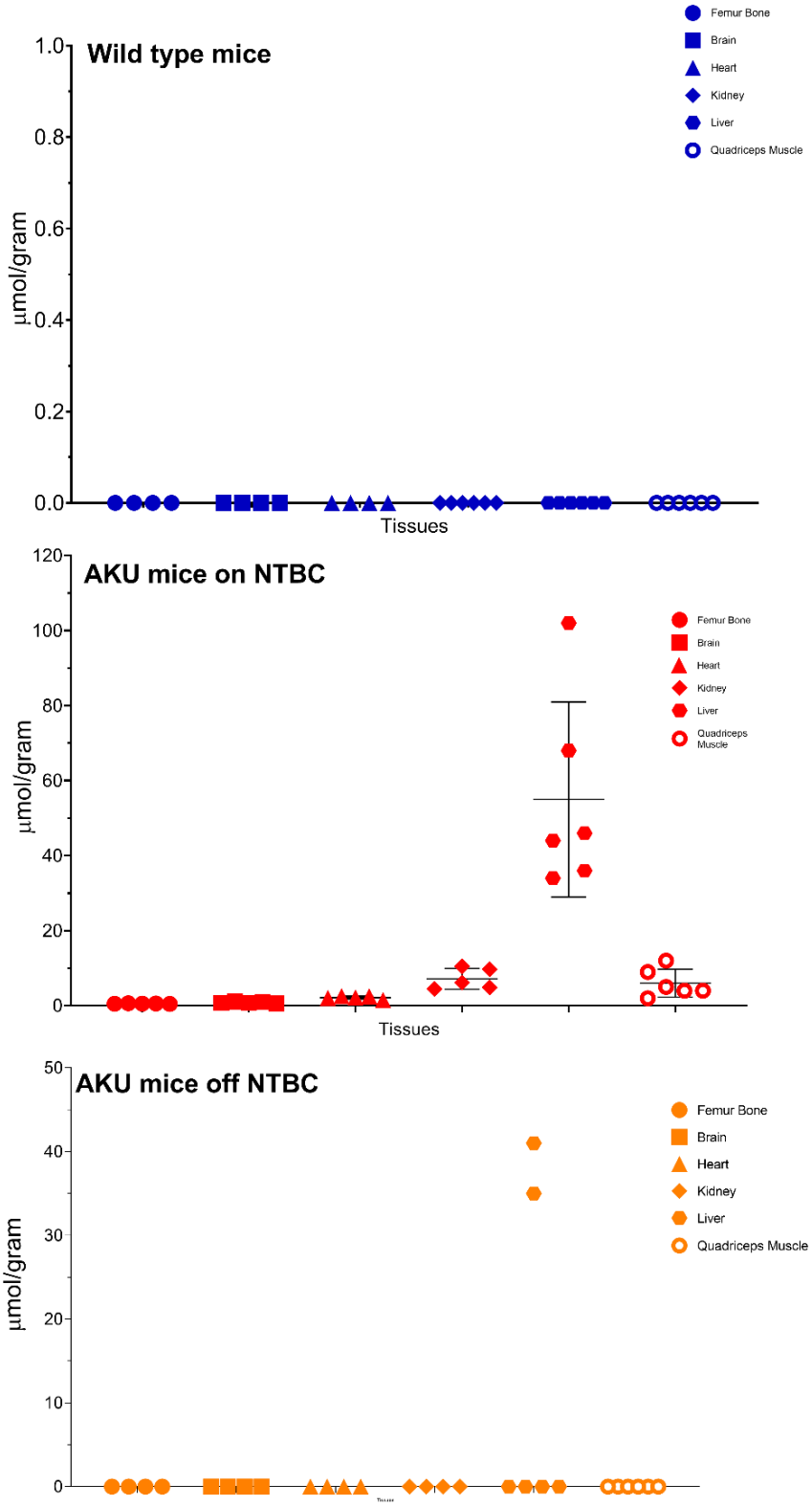

Figure 16: Comparison of NTBC concentrations across all tissue homogenates in mice.
Line and error bars are mean ± SD.

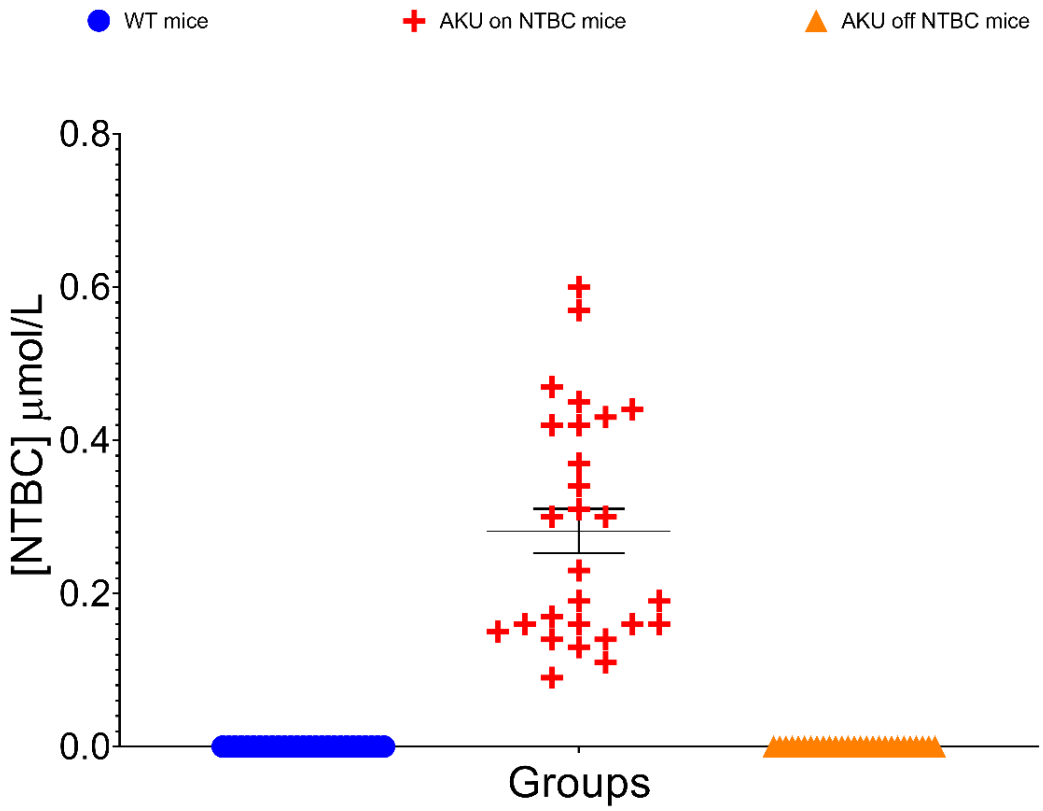

Figure 17: Comparison of plasma NTBC concentrations across the study groups in mice. Line and error bars are mean ± SD.

3.3.6 Comparison of the tyrosine pathway compounds concentrations across all tissue homogenates in mice

3.3.6.1.1 Comparison of phenylalanine concentrations across all tissue homogenates in mice

Across the three groups, phenylalanine concentrations are highest in the liver homogenates with a range of 120-170 µmol/L. This is followed by the kidney, heart, and quadriceps muscle homogenates with a range of 78 to 92 µmol/L in WT mice, 65 to 97 µmol/L in the AKU mice on NTBC and 83 to 99 µmol/L in the AKU mice off NTBC. The femur bone and brain homogenates have the lowest phenylalanine concentrations with a range of 42-53 µmol/L in WT mice, 35 to 44 µmol/L in the AKU mice on NTBC and 50 to 53 µmol/L in the AKU mice off NTBC. This is summarised in Figure 18.

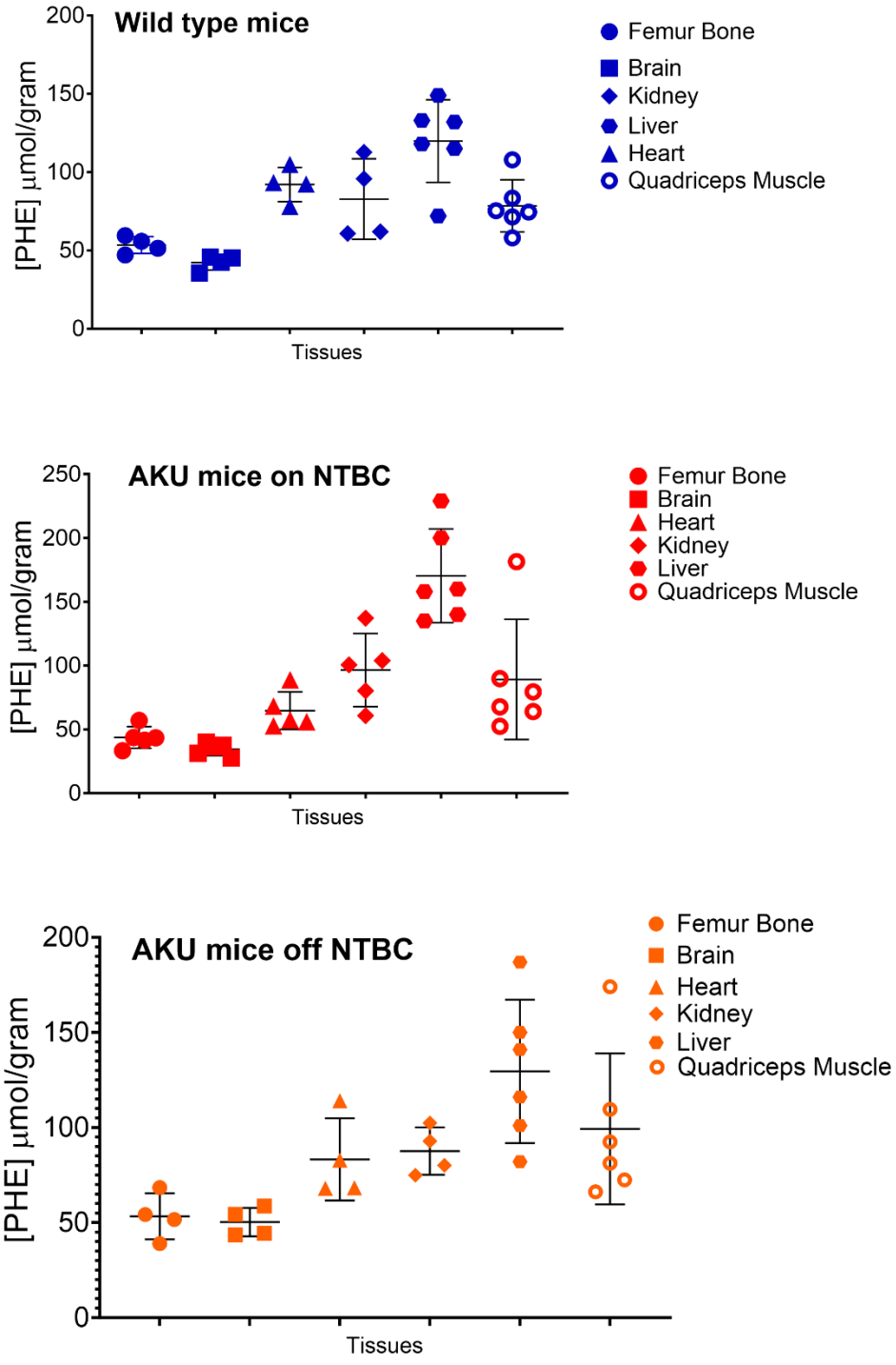

Figure 18: Comparison of phenylalanine concentrations across all murine tissue homogenates in the three groups.
Line and error bars are mean ± SD.

3.3.6.1.2 Comparison of tyrosine concentrations across all tissue homogenates in mice

Across the three groups, tyrosine concentrations are highest in the liver, quadriceps muscle and kidney homogenates with a range of 100-153 μmol/L in WT mice, 787-903 μmol/L in AKU mice on NTBC and 106-189 μmol/L in AKU mice off NTBC. This is followed by the heart homogenates (mean values are 90 μmol/L in WT, 78 μmol/L in AKU mice on NTBC and 689 μmol/L in AKU mice off NTBC. The femur bone and brain homogenates have the lowest tyrosine concentrations with a range of 46-55 μmol/L in WT mice, 421 to 347 μmol/L in the AKU mice on NTBC and 53 to 54 μmol/L in the AKU mice off NTBC. This is summarised in Figure 19 .These differences are likely due to low water content in bones and the presence of the blood brain barrier in the brain.

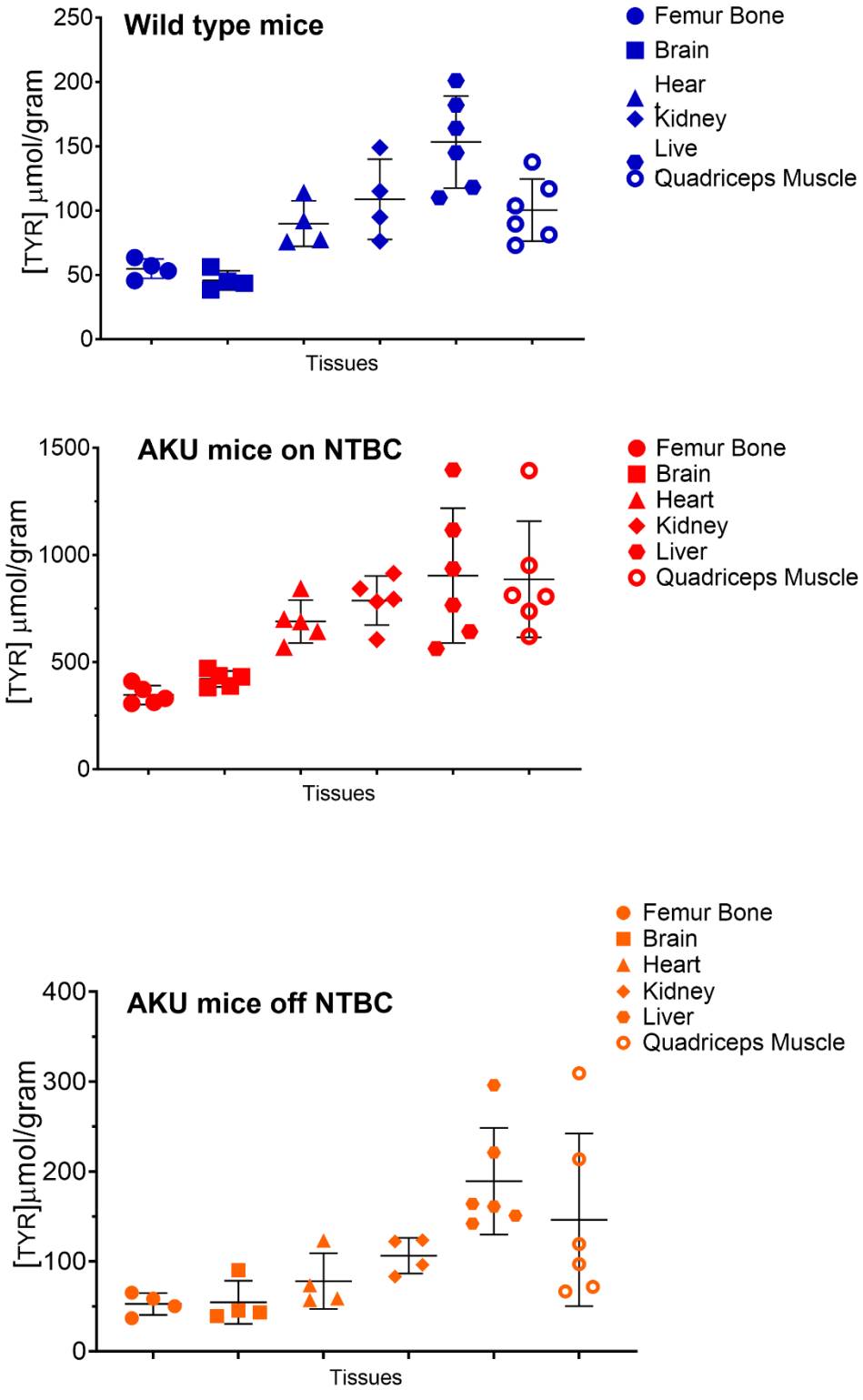

Figure 19: Comparison of tyrosine concentrations across all murine tissue homogenates in the three groups.
Line and error bars are mean ± SD.

3.3.6.1.3 Comparison of HGA concentrations across all tissue homogenates in mice

Homogenates concentrations of HGA are comparable across tissues in WT mice. In NTBC treated mice, HGA concentrations are highest in kidney homogenates, but they are comparable across other tissues. In AKU mice off NTBC, HGA concentrations were highest in kidney homogenates (138 μmol/L). This is followed by the liver (36 μmol/L). Tissue homogenate concentrations of HGA of the quadriceps muscles and femur bone homogenates were comparable (26 μmol/L and 22 μmol/L, respectively). The brain and heart homogenates had the lowest HGA concentrations (6 μmol/L and 12 μmol/L, respectively). This is summarised in Figure 20.

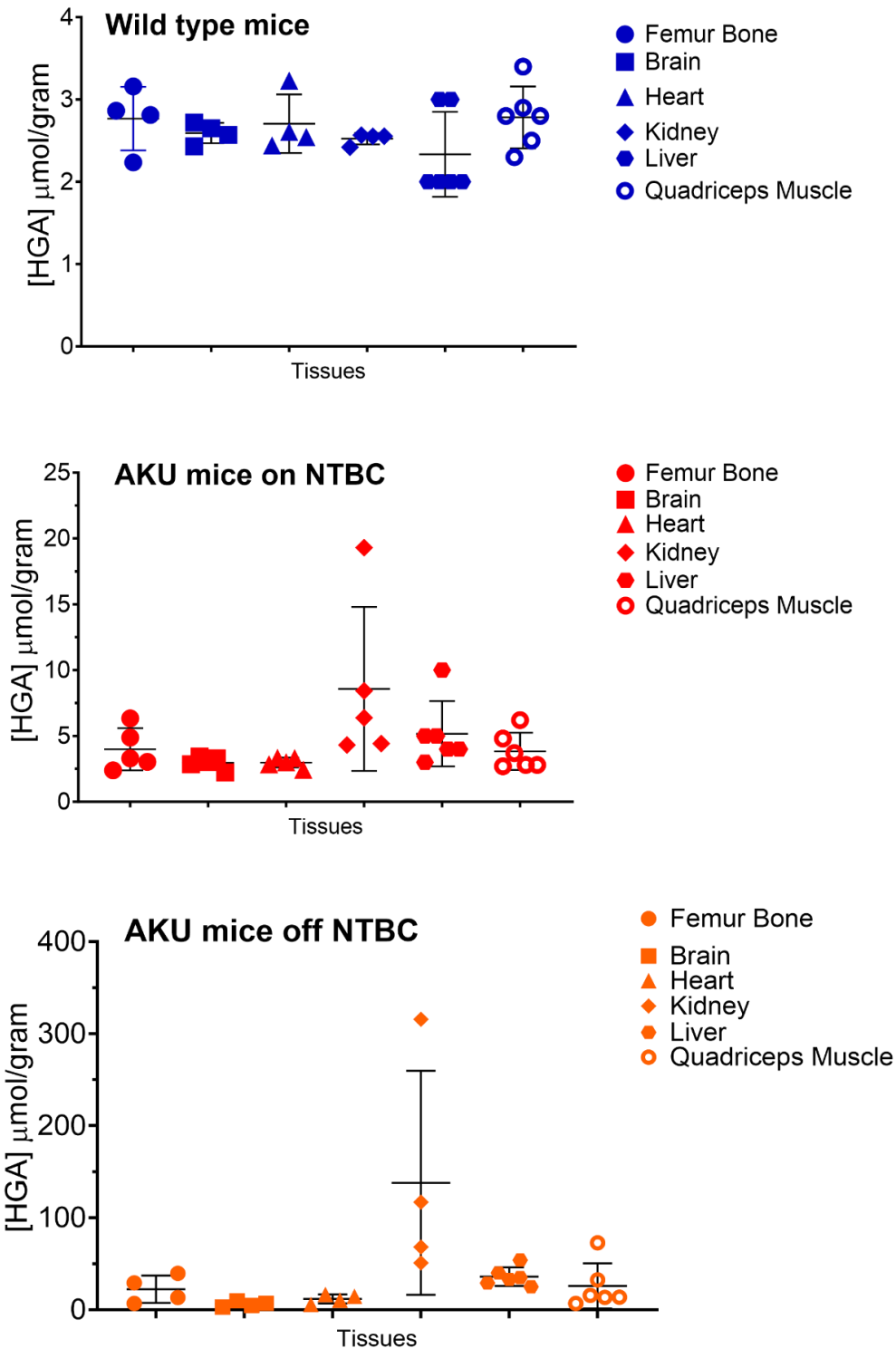

Figure 20: Comparison of HGA concentrations across all murine tissue homogenates in the three groups.
Line and error bars are mean ± SD.

3.3.6.1.4 Comparison of HPLA concentrations across all tissue homogenates in mice

HPLA was not detectable in tissues homogenates of WT mice. In NTBC treated mice, kidney homogenates had the highest HPLA concentration (31μmol/L) ,which can be explained by contamination from residual urine. Heart, liver, and quadriceps homogenates had comparable HPLA concentrations (4 μmol/L, 7 μmol/L and 6 μmol/L, respectively). The lowest HPLA concentrations were in the brain and femur bone homogenates. In the AKU mice off NTBC, there was one outlier that had an HPLA concentration of 25 μmol/L (likely a pre-analytical error). There was no detectable HPLA in any other tissue homogenates in this group. This is summarised in Figure 21.

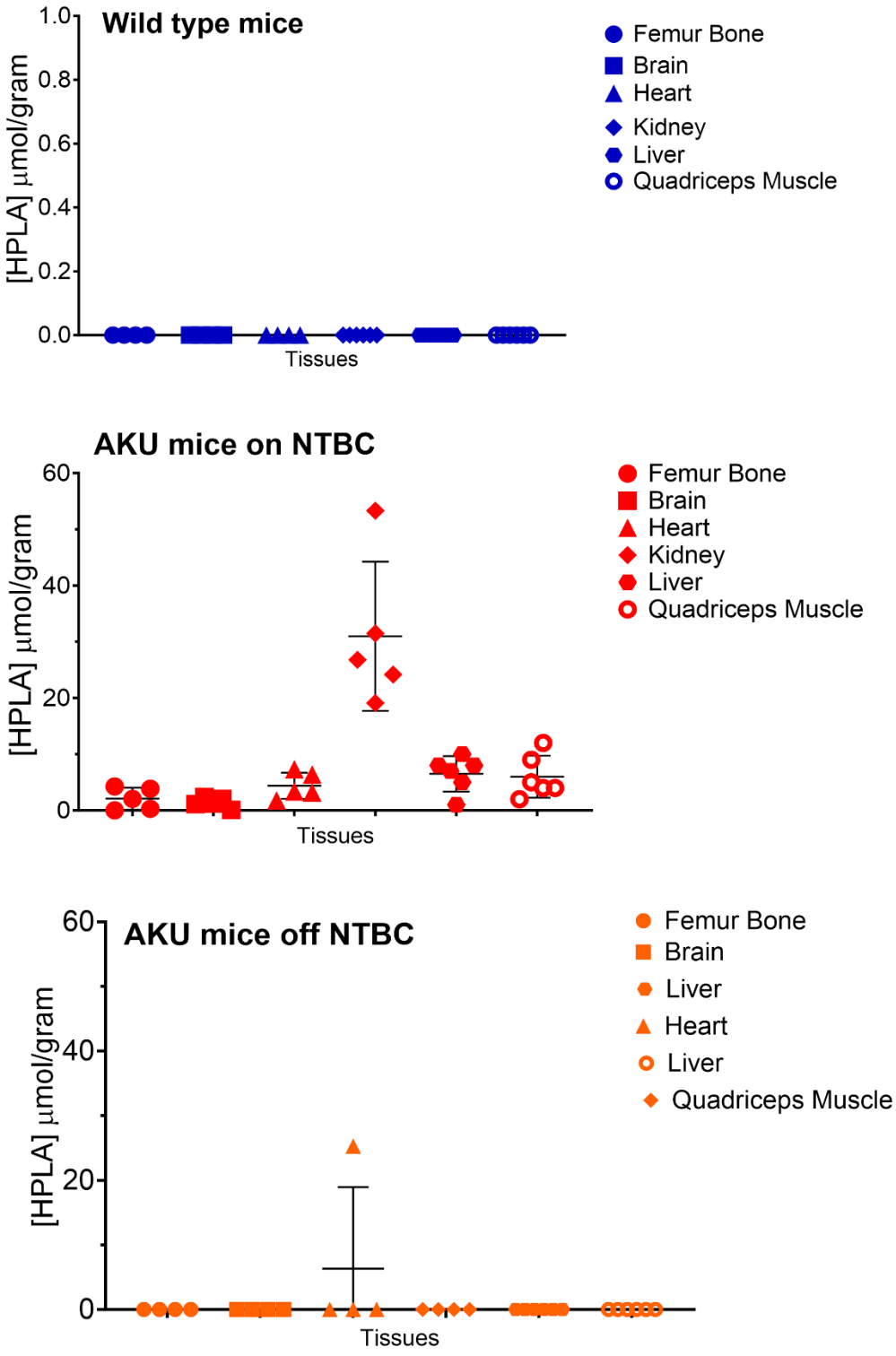

Figure 21: Comparison of HPLA concentrations across all murine tissue homogenates in the three groups.

Line and error bars are mean ± SD.

3.3.6.1.5 Comparison of HPPA concentrations across all tissue homogenates in mice

This is summarised in Figure 22. Homogenates concentrations of HPPA are comparable across tissues in WT and AKU mice off NTBC. In AKU mice on NTBC, HPPA concentrations are highest in kidney homogenates, but they are comparable across other tissues. Notably, HPPA concentrations were highest in kidney homogenates (41 ± 19 μmol/L) and lowest in the brain (14 ± 1 μmol/L). Contamination from urine could explain why the kidney has higher HPPA values, while the presence of the blood brain barrier would probably limit the crossing of HPPA molecule to the brain.

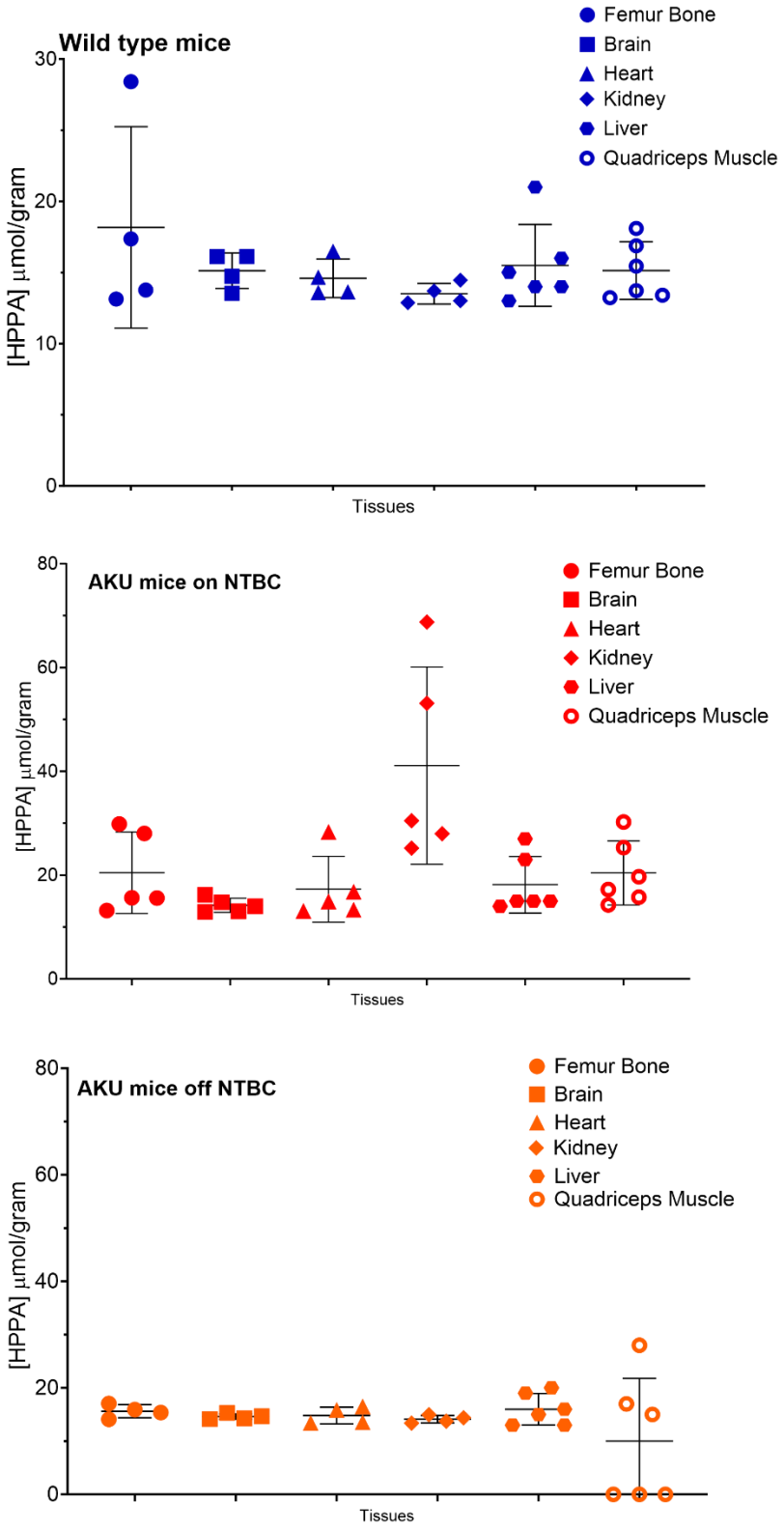

Figure 22: Comparison of HPPA concentrations across all murine tissue homogenates in the three groups.
Line and error bars are mean ± SD.

3.3.7 Effects of NTBC on the urinary concentrations of native compounds in the tyrosine pathway in mice

Descriptive statistics of the urinary concentrations of native compounds in the tyrosine pathway in mice are summarised in Figure 23 and Table 26.

3.3.7.1 *Effects of NTBC on the urinary concentrations of phenylalanine in mice*

The mean urinary phenylalanine (u-Phe) concentration was nearly 4-fold larger in AKU mice off NTBC compared to WT mice ($P< 0.001$). The mean urinary phenylalanine concentration in WT mice was approximately 1.4-fold larger compared to the AKU mice on NTBC but this difference was not statistically significant ($P= 0.870$). In the AKU mice off NTBC, the mean concentration of urinary phenylalanine was 5-fold larger compared to the AKU mice on NTBC ($P< 0.001$).

3.3.7.2 *Effects of NTBC on the urinary concentrations of tyrosine concentrations in mice*

The mean urinary tyrosine (u-Tyr) concentration in the AKU mice on NTBC is 12-fold larger compared to the WT mice ($P< 0.001$). The urinary tyrosine concentration is 8-fold in AKU mice off NTBC compared to WT mice ($P= 0.002$). In the AKU mice off NTBC, the concentration of urinary tyrosine is 1.5-fold larger compared the AKU mice on NTBC; but this difference is not statistically significantly ($P= 0.164$).

3.3.7.3 *Effects of NTBC on the urinary concentrations of HGA in mice*

The mean urinary HGA (u-HGA) concentration is 146008-fold larger in AKU mice off NTBC compared to WT mice ($P< 0.001$). The mean urinary HGA concentration in the AKU mice on NTBC is 6117-fold larger compared to the WT mice ($P< 0.001$). In the AKU mice off NTBC, the mean concentration of urinary HGA is nearly 24-fold larger compared to the AKU mice on NTBC ($P< 0.001$).

3.3.7.4 *Effects of NTBC on the urinary concentrations of HPLA in mice*

The mean urinary HPLA (u-HPLA) concentration in the AKU mice off NTBC is 8-fold-s larger compared to the WT mice; but this difference is not statistically significant ($P= 0.057$). The mean urinary HPLA concentration in the AKU mice on NTBC is 25-fold larger compared the WT mice ($P< 0.001$) and 3-fold larger compared to the AKU mice off NTBC ($P< 0.001$).

3.3.7.5 *Effects of NTBC on the urinary concentrations of HPPA in mice*

The mean urinary HPPA (u-HPLA) concentration in the AKU mice off NTBC is 9-fold larger compared to the WT mice; but this difference is not statistically significant ($P= 0.867$). The mean urinary HPPA concentration in the AKU mice on NTBC is 418-fold larger compared the WT mice ($P< 0.001$) and 44-fold larger compared to the AKU mice off NTBC ($P< 0.001$).

Table 26: Urinary concentrations of native compounds in the tyrosine pathway in mice.
WT: wild type mice, AKU on NTBC: AKU mice treated with NTBC, AKU off NTBC: AKU mice that were not treated with NTBC. (***F1***): the fold change in compound concentration when comparing WT and AKU on NTBC mice (obtained by dividing the concentration of compound in the AKU on NTBC mice by its counterpart in WT mice), (***F2***): the fold change in compound concentration when comparing AKU on NTBC and AKU off NTBC groups (obtained by dividing the concentration of compound in the AKU on NTBC mice by its counterpart in AKU off NTBC mice), (***F3***): the fold change in compound concentration when comparing AKU off NTBC and WT mice (obtained by dividing the concentration of compound in the AKU off NTBC mice by its counterpart in WT mice).

Compound	Urinary concentrations of compounds (µmol/L)											
	WT				AKU on NTBC				AKU off NTBC			
	n	Mean	SD	***F1***	n	Mean	(values)	***F2***	n	Mean	SD	***F3***
[u-Phe]	5	37 ±	20	0.7	2	27	(17, 37)	0.2	6	144 ±	28	4
[u-Tyr]	5	44 ±	25	12	2	525	(385, 665)	1.5	6	352 ±	119	8
[u-HGA]	5	0.6 ±	0.6	6,117	2	3,670	(3528,3811)	0.04	6	87,605 ±	21,849	146,008
[u-HPLA]	5	153 ±	113	25	2	3,839	(3823, 3854)	3	6	1,148 ±	867	8
[u-HPPA]	5	103 ±	45	418	2	43,076	(49053, 37099)	44	6	969 ±	1,095	9

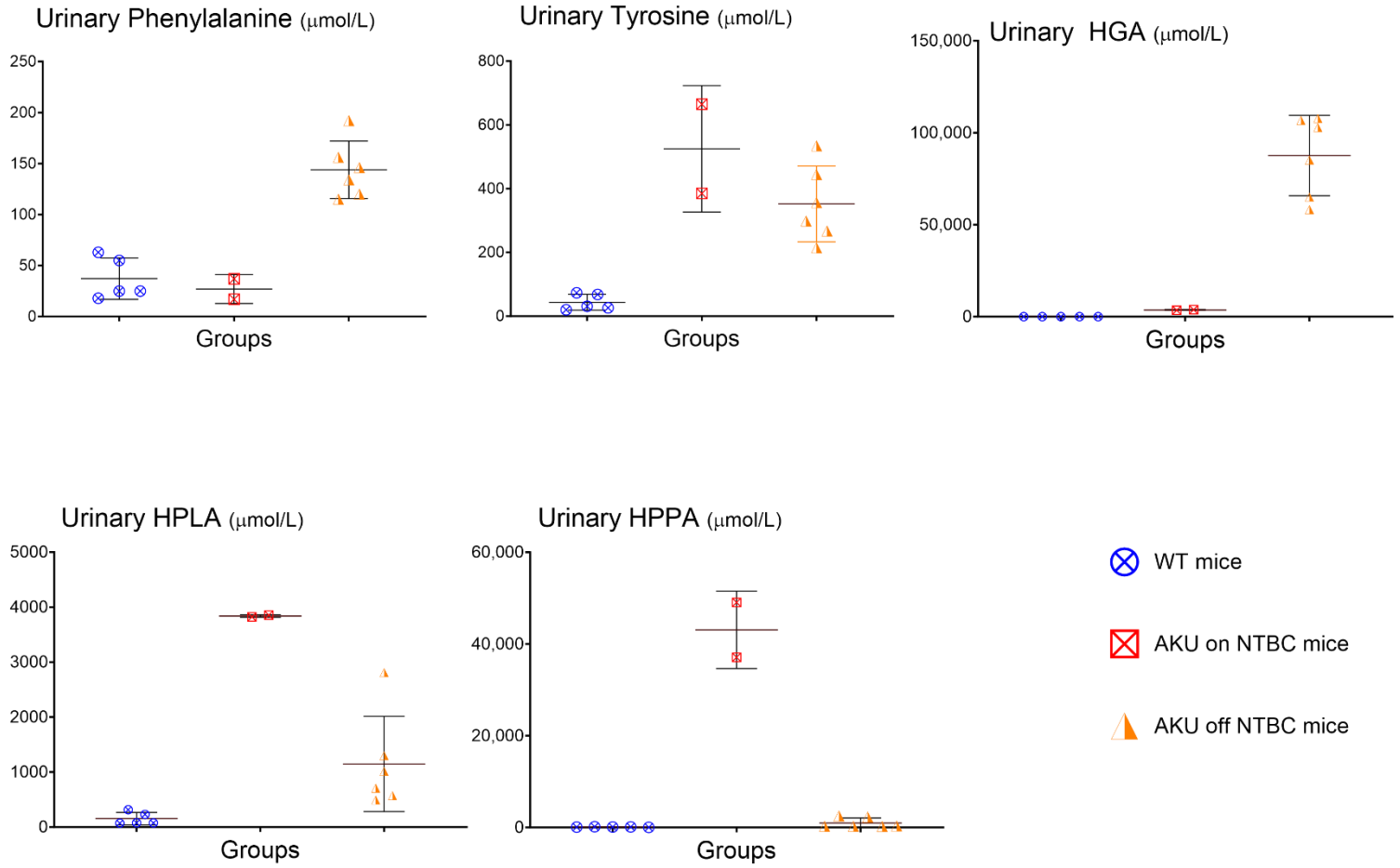

Figure 23: Effects of NTBC on the urinary concentrations of native compounds in the tyrosine pathway.
HGA: homogentisic acid, HPLA: hydroxyphenyllactic acid, HPPA: hydroxyphenylpyruvic acid. Line and error bars are mean ± SD.

3.3.7.6 *Overall comparison of urinary concentrations of native compounds in the tyrosine pathway in mice across the three groups*

Overall changes in the urinary concentrations of native compounds in the tyrosine pathway are summarised in Figure 24 and Table 27. There are two striking findings. Urinary HGA concentration in the AKU off NTBC mice is over 140,000-fold larger compared to WT mice. Even after NTBC it remains over 6000-fold larger compared to WT mice. The second finding is the large rise in urinary HPPA concentrations in the AKU mice on NTBC (418-fold compared to wild type mice and 44-fold compared to AKU mice off NTBC).

When all compounds in each group are viewed separately (Figure 25) these differences become clear by looking at the micromolar concentrations reflected int the scale of Y- axis. The latter extend to 60,000 µmol/L in the AKU mice on NTBC (to accommodate for HGA concentrations) compared to 400 µmol/L in the WT group and 4000 µmol/L in the AKU mice off NTBC.

Table 27: Fold changes in the urinary concentrations of native compounds in the tyrosine pathway in mice following NTBC.

F1: the fold change in the urinary concentration of the native compound in the AKU on NTBC mice compared to WT mice, F2: the fold change in the urinary concentration of the native compound when comparing AKU on NTBC against AKU off NTBC mice, F3: the fold change in the urinary concentration of the native compound in the AKU off NTBC mice compared to WT mice. [u-Phe]: urinary concentration of phenylalanine, [u-Tyr]: urinary concentration of tyrosine, [u-HGA]: urinary concentration of homogentisic acid, [u-HPLA]: urinary concentration of hydroxyphenyllactic acid, [u-HPPA]: urinary concentration of hydroxyphenylpyruvic acid.

Fold changes in the urinary concentrations of compounds			
Compound	***F1***	***F2***	***F3***
[u-Phe]	0.7	0.2	4
[u-Tyr]	12	1.5	8
[u-HGA]	6 117	0.04	146 008
[u-HPLA]	25	3	8
[u-HPPA]	418	44	9

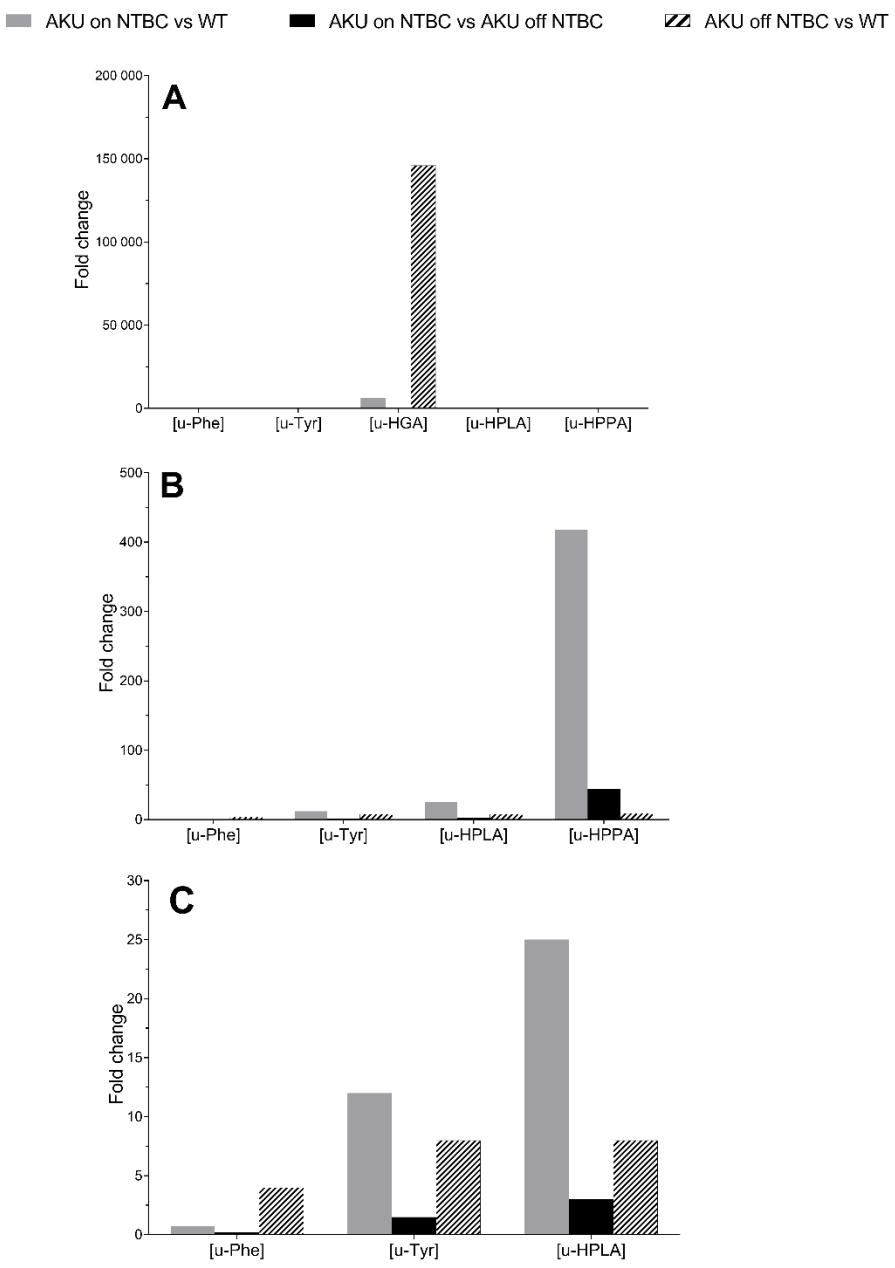

Figure 24: Effect on NTBC on the fold changes in the urinary concentrations of the tyrosine pathway compounds in mice.

HGA was removed from panels B and C to make comparison clearer. HPPA was removed from panel C to make comparison clearer. Panels A, B and C depict the native compounds of the tyrosine pathway in urine. [u-Phe]: urinary concentration of phenylalanine, [u-Tyr]: urinary concentration of tyrosine, [u-HGA]: urinary concentration of homogentisic acid, [u-HPLA]: urinary concentration of hydroxyphenyllactic acid, [u-HPPA]: urinary concentration of hydroxyphenylpyruvic acid.

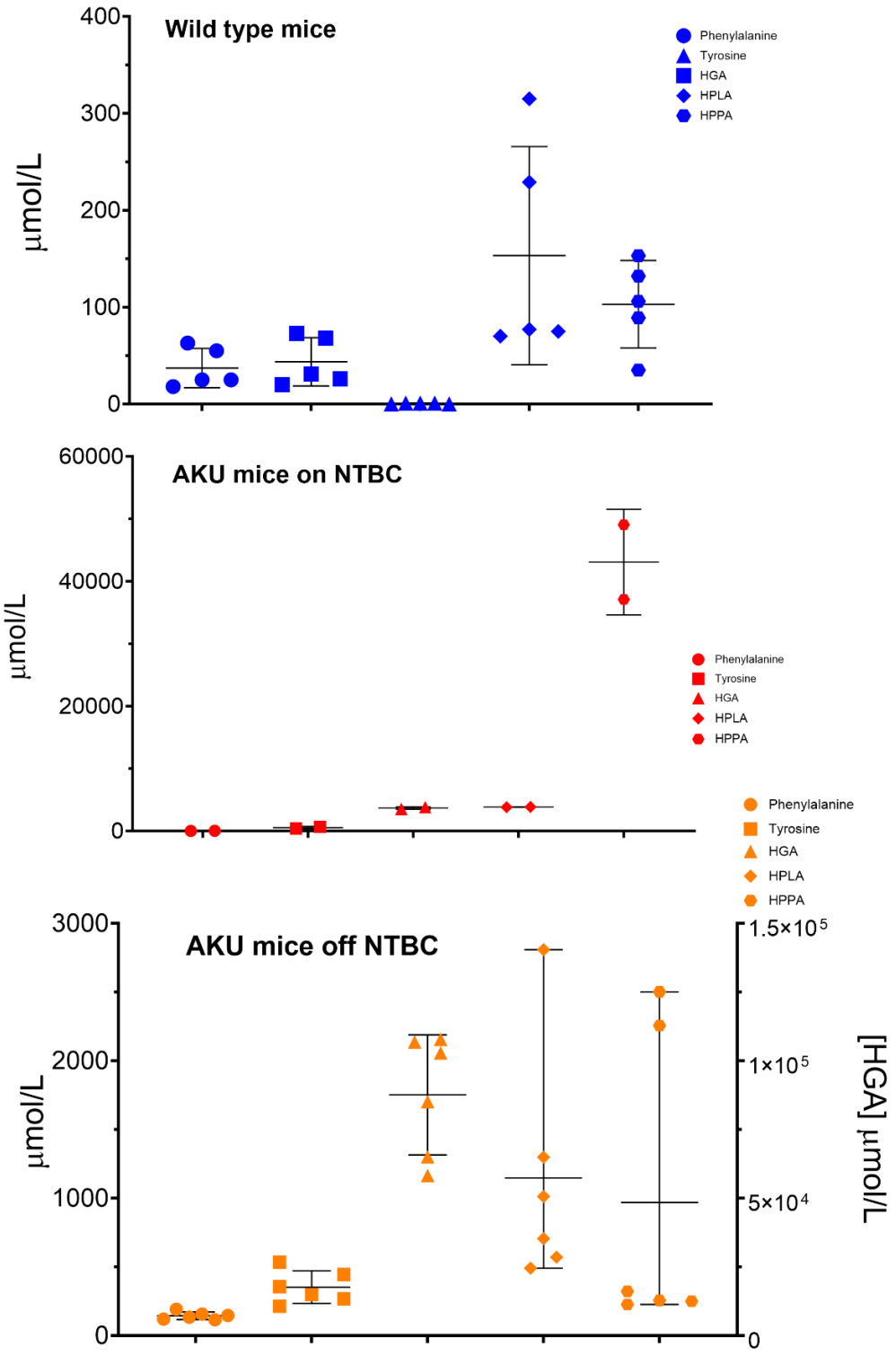

Figure 25: Comparison of urinary concentrations of native compounds in the tyrosine pathway in mice across the three groups.
Line and error bars are mean ± SD. Line and error bars are mean ± SD except for HPPA and HPLA in the AKU mice off NTBC where it indicates mean and range.

3.3.8 Effects of NTBC on phenylalanine and tyrosine stable isotopes change of plasma concentrations against time

Concentrations of stable phenylalanine and tyrosine stable isotopes are plotted against time and illustrated in Figure 26. Differences in Area Under the Curve (AUC) of concentrations against time for each of L-[d_7]tyrosine, L-[d_8]phenylalanine and L-[$^{13}C_9$]tyrosine are not statistically significant amongst the three groups. This is summarised in Table 28.

When assessing the stable isotopes concentrations at 5 and 60 minute, L-[$^{13}C_9$]tyrosine degrades by 34% in the NTBC- treated mice compared to 95%in WT mice ($P< 0.001$) and 93% compared to AKU mice off NTBC ($P< 0.001$). Degradation of L-[d_8]phenylalanine and L-[d_7]tyrosine is not significantly different amongst the three groups. This is summarised in Table 30 and Figure 27.

When assessing the stable isotopes concentrations at 20 and 60 minute, L-[$^{13}C_9$]tyrosine degrades by 19% in the NTBC- treated mice compared to 83% in WT mice ($P< 0.001$) and 83% compared to AKU mice off NTBC ($P< 0.001$). L-[d_7]tyrosine concentration in AKU mice on NTBC is reduced by 55% between the 20 and 60 min time points compared to 78% in WT mice ($P= 0.039$) and 75% compared to AKU mice off NTBC ($P= 0.939$). Degradation of L-[d_8]phenylalanine is not significantly different amongst the three groups. This is summarised in Figure 28 and Table 31.

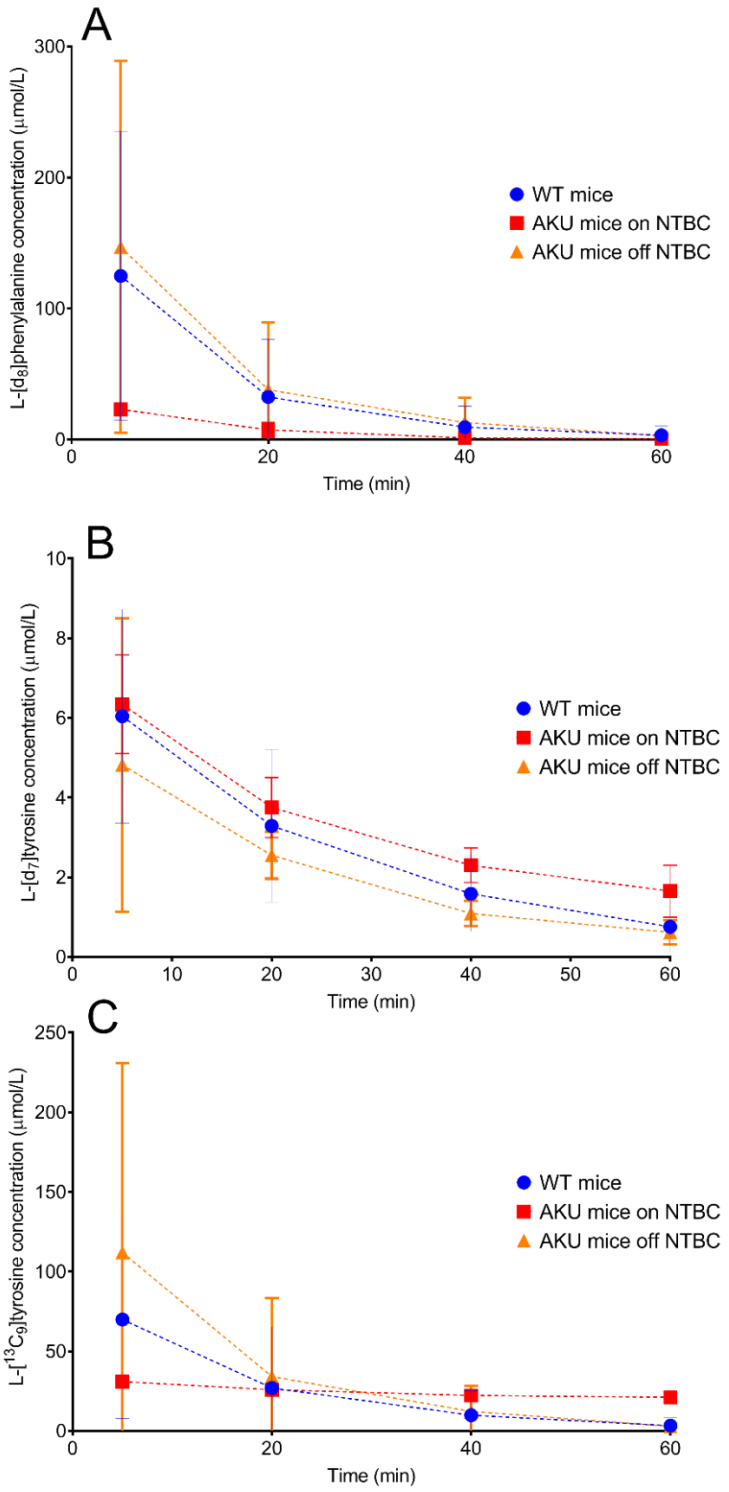

Figure 26: Tyrosine and phenylalanine stable isotopes plasma concentrations versus time following a bolus injection of 1.05 µmol L-[$^{13}C_9$]tyrosine and 2.70 µmol of L-[d_8]phenylalanine.
A: L-[d_8]phenylalanine concentrations versus time, B: L-[d_7]tyrosine concentrations versus time, C: L-[$^{13}C_9$]tyrosine concentrations versus time. Line and error bars are mean ± SD.

Table 28: Comparison of AUCs for the curves representing tracer's plasma concentrations against time in the study mice. WT: wild type mice, AKU on NTBC: AKU mice treated with NTBC, AKU off NTBC: AKU mice that were not treated with NTBC. Ordinary one-way ANOVA with Tukey's multiple comparisons test was used to compare data in the three groups. †Comparison of WT and AKU on NTBC mice. ††Comparison of AKU on NTBC and AKU off NTBC mice. †††Comparison of WT and AKU off NTBC mice. (F1): the fold change in AUC when comparing WT and AKU on NTBC mice (obtained by dividing AUC in AKU on NTBC by that of WT mice) . (F2): the fold change in AUC when comparing AKU on NTBC and AKU off NTBC mice (obtained by dividing AUC in the AKU on NTBC mice by that of AKU off NTBC mice). (F3): the fold change in AUC when comparing WT and AKU off NTBC mice (obtained by dividing AUC in AKU off NTBC by that of WT mice). SEM: standard error of the mean.

Tracer	Area under the curve (AUC) of tracer's plasma concentrations against time																
	WT						AKU on NTBC						AKU off NTBC				
	n	Mean		SEM	$p^{\dagger}$	F1	n	Mean		SEM	$p^{\dagger\dagger}$	F2	n	Mean		SEM	$p^{\dagger\dagger\dagger}$	F3
L-[d_8]phenylalanine	6	1724	±	1021	0.537	0.2	6	329	±	69	0.389	0.2	6	2053	±	1272	0.966	1.2
L-[$^{13}C_9$]tyrosine	6	1227	±	704	0.994	1.1	6	1341	±	55	0.934	0.8	6	1709	±	1107	0.893	1.4
L-[d_7]tyrosine	6	142	±	35	0.660	1.2	6	176	±	16	0.194	1.6	6	108	±	29	0.659	0.8

Table 29: Changes in stable isotopes plasma concentrations during serial sampling.

Concentrations-Ratio 60 to 5 min is: [stable isotope]at 5 min/ [stable isotope]at 60 min, Concentrations-Ratio 60 to 20 min is: [stable isotope]at 20 min/ [stable isotope]at 60 min, Concentration-% decrease 5 to 60 min: percentage decrease in [stable isotope] from 5 min timepoint to 60 min timepoint, Concentration-% decrease 20 to 60 min: percentage decrease in [stable isotope] from 20 min timepoint to 60 min timepoint.

		Changes in L-[d_7]tyrosine plasma concentrations during serial sampling					
		BALB/c Wild type		BALB/c HGD-/- on NTBC		BALB/c HGD-/- off NTBC	
	n	Mean	± SD	n	Mean ± SD	n	Mean ± SD
Concentrations-Ratio *60 to 5 min*	4	0.11	± 0.1236	5	0.348 ± 0.07	4	0.16 ± 0.11
Concentrations-Ratio *60 to 20 min*	6	0.22	± 0.1345	6	0.448 ± 0.17	6	0.25 ± 0.12
Concentration-% decrease *5 to 60 min*	4	89	± 12	5	77 ± 7	4	85 ± 11
Concentration-% decrease *20 to 60 min*	6	78	± 14	6	55 ± 17	6	75 ± 12
	Changes in L-[$^{13}C_9$]tyrosine plasma concentrations during serial sampling						
		BALB/c Wild type		BALB/c HGD-/- on NTBC		BALB/c HGD-/- off NTBC	
Concentrations-Ratio *60 to 5 min*	4	0.05	± 0.04	5	0.718 ± 0.15	4	0.07 ± 0.09
Concentrations-Ratio *60 to 20 min*	6	0.17	± 0.09	6	0.812 ± 0.10	6	0.17 ± 0.12
Concentration-% decrease *5 to 60 min*	4	95	± 4	5	34 ± 15	4	93 ± 9
Concentration-% decrease *20 to 60 min*	6	83	± 10	6	19 ± 10	6	83 ± 11
	Changes in L-[d_8]phenylalanine plasma concentrations during serial sampling						
		BALB/c Wild type		BALB/c HGD-/- on NTBC		BALB/c HGD-/- off NTBC	
Concentrations-Ratio *60 to 5 min*	4	0.03	± 0.0362	5	0.038 ± 0.01	4	0.04 ± 0.05
Concentrations-Ratio *60 to 20 min*	6	0.06	± 0.0556	6	0.065 ± 0.02	6	0.08 ± 0.07
Concentration-% decrease *5 to 60 min*	4	97	± 4	5	98 ± 1	4	96 ± 5
Concentration-% decrease *20 to 60 min*	6	94	± 6	6	94 ± 2	6	92 ± 7

Table 30: Effects of NTBC on phenylalanine and tyrosine stable isotopes change of plasma concentrations at the 5- and 60-minute time points. Ordinary one-way ANOVA with Tukey's multiple comparisons test was used to compare data in the three groups. †Comparison of WT and AKU on NTBC mice. ††Comparison of AKU on NTBC and AKU off NTBC mice. †††Comparison of WT and AKU off NTBC mice. (***F1***): the fold change in percentage decrease in compound concentration when comparing WT and AKU on NTBC mice (obtained by dividing the percentage decrease in compound concentration in the AKU on NTBC mice by its counterpart in WT mice), (***F2***): the fold change in compound concentration when comparing AKU on NTBC and AKU off NTBC groups (obtained by dividing the percentage decrease in compound concentration in the AKU on NTBC mice by its counterpart in AKU off NTBC mice), (***F3***): the fold change in compound concentration when comparing AKU off NTBC and WT mice (obtained by dividing the percentage decrease in compound concentration in the AKU off NTBC mice by its counterpart in WT mice).

Tracer	Percentage decrease in tracer's plasma concentrations between 5 and 60 min															
	WT						AKU on NTBC						AKU off NTBC					
	n	Mean		SD	$p^{\dagger}$	F1	n	Mean		SD	$p^{\dagger\dagger}$	F2	n	Mean		01	$p^{\dagger\dagger\dagger}$	F3
L-[d_8]phenylalanine	4	97	±	4	0.917	1.0	5	98	±	1	0.518	1.0	4	96	±	5	0.771	1.0
L-[$^{13}C_9$]tyrosine	4	95	±	4	<0.001	0.4	5	34	±	15	<0.001	0.4	4	93	±	9	0.972	1.0
L-[d_7]tyrosine	4	89	±	12	0.245	0.9	5	77	±	7	0.523	0.9	4	85	±	11	0.840	1.0

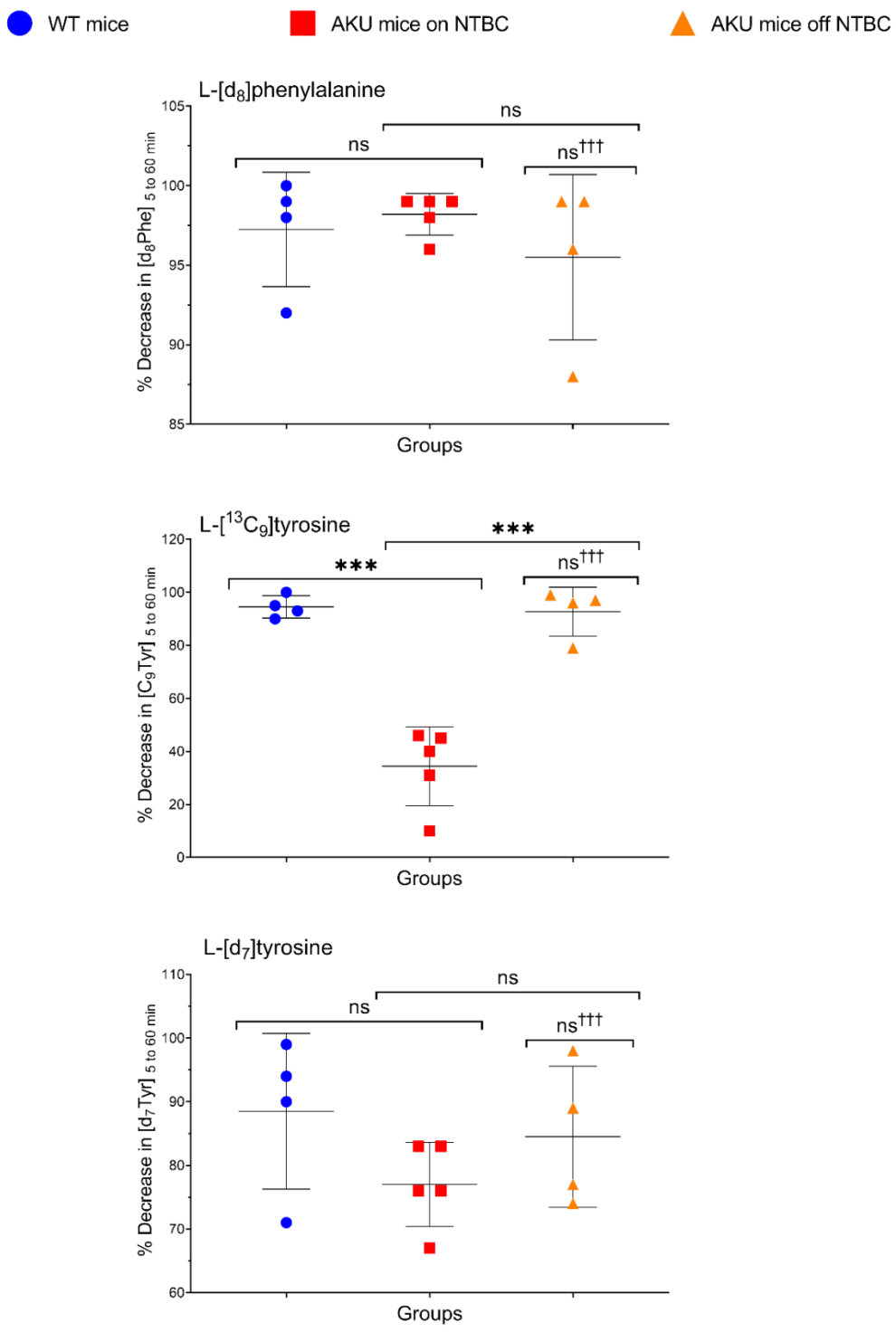

Figure 27: Effects of NTBC on phenylalanine and tyrosine stable isotopes change of plasma concentrations at the 5- and 60-minute time points.

Ordinary one-way ANOVA with Tukey's multiple comparisons test was used to compare data in the three groups. ns: $P \geq 0.05$, ***$P < 0.001$.††† AKU off NTBC compared to WT mice.Line and error bars are mean ± SD.

Table 31: Effects of NTBC on phenylalanine and tyrosine stable isotopes change of plasma concentrations at the 20- and 60-minute time points.

Ordinary one-way ANOVA with Tukey's multiple comparisons test was used to compare data in the three groups. †Comparison of WT and AKU on NTBC mice. ††Comparison of AKU on NTBC and AKU off NTBC mice. †††Comparison of WT and AKU off NTBC mice. (***F1***): the fold change in percentage decrease in compound concentration when comparing WT and AKU on NTBC mice (obtained by dividing the percentage decrease in compound concentration in the AKU on NTBC mice by its counterpart in WT mice), (***F2***): the fold change in compound concentration when comparing AKU on NTBC and AKU off NTBC groups (obtained by dividing the percentage decrease in compound concentration in the AKU on NTBC mice by its counterpart in AKU off NTBC mice), (***F3***): the fold change in compound concentration when comparing AKU off NTBC and WT mice (obtained by dividing the percentage decrease in compound concentration in the AKU off NTBC mice by its counterpart in WT mice).

Tracer	Percentage decrease in tracer's plasma concentrations between 20 and 60 min															
	WT						AKU on NTBC						AKU off NTBC					
	n	Mean		SD	$p^{\dagger}$	F1	n	Mean		SD	$p^{\dagger\dagger}$	F2	n	Mean		SD	$p^{\dagger\dagger\dagger}$	F3
L-[d_8]phenylalanine	6	94	±	6	0.986	1.0	6	94	±	2	0.858	1.0	6	92	±	7	0.774	1.0
L-[$^{13}C_9$]tyrosine	6	83	±	10	<0.001	0.2	6	19	±	10	<0.001	0.2	6	83	±	11	0.996	1.0
L-[d_7]tyrosine	6	78	±	14	0.039	0.7	6	55	±	17	0.074	0.7	6	75	±	12	0.939	1.0

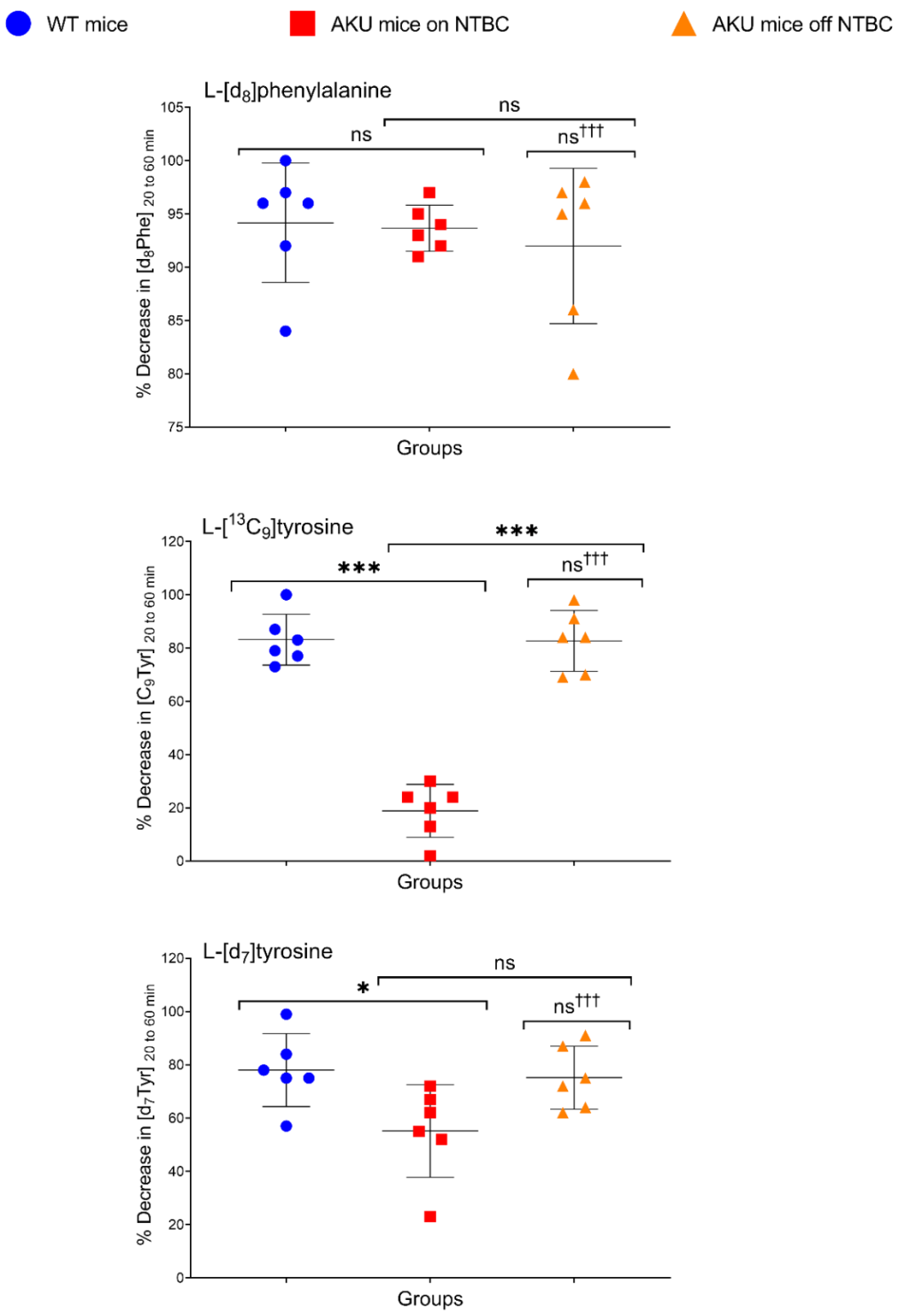

Figure 28: Effects of NTBC on phenylalanine and tyrosine stable isotopes change of plasma concentrations at the 20- and 60-minute time points.

Ordinary one-way ANOVA with Tukey's multiple comparisons test was used to compare data in the three groups. ns: $P \geq 0.05$, ***$P < 0.001$.ˆˆˆ AKU off NTBC compared to WT mice. Line and error bars are mean ± SD.

3.3.9 Effects of NTBC on phenylalanine and tyrosine stable isotopes enrichment (TTR) curves in mice

L-[d_8]phenylalanine degradation is almost identical in AKU mice regardless of NTBC therapy (Figure 29, panel A). In contrast, L-[d_7]tyrosine and L-[$^{13}C_9$]tyrosine undergo a minimal degree of degradation in AKU mice on NTBC (Figure 29, panels B and C) with TRR curves are represented by a nearly flat line.

AUC for L-[d_8]phenylalanine Tracer to tracee ratio (TTR) curve in NTBC-treated AKU mice was not significantly different compared to the AKU mice off NTBC (p >0.999). However, AUC for L-[d_8]phenylalanine TTR curve in WT times was five times larger than in AKU mice on NTBC (P=0.044) and AKU mice off NTBC (P=0.044) reflecting the reduced efficiency of the tyrosine pathway in AKU mice .

Despite the lack of statistical significance, AUC for L-[$^{13}C_9$]tyrosine TTR curve was 7-fold larger in AKU mice off NTBC compared to the NTBC- treated AKU mice (P = 0.628); while in the WT mice, AUC for L-[$^{13}C_9$]tyrosine TTR curve was 12-fold larger compared to NTBC- treated AKU mice (P= 0.208), Furthermore, for L-[$^{13}C_9$]tyrosine TTR curve was nearly 2-fold larger in the WT mice compared to the AKU mice off NTBC (p =0.715).

AUC for L-[d_7]tyrosine TTR curve in the AKU mice on NTBC is reduced to 10% of that in WT mice (P<0.001), and 20% to that in the AKU mice off NTBC although this difference is not statistically significant (P= 0.123). AUC for L-[d_7]tyrosine TTR curve in WT mice is double that of AKU mice off NTBC (P= 0.053).This is summarised in Table 32.

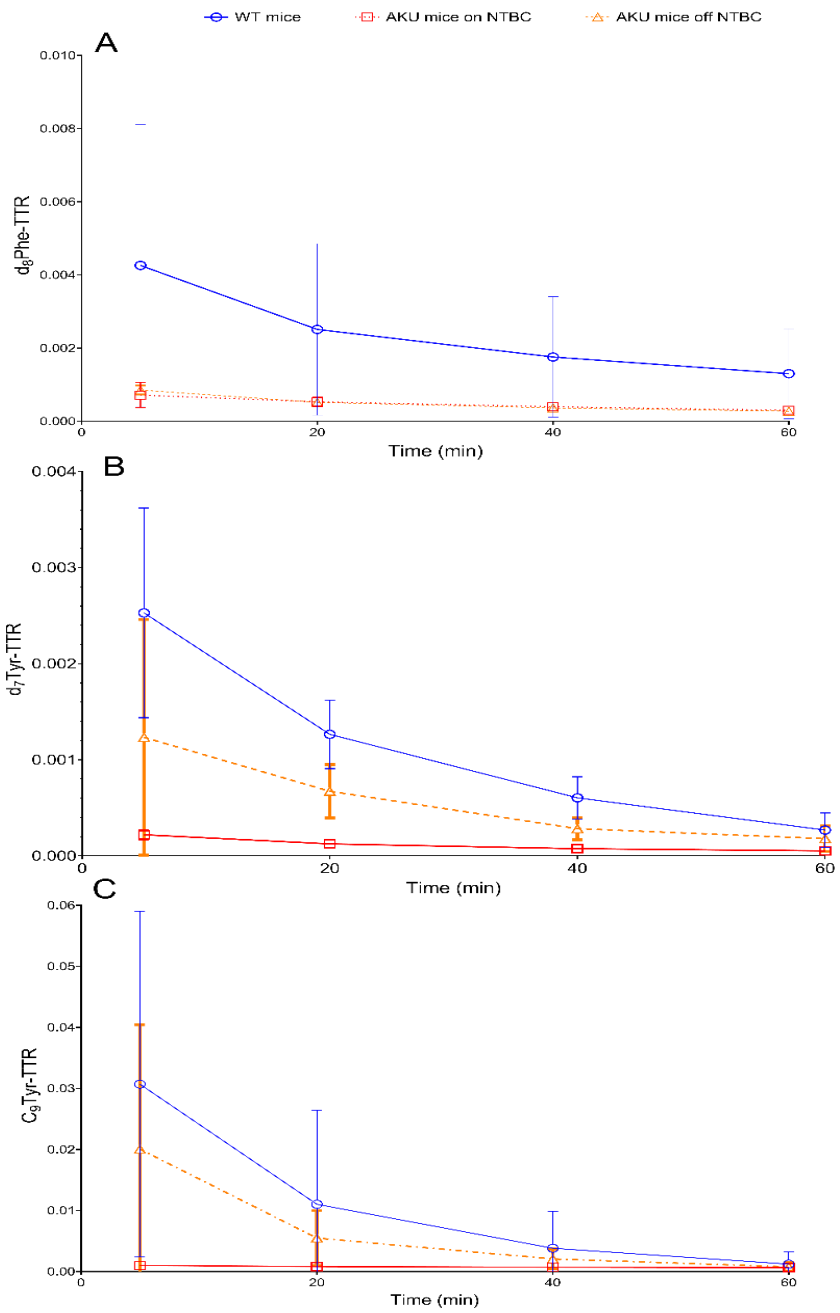

Figure 29: Tyrosine and phenylalanine enrichment versus time following a bolus injection of 1.05 µmol L-[$^{13}C_9$]tyrosine and 2.70 µmol of L-[d_8]phenylalanine.

A: L-[d_8]phenylalanine enrichment versus time, d_8Phe-TTR is [L-[d_8]phenylalanine]/[median of native phenylalanine]; B: L-[d_7]tyrosine enrichment versus time, d_7Tyr-TTR is [L-[d_7]tyrosine]/[median of native tyrosine]; C: L-[$^{13}C_9$]tyrosine enrichment versus time. C_9Tyr-TTR is [L-[$^{13}C_9$]tyrosine]/[median of native tyrosine]; Line and error bars are mean ± SD

Table 32: Comparison of AUCs for Tracer-Tracee Ratio (TTR) curves in the study mice. WT: wild type mice, AKU on NTBC: AKU mice treated with NTBC, AKU off NTBC: AKU mice that were not treated with NTBC. Ordinary one-way ANOVA with Tukey's multiple comparisons test was used to compare data in the three groups. †Comparison of WT and AKU on NTBC mice. ††Comparison of AKU on NTBC and AKU off NTBC mice. †††Comparison of WT and AKU off NTBC mice. (F1): the fold change in AUC when comparing WT and AKU on NTBC mice (obtained by dividing AUC in AKU on NTBC by that of WT mice) . (F2): the fold change in AUC when comparing AKU on NTBC and AKU off NTBC mice (obtained by dividing AUC in the AKU on NTBC mice by that of AKU off NTBC mice). (F3): the fold change in AUC when comparing WT and AKU off NTBC mice (obtained by dividing AUC in AKU off NTBC by that of WT mice). SEM: standard error of the mean.

Tracer used for TTR	Area under the curve of TTR (min)															
	WT						AKU on NTBC						AKU off NTBC			
	n	Mean	±	SEM	$p^{\dagger}$	F1	n	Mean	±	SEM	$p^{\dagger\dagger}$	F2	n	Mean	±	SEM	$p^{\dagger\dagger\dagger}$	F3
L-[d_8]phenylalanine	6	0.124	±	0.049	0.044	0.2	6	0.026	±	0.004	>0.999	1.0	6	0.026	±	0.001	0.044	0.2
L-[$^{13}C_9$]tyrosine	6	0.512	±	0.300	0.208	0.1	6	0.042	±	0.002	0.628	0.1	6	0.295	±	0.165	0.715	0.6
L-[d_7]tyrosine	6	0.056	±	0.010	<0.001	0.1	6	0.006	±	0.001	0.123	0.2	6	0.028	±	0.010	0.053	0.5

3.3.10 Effects of NTBC on tissue homogenates concentrations of phenylalanine and tyrosine stable isotopes in mice

3.3.10.1 *Effects of NTBC on tissue homogenates concentrations of L-[d_8]phenylalanine in mice*

There are no statically significant differences in L-[d_8]phenylalanine concentrations across tissue homogenates amongst the three groups. This is summarised Table 33 and Figure 33. However, the following observations can be made:

- Tissue homogenates concentrations of L-[d_8]phenylalanine in WT mice were higher (2-folds higher in femur bone homogenates, 5-fold higher in the kidney , liver and quadriceps; 50 fold- higher in the brain and the heart) when compared to NTBC- treated mice.
- Quadriceps muscle homogenates concentration of L-[d_8]phenylalanine in AKU mice off NTBC is comparable to that in WT mice. In other tissue homogenates, WT mice have higher concentrations of L-[d_8]phenylalanine (up to 10-fold in brain and heart homogenates).
- When compared to AKU mice off NTBC, homogenate concentrations of L-[d_8]phenylalanine in NTBC- treated AKU mice are 2-fold larger in the femur bone and almost comparable in the kidney. In contrast, the other tissue homogenates in the AKU mice off NTBC have higher concentrations of L-[d_8]phenylalanine (up to 5-fold in brain and quadriceps homogenates).

Table 33: Tissue homogenates concentrations of L-[d_8]phenylalanine in mice.

Plasma concentrations are at 60 minWT: wild type mice, AKU on NTBC: AKU mice treated with NTBC, AKU off NTBC: AKU mice that were not treated with NTBC. Ordinary one-way ANOVA with Tukey's multiple comparisons test was used to compare data in the three groups. F1: the fold change in compound concentrations in the AKU on NTBC mice compared to WT mice, F2: the fold change in compound concentration when comparing AKU on NTBC and AKU off NTBC mice, F3: the fold change in compound concentrations in the AKU off NTBC mice compared to WT mice. †Comparison of WT and AKU on NTBC mice. ††Comparison of AKU on NTBC and AKU off NTBC mice. †††Comparison of WT and AKU off NTBC mice.

Tissues	L-[d_8]phenylalanine concentrations (µmol/gram)															
	WT						AKU on NTBC						AKU off NTBC					
	n	Mean		SD	$p^{\dagger}$	F1	n	Mean		SD	$p^{\dagger\dagger}$	F2	n	Mean		SD	$p^{\dagger\dagger\dagger}$	F3
Femur bone	4	2	±	3	0.485	0.5	5	1	±	1	0.921	1.7	4	0.6	±	0.5	0.333	0.3
Brain	4	1	±	2	0.335	0.02	5	0.02	±	0.04	0.982	0.2	4	0.1	±	0.1	0.457	0.1
Heart	4	5	±	9	0.323	0.02	5	0.1	±	0.2	0.999	0.3	4	0.3	±	0.2	0.378	0.1
Kidney	4	3	±	5	0.388	0.2	5	0.5	±	0.3	0.997	0.8	4	0.6	±	0.1	0.457	0.2
Liver	6	3	±	5	0.573	0.2	6	0.6	±	0.4	0.678	0.3	6	2	±	3	0.983	0.7
Quadriceps	6	2	±	2	0.445	0.2	6	0.4	±	0.3	0.397	0.2	6	2	±	2	0.996	1.0
	L-[d_8]phenylalanine concentrations at 60 min (µmol/gram)																	
Plasma	6	0.003	±	0.007	0.553	0.13	6	0.0004	±	0.0003	0.67	0.2	6	0.003	±	0.003	0.979	0.8

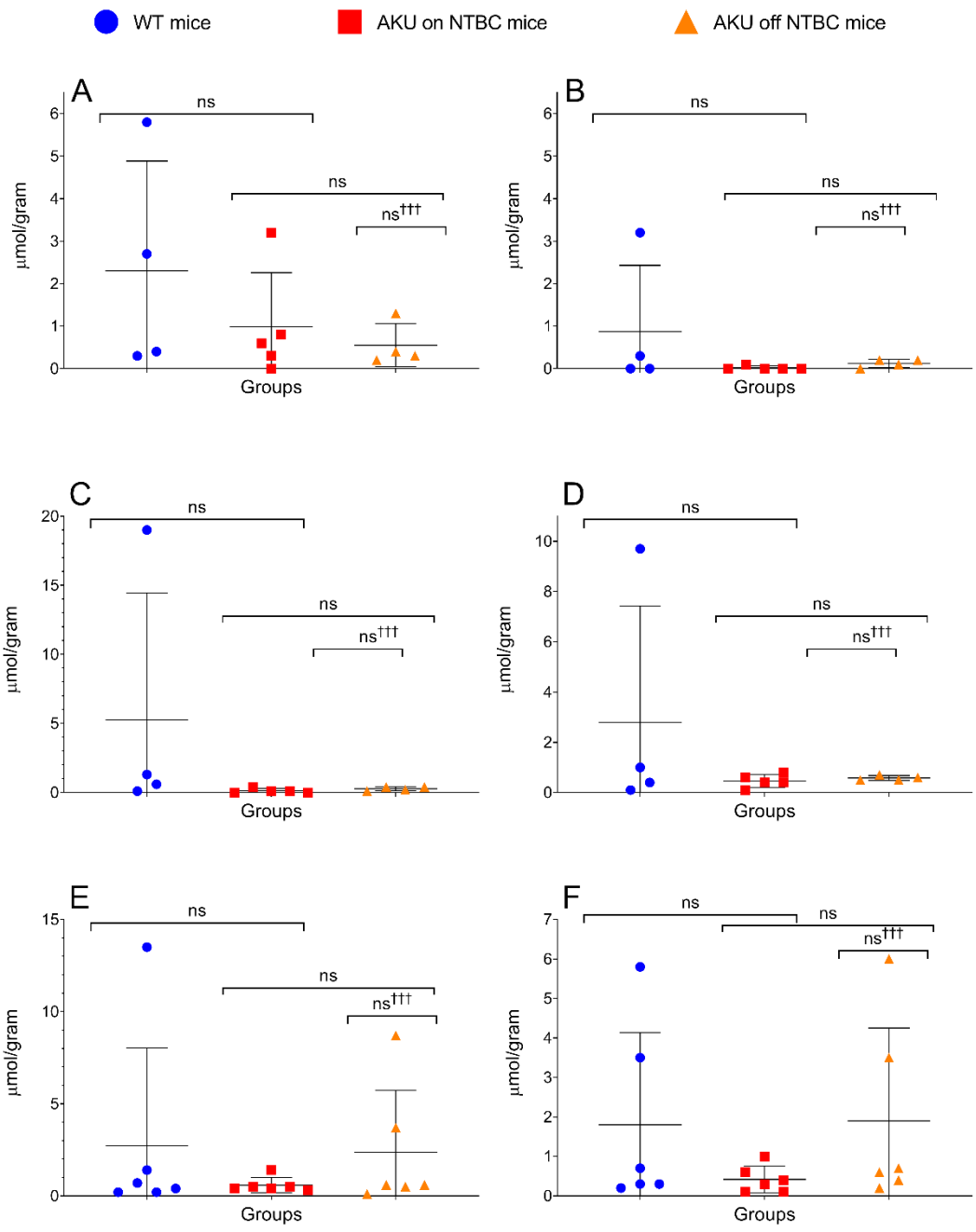

Figure 30: Effects of NTBC on tissue homogenates concentrations of L-[d_8]phenylalanine in mice.
(A) femur bone, (B) brain, (C) heart, (D) kidney, (E) liver, (F) quadriceps muscle. Line and error bars are mean ± SD. ns: $P \geq 0.05$. Ordinary one-way ANOVA with Tukey's multiple comparisons test was used to compare data among the three groups. †††Comparison of WT and AKU off NTBC mice.

3.3.10.2 *Effects of NTBC on tissue homogenates concentrations of L-[d_7]tyrosine in mice*

Despite the variations in L-[d_7]tyrosine concentrations across tissue homogenates between WT mice and AKU mice off NTBC, these differences are not statistically significant. This is summarised in Figure 31 and Table 34. In AKU mice on NTBC, the following observations can be made:

- Tissue homogenates concentrations of L-[d_7]tyrosine are 1.4 to 3-fold larger compared to that in WT mice. However, it is only statistically significant in the brain homogenates (3-fold, *P*=0.20)
- Tissue homogenates concentrations of L-[d_7]tyrosine in the liver and kidney homogenates are nearly 3-fold larger compared to AKU mice off NTBC. Nonetheless, this difference is not statistically significant.
- Statistically significant differences are note in the other tissue homogenates compared to AKU mice off NTBC: bone homogenates (5-fold larger, *P*=0.004), brain (3-fold larger, *P*=0.004), heart (11-fold larger, *P*=0.004), and quadriceps (5-fold larger, *P*=0.004).

Table 34: Tissue homogenates concentrations of L-[d_7]tyrosine in mice.
Plasma concentrations are at 60 min.WT: wild type mice, AKU on NTBC: AKU mice treated with NTBC, AKU off NTBC: AKU mice that were not treated with NTBC. Ordinary one-way ANOVA with Tukey's multiple comparisons test was used to compare data in the three groups. F1: the fold change in metabolite concentrations in the AKU on NTBC compared to WT mice, F2: the fold change in L-[d_7]tyrosine concentration when comparing AKU on NTBC and AKU off NTBC mice, F3: the fold change in L-[d_7]tyrosine concentrations in the AKU off NTBC compared to WT mice. †Comparison of WT and AKU on NTBC mice. ††Comparison of AKU on NTBC and AKU off NTBC mice. †††Comparison of WT and AKU off NTBC mice.

Tissues	L-[d_7]tyrosine concentrations (µmol/gram)																
	WT						AKU on NTBC						AKU off NTBC				
	n	Mean		SD	$p^{\dagger}$	F1	n	Mean		SD	$p^{\dagger\dagger}$	F2	n	Mean		SD	$p^{\dagger\dagger\dagger}$	F3
Femur bone	4	0.3	±	0.08	0.129	1.7	5	0.5	±	0.1	0.004	5.0	4	0.1	±	0.1	0.166	0.3
Brain	4	0.2	±	0.2	0.020	3.0	5	0.6	±	0.2	0.014	3.0	4	0.2	±	0.2	0.976	1.0
Heart	4	0.5	±	0.3	0.086	1.8	5	0.9	±	0.3	0.003	11.3	4	0.1	±	0.2	0.171	0.2
Kidney	4	0.8	±	1.0	0.822	0.8	5	0.6	±	0.2	0.687	3.0	4	0.2	±	0.05	0.391	0.3
Liver	6	0.4	±	0.4	0.366	2.0	6	0.8	±	0.5	0.197	2.7	6	0.3	±	0.3	0.908	0.8
Quadriceps	6	0.7	±	0.4	0.095	1.4	6	1.0	±	0.5	<0.001	5.0	6	0.2	±	0.2	0.061	0.3
	L-[d_7]tyrosine concentrations (µmol/gram)																	
Plasma	6	0.0008	±	0.0007	0.037	2.3	6	0.002	±	0.0007	0.017	2.7	6	0.0006	±	0.0003	0.913	0.8

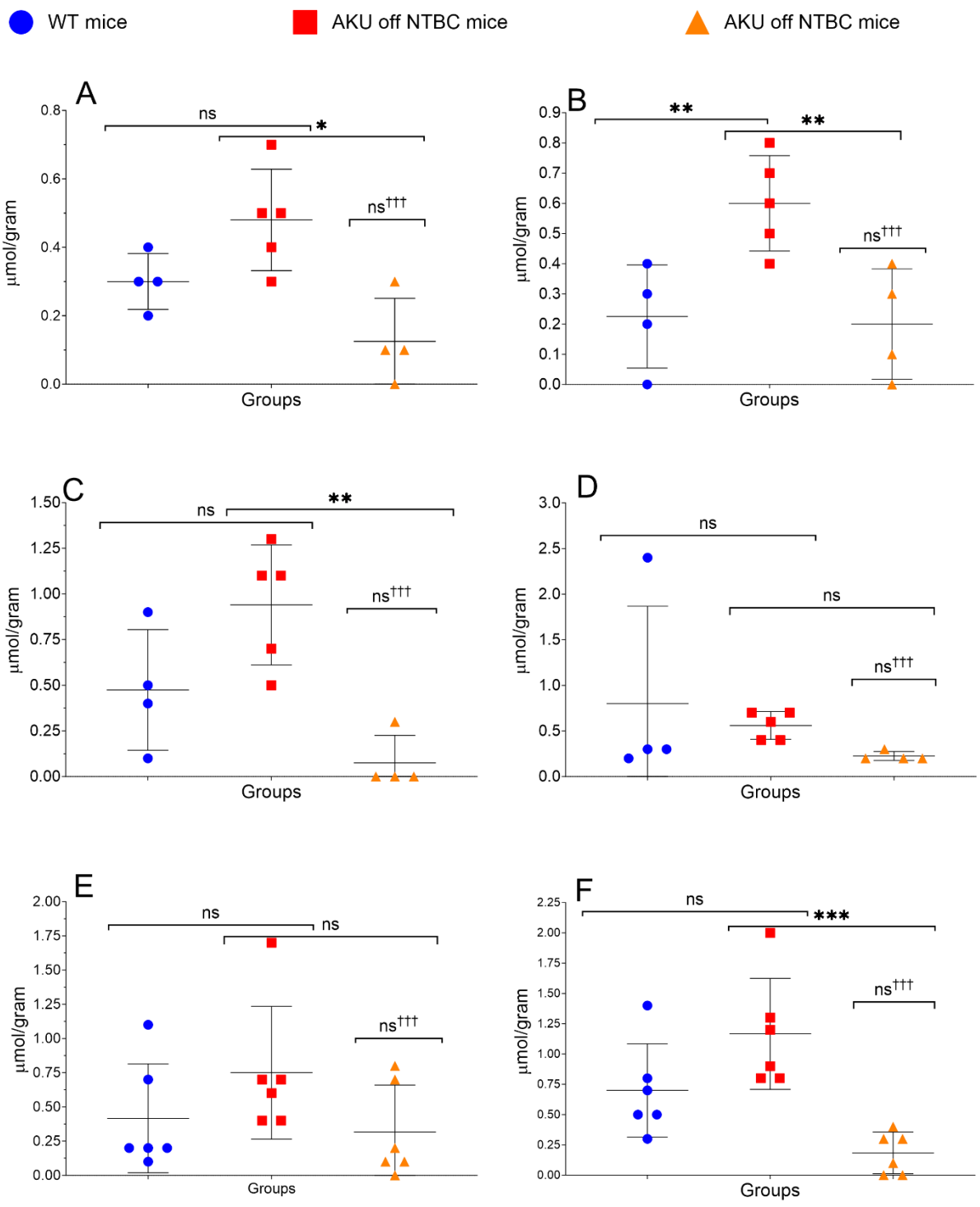

Figure 31: Effects of NTBC on tissue homogenates concentrations of L-[d_7]tyrosine in mice.

(A) femur bone, (B) brain, (C) heart, (D) kidney, (E) liver, (F) quadriceps muscle. Line and error bars are mean ± SD. Ordinary one-way ANOVA with Tukey's multiple comparisons test was used to compare data among the three groups. †††Comparison of WT and AKU off NTBC mice. ns: $P \geq 0.05$, *P <0.05, **P <0.01, ***P< 0.001.

3.3.10.3 *Effects of NTBC on tissue homogenates concentrations of L-[$^{13}C_9$]tyrosine in mice*

Despite the variations in L-[$^{13}C_9$]tyrosine concentrations across tissue homogenates between WT mice and AKU mice off NTBC, these differences are not statistically significant. This is summarised in Figure 32 and Table 35. In AKU mice on NTBC, the following observations can be made:

- Tissue homogenates concentrations of L-[$^{13}C_9$]tyrosine are 5 to 10-fold larger compared to that in WT mice (P<0.001 across all tissues homogenates).
- Tissue homogenates concentrations of L-[$^{13}C_9$]tyrosine are 7.5 to16.7-fold larger compared to AKU mice off NTBC (P<0.001 across all tissues homogenates).

Table 35: Tissue homogenates concentrations of L-[$^{13}C_9$]tyrosine in mice.

Plasma concentrations are at 60 min.WT: wild type mice, AKU on NTBC: AKU mice treated with NTBC, AKU off NTBC: AKU mice that were not treated with NTBC. Ordinary one-way ANOVA with Tukey's multiple comparisons test was used to compare data in the three groups. F1: the fold change in metabolite concentrations in the AKU on NTBC compared to WT mice, F2: the fold change in metabolite concentration when comparing AKU on NTBC and AKU off NTBC mice, F3: the fold change in metabolite concentrations in the AKU off NTBC compared to WT mice. †Comparison of WT and AKU on NTBC mice. ††Comparison of AKU on NTBC and AKU off NTBC mice. †††Comparison of WT and AKU off NTBC mice.

Tissues	L-[$^{13}C_9$]tyrosine concentrations (µmol/gram)																
	WT						AKU on NTBC						AKU off NTBC				
	n	Mean		SD	$p^{\dagger}$	F1	n	Mean		SD	$p^{\dagger\dagger}$	F2	n	Mean		SD	$p^{\dagger\dagger\dagger}$	F3
Femur bone	4	1.0	±	0.8	<0.001	7.0	5	7	±	0.7	<0.001	10.0	4	0.7	±	0.1	0.388	0.7
Brain	4	1.0	±	0.4	<0.001	10.0	5	10	±	1	<0.001	11.1	4	0.9	±	0.5	0.997	0.9
Heart	4	3.0	±	3.0	<0.001	5.0	5	15	±	1	<0.001	15.0	4	1.0	±	0.4	0.507	0.3
Kidney	4	2.0	±	3.0	<0.001	7.5	5	15	±	1	<0.001	16.7	4	0.9	±	0.2	0.498	0.5
Liver	6	2.0	±	1.0	<0.001	7.5	6	15	±	4	<0.001	7.5	6	2.0	±	1.0	0.996	1.0
Quadriceps	6	3.0	±	1.0	<0.001	6.7	6	20	±	3	<0.001	10.0	6	2.0	±	1.0	0.900	0.7
	L-[13C9]tyrosine concentrations (µmol/gram) at the 60-min time point																	
Plasma	6	0.003	±	0.005	<0.001	6.4	6	0.021	±	0.004	<0.001	7.5	6	0.003	±	0.002	0.974	0.8

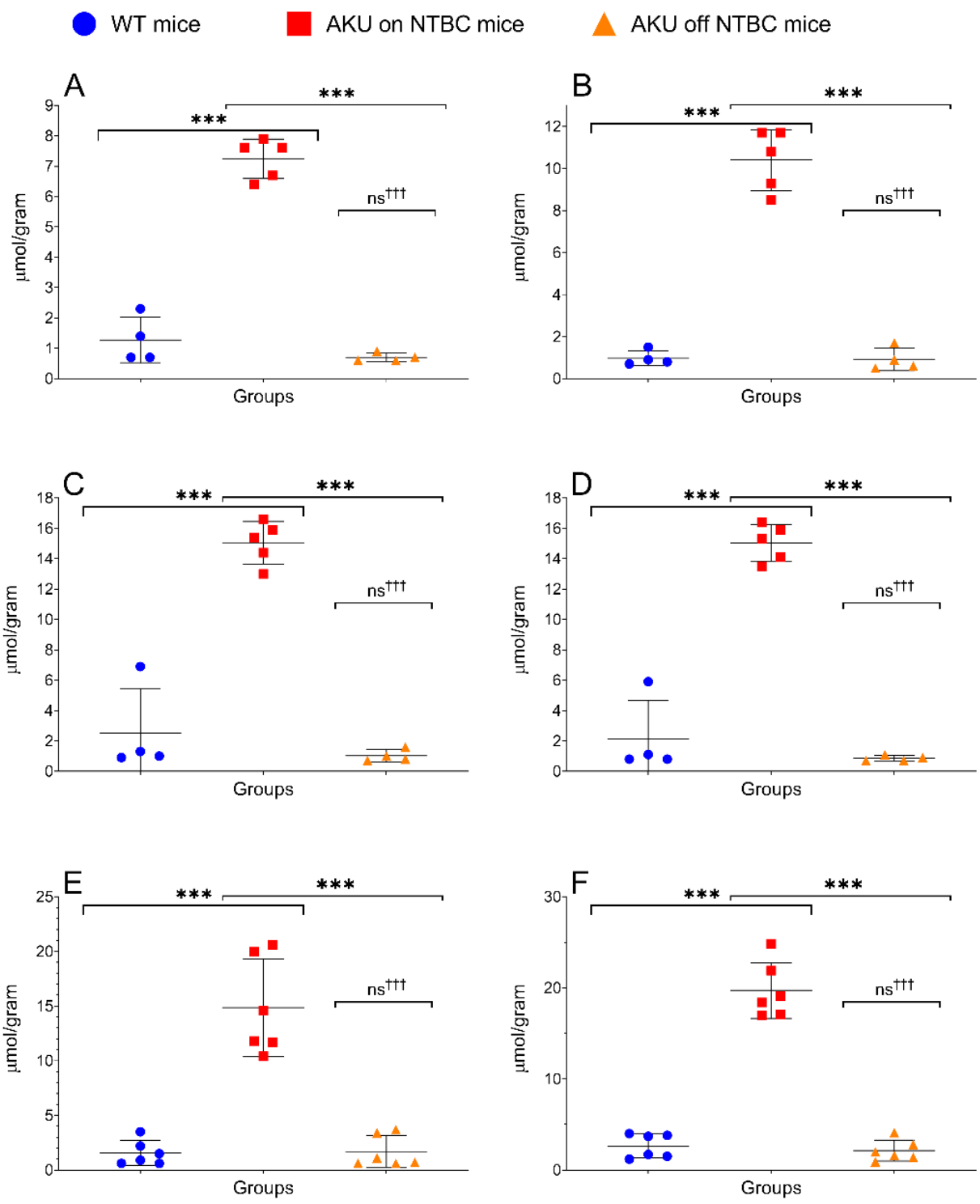

Figure 32: Effects of NTBC on tissue homogenates concentrations of L-[$^{13}C_9$]tyrosine in mice.
(A) femur bone, (B) brain, (C) heart, (D) kidney, (E) liver, (F) quadriceps muscle. Line and error bars are mean ± SD. Ordinary one-way ANOVA with Tukey's multiple comparisons test was used to compare data among the three groups. †††Comparison of WT and AKU off NTBC mice. ns: $P \geq 0.05$, ***$P < 0.001$.

3.3.11 Effects of NTBC on the urinary concentrations of phenylalanine and tyrosine stable isotopes in mice

Urinary concentrations of L-[$^{13}C_9$]tyrosine in AKU mice on NTBC are 16-fold larger compared to WT mice and approximately five-fold larger compared to AKU mice off NTBC . Urinary concentrations of L-[$^{13}C_9$]tyrosine are 3.4-fold larger in AKU mice off NTBC compared WT mice although this is not statistically significant .

Urinary concentrations of L-[d_8]phenylalanine in AKU mice off NTBC are 5.4-fold larger compared to WT mice . In NTBC treated mice, L-[d_8]phenylalanine is comparable to that in AKU mice off NTBC, and 4.7-fold larger compared to WT mice although this is not statistically significant .

Urinary concentrations of L-[d_7]tyrosine in AKU mice on NTBC are 4.3-fold larger compared to WT mice. In AKU mice off NTBC, they are3.7-fold larger compared to WT mice. Urinary concentrations of L-[d_7]tyrosine are comparable between untreated and AKU mice on NTBC. This is summarised in Figure 33 and Table 36.

When comparing the urinary concentrations of native compounds and tracers, in micromolar terms, the former are ten to twenty-six times larger in WT mice, four to twenty-six times larger in the NTBC-treated group, and nineteen to seventy-three times higher in the AKU mice off NTBC. An overall summary is provided is provided in Figure 34 and Table 37.

Table 36: Summary of the urinary concentrations of phenylalanine and tyrosine stable isotopes in mice. WT: wild type mice, AKU on NTBC: AKU mice treated with NTBC, AKU off NTBC: AKU mice that were not treated with NTBC. Ordinary one-way ANOVA with Tukey's multiple comparisons test was used to compare data in the three groups. (F1): the fold change in compound concentrations in the AKU on NTBC mice compared to WT mice, (F2): the fold change in compound concentration when comparing AKU on NTBC and AKU off NTBC mice, (F3): the fold change in compound concentrations in the AKU off NTBC mice compared to WT mice. †Comparison of WT and AKU on NTBC mice. ††Comparison of AKU on NTBC and AKU off NTBC mice. †††Comparison of WT and AKU off NTBC mice.

Compound	Urinary concentrations of compounds (µmol/L)											
	WT					AKU on NTBC				AKU off NTBC				
	n	Mean	±	SD	***F1***	n	Mean	Values	***F2***	n	Mean	±	SD	***F3***
[u-d8-Phe]	4	1.4	±	0.17	4.7	2	6.6	(4.6, 8.5)	0.9	6	7.6	±	3.9	5.4
[u-C9-Tyr]	4	1.4	±	0.33	16.4	2	23.0	(18.3, 27.4)	4.8	6	4.8	±	1.2	3.4
[u-d7-Tyr]	4	4.6	±	2.4	4.3	2	20.0	(20.6,18.7)	1.2	6	17.0	±	4.4	3.7

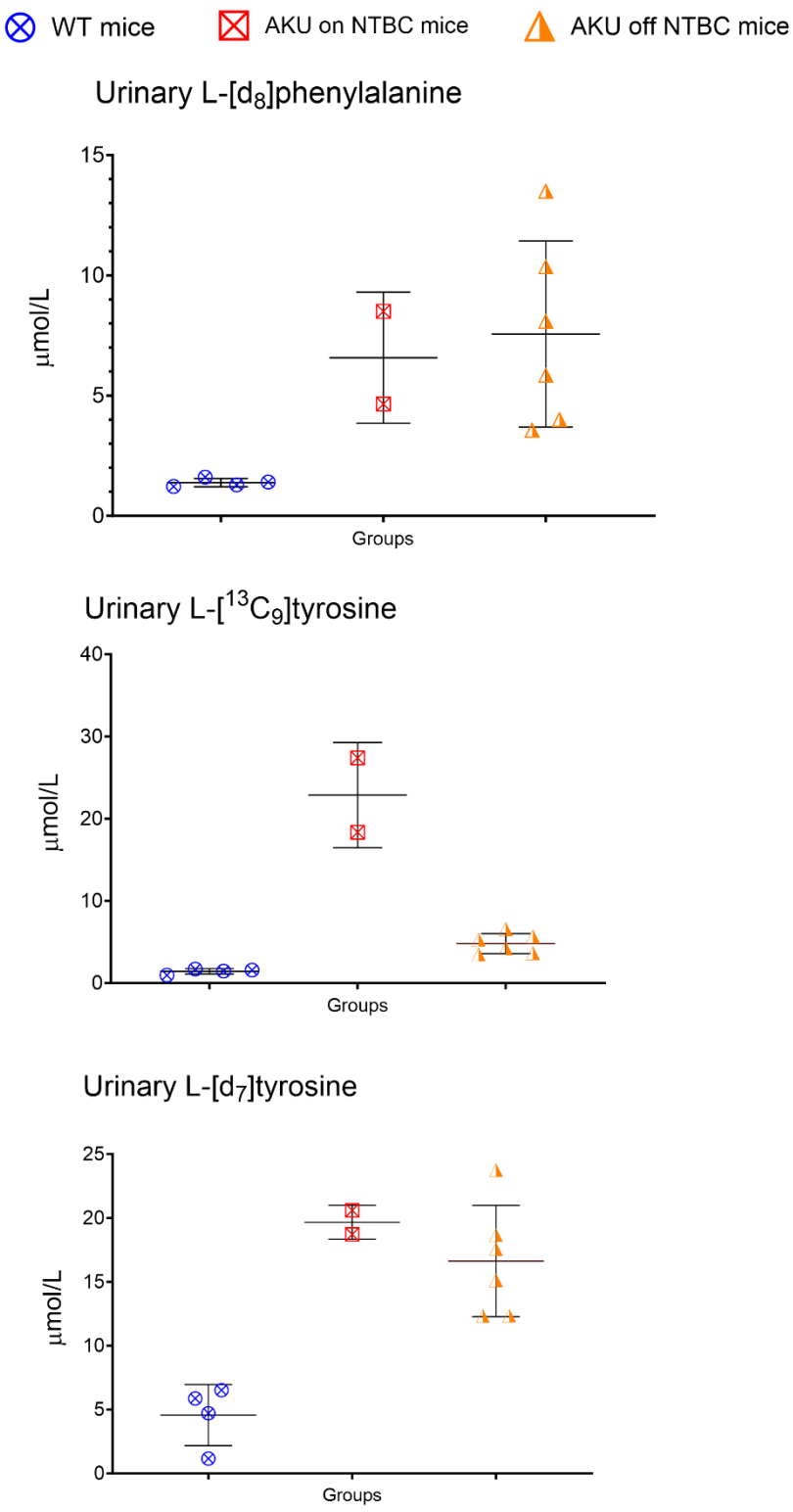

Figure 33: Effects of NTBC on the urinary concentrations of tyrosine and phenylalanine stable isotopes.
Line and error bars are mean ± SD.

Table 37: Fold changes in concentrations of the native compounds and stable isotopes of tyrosine and phenylalanine.
[u-Phe]: urinary concentrations of native phenylalanine, [u-d_8-Phe]: urinary concentrations of L-[d_8]phenylalanine, [u-Tyr]: urinary concentrations of tyrosine, [u-C_9-Tyr]: urinary concentrations of L-[$^{13}C_9$]tyrosine, [u-Tyr]: urinary concentrations of native tyrosine, [u-d_7-Tyr]: urinary concentrations of L-[d_7]tyrosine.

Native compound/Tracer	WT mice	AKU on NTBC mice	AKU off NTBC mice
[u-Phe]/[u-d_8-Phe]	26	4	19
[u-Tyr]/[u-C_9-Tyr]	31	23	73
[u-Tyr]/[u-d_7-Tyr]	10	26	21

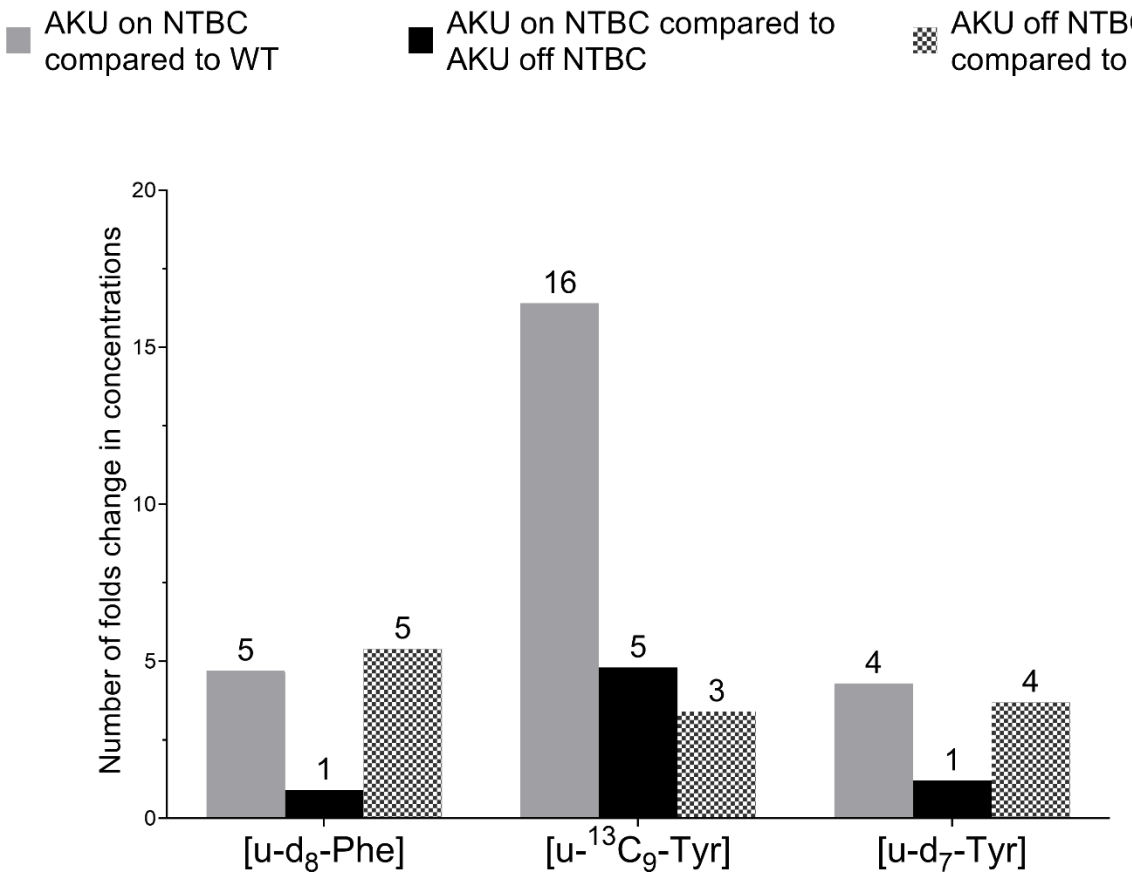

Figure 34: Fold change in the urinary concentrations of tyrosine and phenylalanine stable isotopes.
Number of folds has been rounded for ease of comparison

3.3.11.1 *Effects of NTBC on the urinary concentrations of L-[d_8]phenylalanine mice*

Urinary L-[d_8]phenylalanine concentration was approximately 2-fold larger in the AKU mice on NTBC compared to WT mice despite the lack of statistical significance. Similarly, Urinary L-[d_8]phenylalanine concentration was about 5-fold larger in the AKU mice off NTBC compared to WT mice. There was no significant difference in the urinary L-[d_8]phenylalanine concentrations between non-treated AKU and AKU mice on NTBC .

3.3.11.2 *Effects of NTBC on the urinary concentrations of L-[$^{13}C_9$]tyrosine concentrations in mice*

The urinary L-[$^{13}C_9$]tyrosine concentration in the AKU mice on NTBC was 16-fold larger compared to WT mice ; and nearly 5-fold larger compared to the AKU mice off NTBC . In contrast, there was no significant difference between WT and AKU mice off NTBC despite a 3-fold increase in the latter group.

3.3.11.3 *Effects of NTBC on the urinary concentrations of L-[d_7]tyrosine concentrations in mice*

Urinary L-[d_7]tyrosine concentration was 4-fold larger in the AKU mice on NTBC compared to WT mice . Similarly, urinary L-[d_7]tyrosine concentration was nearly 4-fold larger in the AKU mice off NTBC compared to WT mice . In contrast, there was no significant difference in the urinary L-[d_7]tyrosine concentrations between non-treated AKU and AKU mice on NTBC .

3.3.11.4 *Comparison of the urinary concentrations of phenylalanine and tyrosine stable isotopes within each of the three groups*

In the WT mice group, the concentration of L-[d_7]tyrosine is approximately 3-fold larger than that of each of L-[$^{13}C_9$]tyrosine and L-[d_8]phenylalanine. Similarly, in the AKU mice on NTBC, the concentration of L-[d_7]tyrosine is approximately 3-fold larger than that of L-[d_8]phenylalanine but is comparable to that of each of L-[$^{13}C_9$]tyrosine. In the AKU mice off NTBC, the concentration of L-[$^{13}C_9$]tyrosine is lower than that of L-[d_8]phenylalanine (~60%) while the concentration L-[d_7]tyrosine is approximately 2-fold higher. This is illustrated in Figure 35.

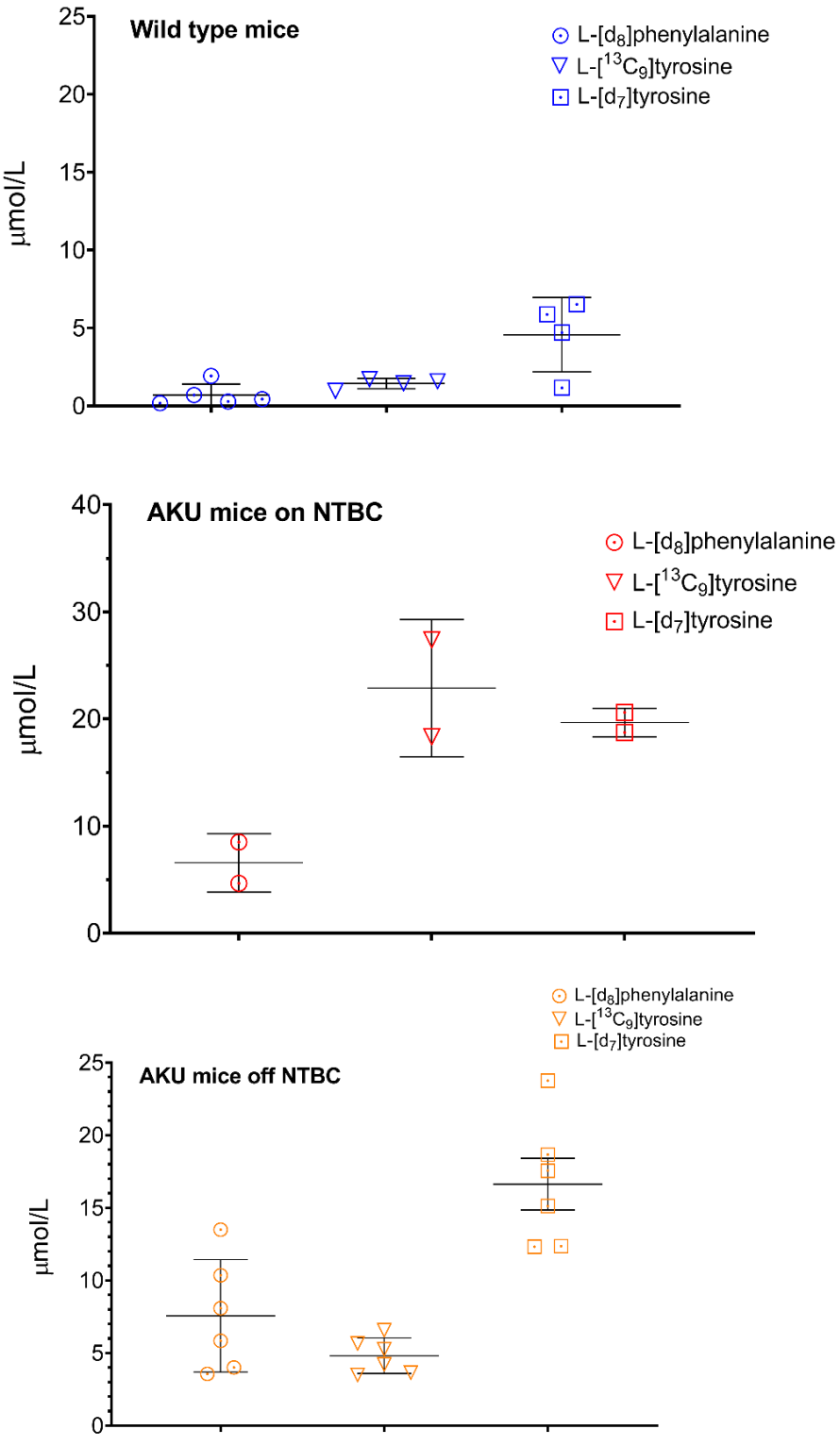

Figure 35: Summary of changes in the urinary concentrations of phenylalanine and tyrosine stable isotopes in the three groups.
Line and error bars are mean ± SD.

3.3.12 Effects of NTBC on protein turnover in mice

Data on whole-body protein breakdown (WB-PB), whole-body protein synthesis (WB-PS) and phenylalanine hydroxylation to tyrosine (Phe >Tyr) is summarised in Table 38.

There is no difference in WB-PB between NTBC-treated and AKU mice off NTBC. Both groups have 1.4-fold higher WB-PB compared to WT mice although it is not of statistical significance (*P*= 0.525 compared to AKU mice on NTBC, *P*= 0.630 compared to AKU mice off NTBC).

WB-PS is 2.5-fold higher in AKU mice on NTBC compared to WT (*P*= 0.008), and 2.1-fold higher in AKU mice off NTBC compared to WT mice although it is not of statistical significance (*P*= 0.060). WB-PS is comparable between NTBC-treated and AKU mice off NTBC (*P*= 0.561).

Compared to WT mice, Phe >Tyr is reduced by ~60% in NTBC-treated AKU(*P*= 0.299), and by ~36% in AKU mice off NTBC (*P*= 0.689). Compared to AKU mice off NTBC, Phe >Tyr is reduced by~40% compared to AKU mice off NTBC (*P*= 0.760).

Table 38: Summary of protein turnover data in mice.WB-PB: whole-body protein breakdown, WB-PS: whole-body protein synthesis, Phe >Tyr: phenylalanine hydroxylation to tyrosine. (***F1***): the fold change in the protein turnover parameter in the AKU on NTBC mice compared to WT mice, (***F2***): the fold change in the protein turnover parameter when comparing AKU on NTBC and AKU off NTBC mice, (***F3***): the fold change in the protein turnover parameter in the AKU off NTBC mice compared to WT mice. †Comparison of WT and AKU on NTBC mice. ††Comparison of AKU on NTBC and AKU off NTBC mice. †††Comparison of WT and AKU off NTBC mice.

Parameter	Mice study groups															
(µmol/Kg/min)	WT						AKU on NTBC						AKU off NTBC					
	n	Mean		SD	$p^{\dagger}$	***F1***	n	Mean		SD	$p^{\dagger\dagger}$	***F2***	n	Mean		SD	$p^{\dagger\dagger\dagger}$	***F3***
WB-PB	6	27	±	27	0.525	1.4	6	38	±	12	0.983	1.0	6	37	±	6	0.630	1.4
WB-PS	6	13	±	12	0.008	2.5	6	33	±	10	0.561	1.2	6	27	±	6	0.060	2.1
Phe > Tyr	6	14	±	15	0.299	0.4	6	5.2	±	1.3	0.689	0.6	6	9	±	8	0.760	0.7

3.3.12.1 *Effects of NTBC on whole-body protein breakdown in mice*

There is no significant difference in whole-body protein breakdown amongst the three groups in mice (Figure 36). When data examined from age perspective, three male mice in the WT group had the lowest WB-PB rate (Figure 37). From a weight perspective, three male mice with the highest weight had the lowest WB-PB (Figure 38). No firm conclusion can be drawn as one male mouse falls in the yellow zone despite its age and weight.

A group of 4 male AKU mice (3 on NTBC, 1 off NTBC) has an age of 6-8 weeks appear to have comparable PB. Also, a group of 5 female AKU mice (2 on NTBC, 3 off NTBC) aged ~25 weeks appear to have comparable WB-PB.

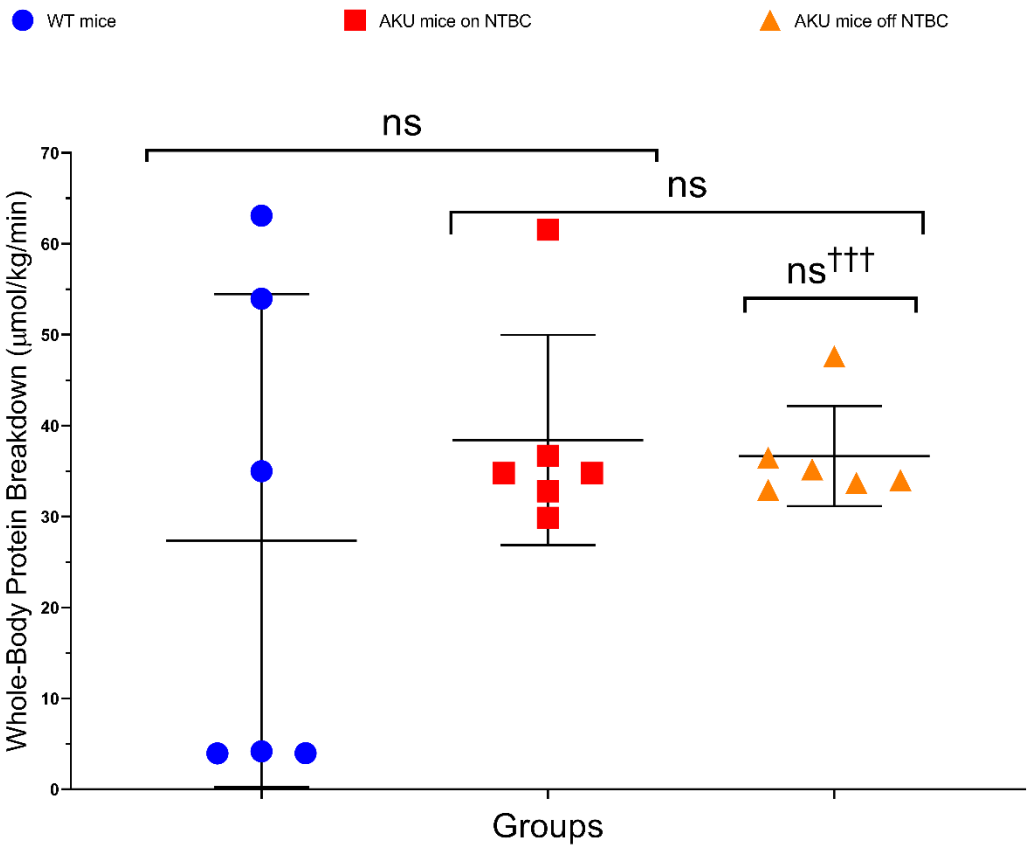

Figure 36: Effects of NTBC on protein breakdown in mice.
Line and error bars are mean ± SD.

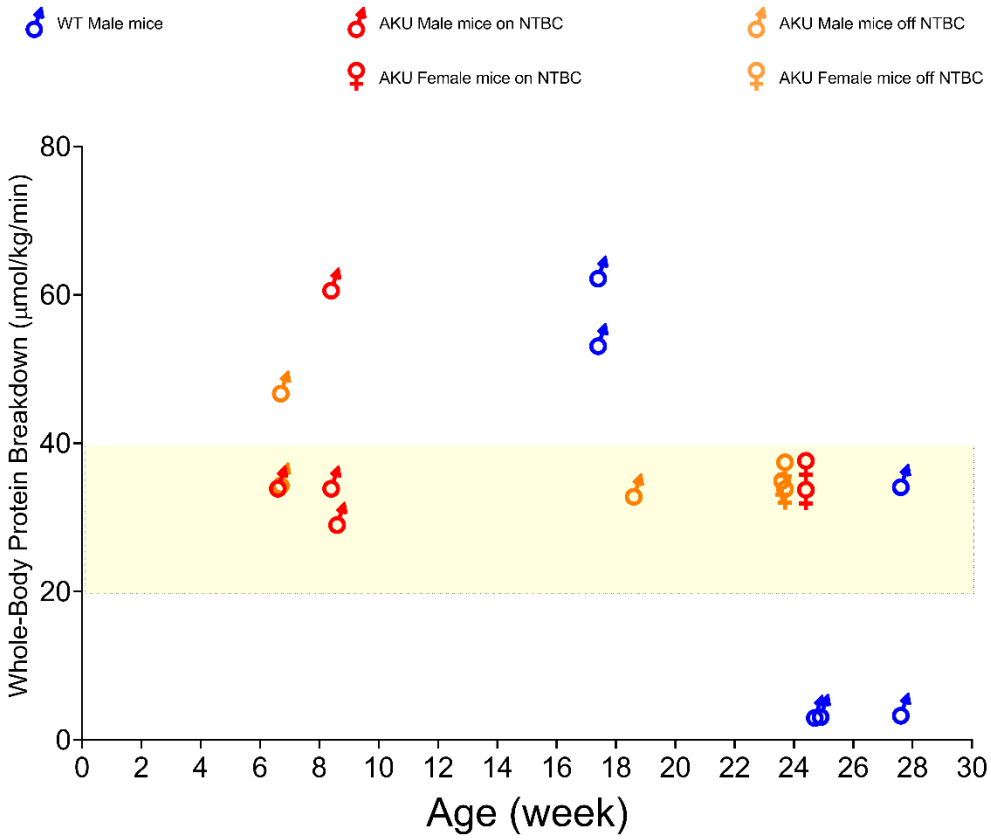

Figure 37: Effects of NTBC on whole-body protein breakdown in mice-detailed by gender and age.

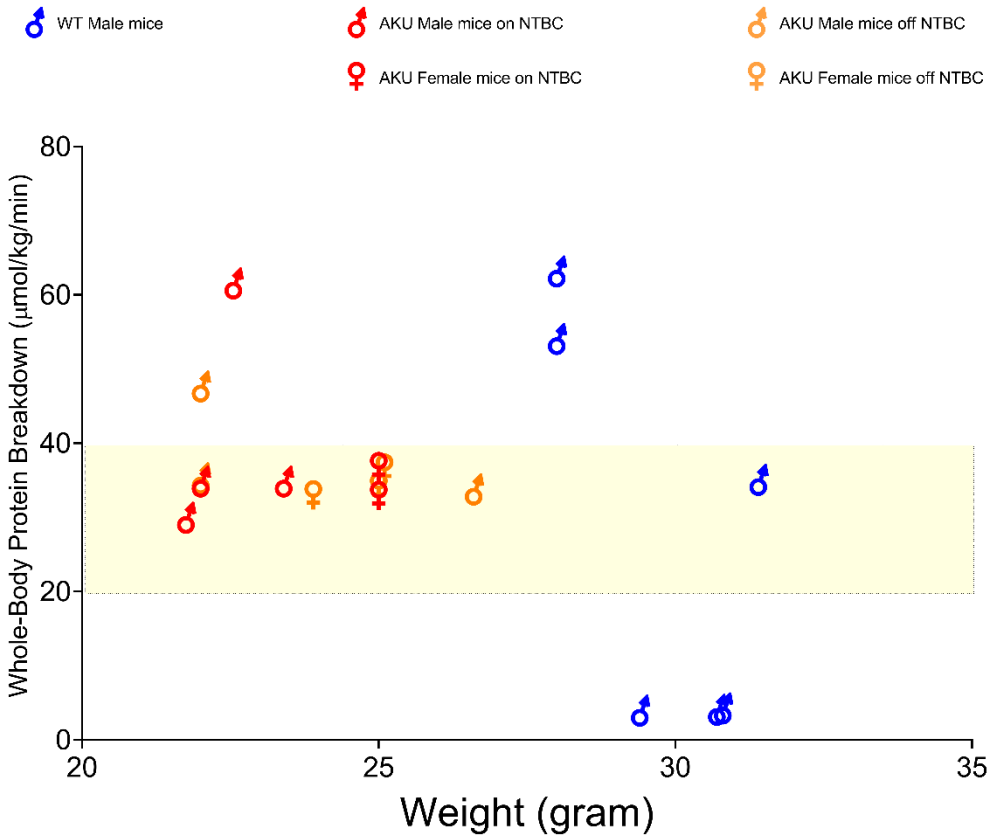

Figure 38: Effects of NTBC on whole-body protein breakdown in mice- detailed by gender and weight.*Effects of NTBC on whole-body protein synthesis in mice*

WB-PS rate in AKU mice on NTBC was almost comparable to AKU mice off NTBC (P=0.561), in contrast to being 2.5- fold higher compared to WT mice (P=0.008) and 1.2-fold higher than AKU mice off NTBC (P=0.060). WB-PS rate in AKU mice off NTBC was 2- fold higher compared to WT mice (P=0.060). This is demonstrated in Figure 39. When data examined from age perspective, three male mice in the WT group had the lowest WB-PS rate (Figure 40). From a weight perspective, three WT male mice with the highest weight had the lowest WB-PB (Figure 41).

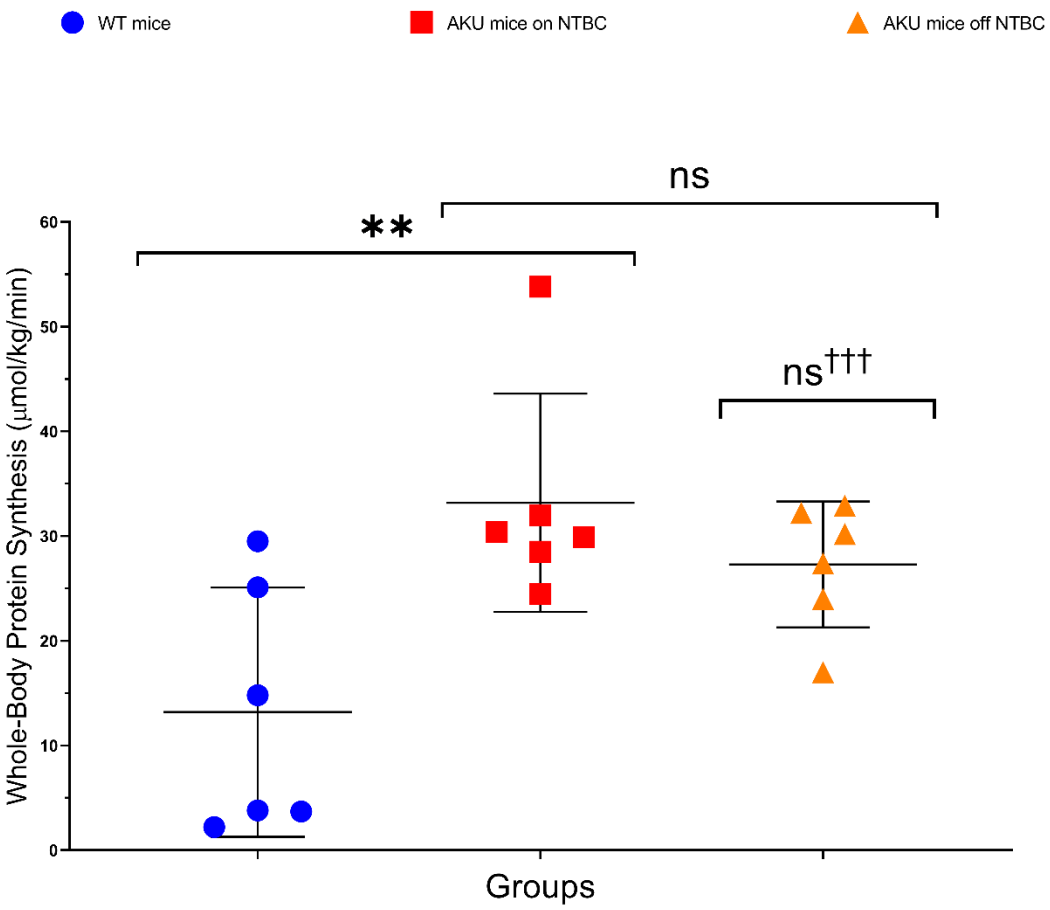

Figure 39: Effects of NTBC on whole-body protein synthesis in mice. Line and error bars are mean ± SD.

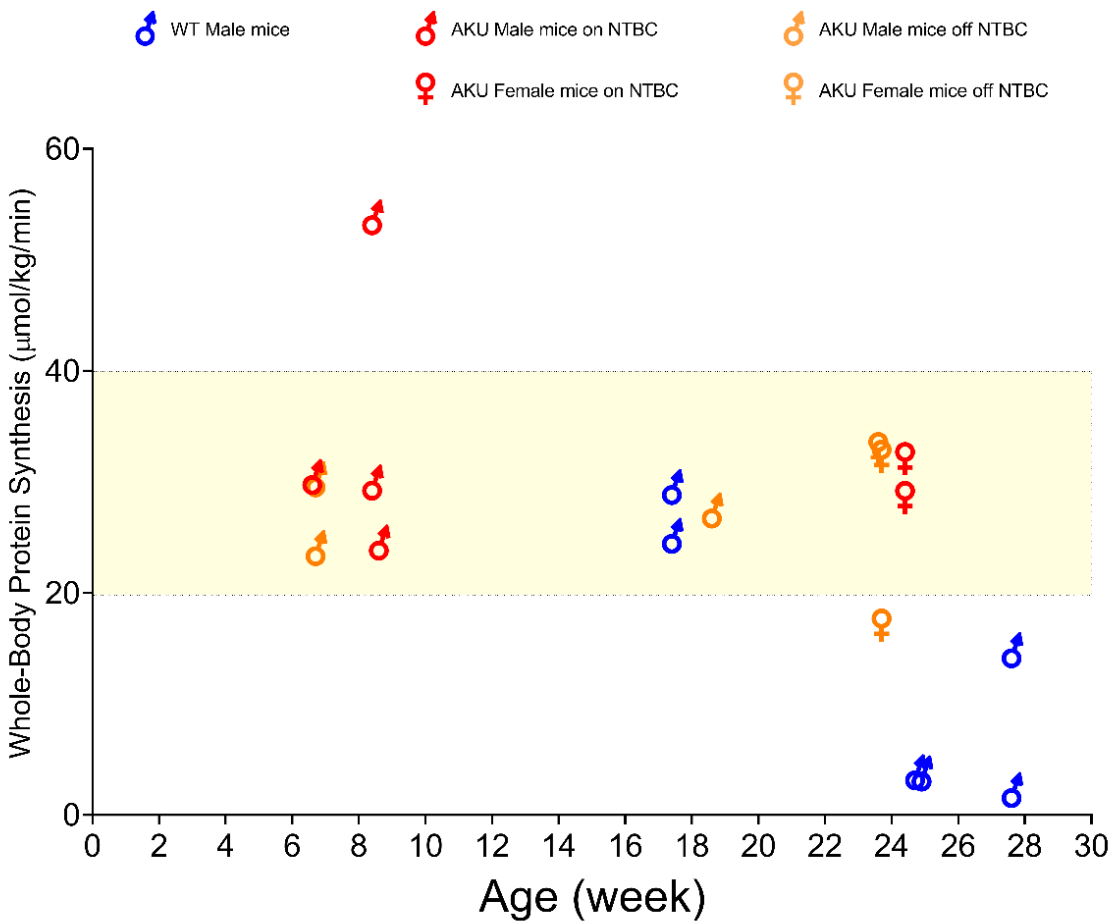

Figure 40: Effects of NTBC on whole-body protein synthesis in mice- detailed by gender and age.

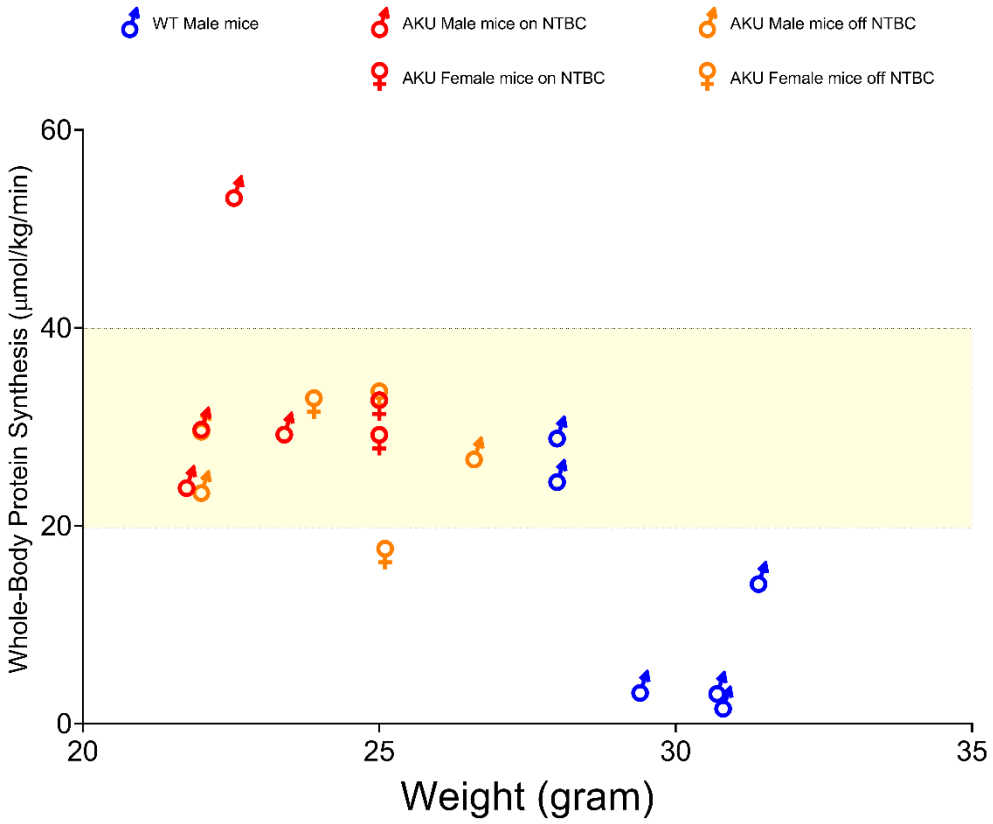

Figure 41: Effects of NTBC on whole-body protein synthesis in mice-detailed by gender and weight.

3.3.12.3 *Effects of NTBC on phenylalanine hydroxylation to tyrosine in mice*

There is no significant difference in phenylalanine hydroxylation to tyrosine amongst the three groups in mice (Figure 42).

When Phe>Tyr rate is assessed by age (Figure 43), it appears that oldest mice, regardless of which group they belong to, have the lowest hydroxylation rate.

In contrast, when assessed by weight (Figure 44), the male mice in WT group are split in two sub-groups with three eldest mice having the lowest hydroxylation rate in the study and the other three having the highest rate despite very little difference in weight.

Overall, it is difficult to make any firm conclusion on what effects age or gender have on phenylalanine hydroxylation to tyrosine following NTBC therapy in mice. Matching the study mice in future experiments will facilitate better evaluation and understanding.

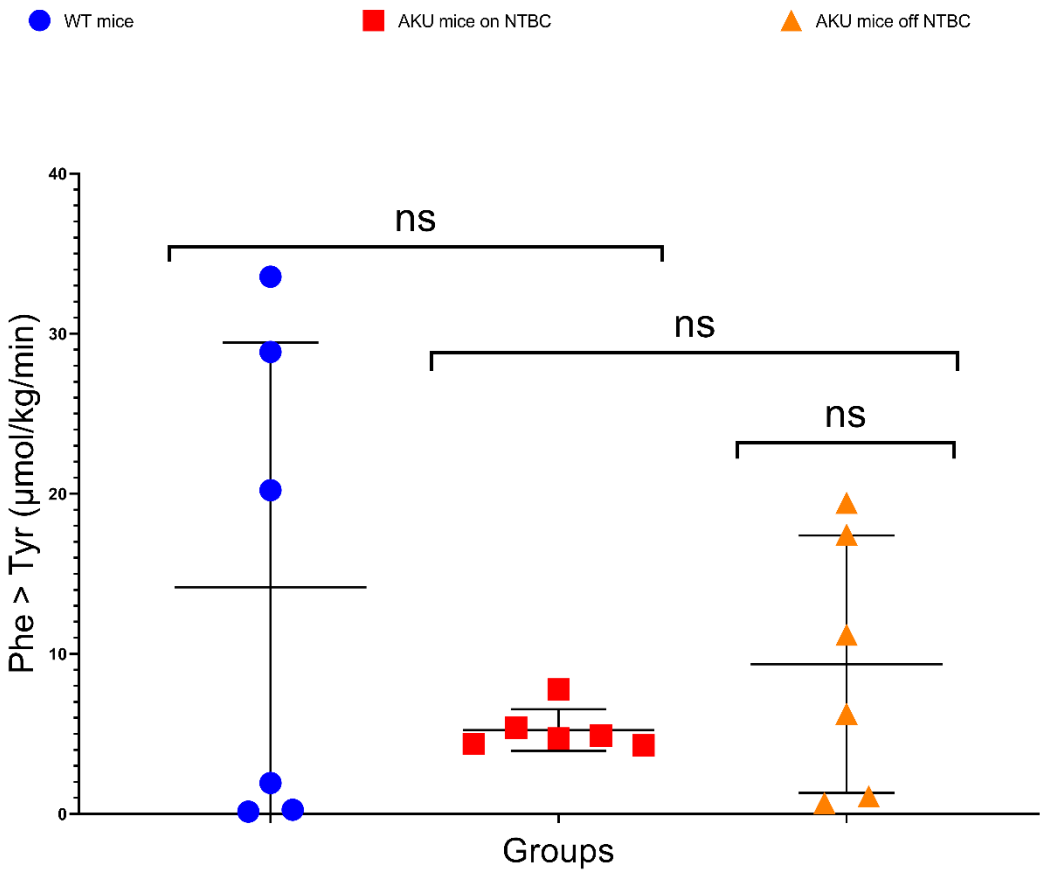

Figure 42: Effects of NTBC on phenylalanine hydroxylation to tyrosine in mice.
Line and error bars are mean ± SD.

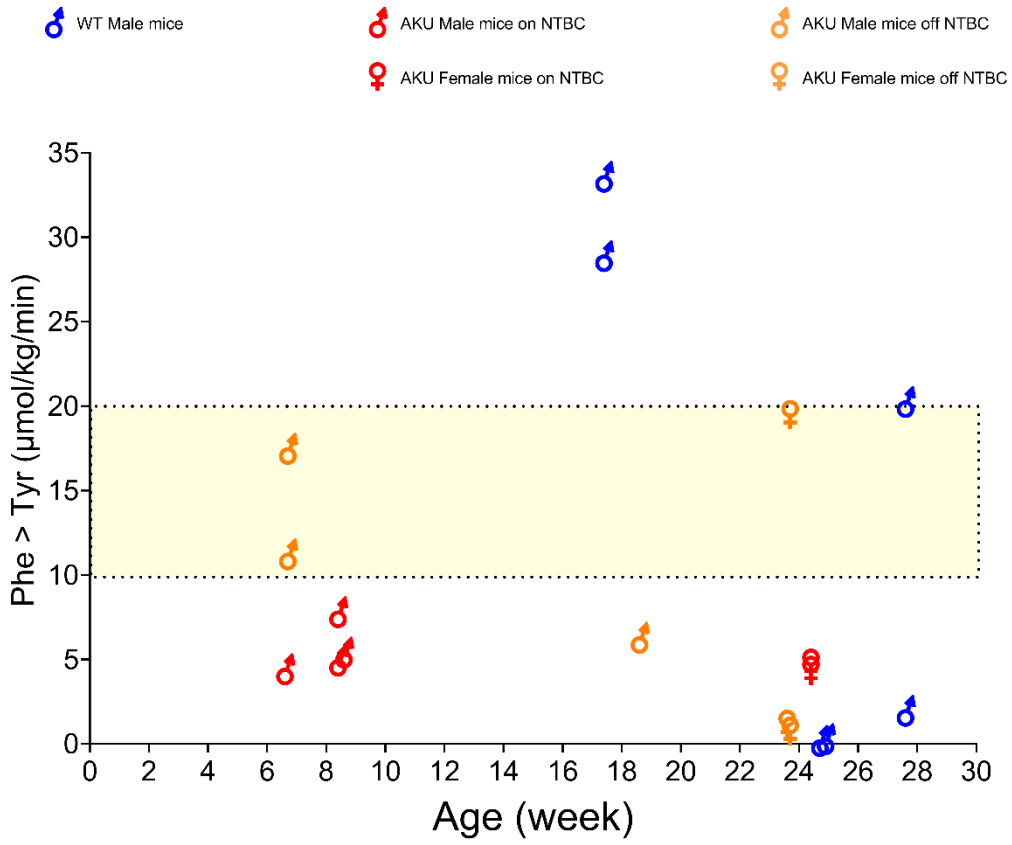

Figure 43: Effects of NTBC on phenylalanine hydroxylation to tyrosine in mice- detailed by age and gender.

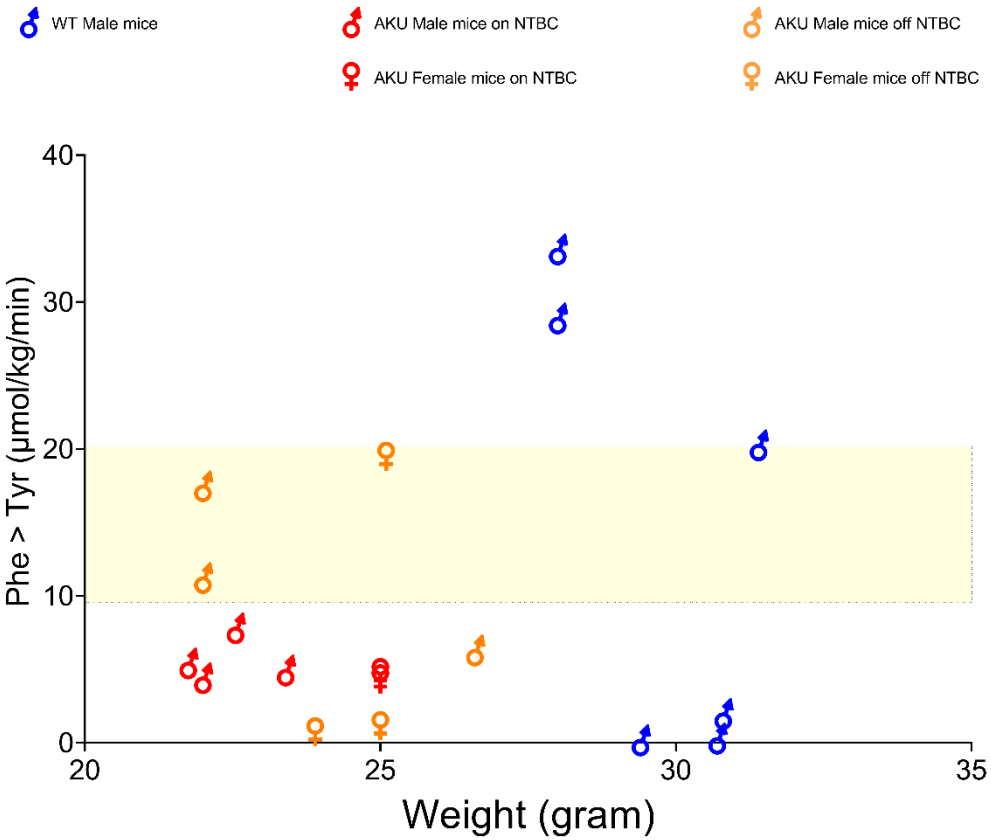

Figure 44: Effects of NTBC on phenylalanine hydroxylation to tyrosine in mice- detailed by gender and weight.

3.4 Discussion

This study presents a comprehensive examination of the NTBC effects on the tyrosine degradation pathway in urine, plasma, and tissues in mice. It also, for the first time in AKU, deploys stable isotopes methodology to assess the effects of NTBC on protein turnover in mice.

The inclusion of three groups of mice, namely, wild type, AKU mice on NTBC and AKU mice off NTBC has allowed the comparison of the changes in tyrosine pathway resulting from HGD deficiency in the case of AKU mice off NTBC and the effects of HPPD inhibition in the case of AKU mice on NTBC. Studies have reported data on plasma and urine, but this study provides tissues level data for the first time.

3.4.1 Choice of NTBC dose

There is evidence that low doses of NTBC (0.5 and 2.0 mg/L) can reduce plasma HGA concentration and ochronotic pigmentation of chondrocyte within the mouse tibio-femoral joint (Lewis, 2018). Tyrosinaemia was less marked in mice receiving 0.5 mg/L.

This study used an NTBC dose of 4mg/L which is equivalent to 0.8 mg/kg/day based on a 30 g mouse drinking approximately 6 mL/day. This dose was derived from previous work on hereditary tyrosinaemia type 1 (HT-1) mice model which showed that an NTBC dose of 1 mg/kg/day dose was efficacious in preventing liver damage (Grompe et al., 1995). This dose is consistent with previous AKU studies in mice (Taylor et al., 2012, Preston et al., 2014, Keenan et al., 2015, Hughes et al., 2020). In the NAC, AKU patients are treated with a daily dose of 2 mg (Ranganath et al., 2018) whereas in HT-1 patients are given

a daily dose of 1-2 mg/kg/day (McKiernan, 2013). In SONIA 2, the 10 mg dose of NTBC was used and subsequently approved for treatment of AKU adults by the EMA (Ranganath et al., 2020d, EMA, 2020).

3.4.2 Changes in the urinary concentrations of compounds in the tyrosine pathway

3.4.2.1 *Changes in the urinary concentrations of native compounds in the tyrosine pathway*

Overall, there are two striking findings. The first is the elevated urinary HGA in AKU mice off NTBC. The second is the massive rise in the HPPA concentrations in the AKU mice on NTBC. The y-axis scale extends to 60,000 µmol/L compared to 400 µmol/L in the WT mice and 5,000 µmol/L in the AKU mice off NTBC (Figure 25). This results from NTBC-induced HPPD enzyme inhibition and accumulation of compounds above the NTBC -induced block.

When comparing AKU mice off NTBC to WT, there is an increase of 4 to 9-fold in excretion of tyrosine, phenylalanine, HPLA and HPPA. Because of HGD deficiency, the tyrosine degradation pathway becomes less efficient and excess metabolites are excreted, via the kidneys, as they are as opposed to been broken down to fumaric acid, an intermediary in citric acid cycle, and acetoacetic acid. The latter, a weak water soluble acid, can be transformed back to acetyl Co to be utilised in citric acid cycle (Paoli, 2014)

In the NTBC -treated mice the decrease in urinary HGA and the increase in urinary tyrosine HPPA and HPLA is similar to what has been found in human studies (Milan et al., 2019). Interestingly, the post NTBC increase in urinary

HPPA and HPLA is similar to what has been reported in HPPD deficient mice with type III tyrosinaemia (Endo et al., 1991).

3.4.2.2 *Changes in the urinary concentrations of tyrosine and phenylalanine stable isotopes*

When comparing the urinary concentrations of native compounds and tracers, in micromolar terms, the former are seven to fourteen times larger in WT mice, four- twenty-six times larger in AKU mice on NTBC, and nineteen to seventy-three times higher in AKU mice off NTBC (Table 37).

Changes in these tracers' concentrations do not exactly mirror what is seen in the native compounds. Urinary L-[d_8]phenylalanine concentrations are not significantly different in AKU mice regardless of NTBC therapy (Table 36). In contrast, urinary phenylalanine in AKU mice off NTBC is 5-fold larger compared to AKU mice on NTBC, and 4-fold larger compared to WT mice (Table 27). Urinary L-[$^{13}C_9$]tyrosine in AKU mice on NTBC is 5-fold larger when compared to AKU mice off NTBC; and approximately 16-fold larger compared WT mice. On the other hand, urinary L-[d_7]tyrosine in AKU mice on NTBC is comparable to that in the AKU mice off NTBC, but it is 4-fold larger compared to WT mice. Furthermore, urinary L-[d_7]tyrosine in AKU mice off NTBC is 3.7-fold larger compared to WT mice.

The diet in this study contained 36 mg tyrosine and 55 mg phenylalanine per 5 grams (average consumption of 30-gram mouse) of mice food. In contrast, the tracers were given in a bolus of 0.5 mL of 0.9% saline containing 0.2 mg (1.05 µmol) of L-[$^{13}C_9$]tyrosine and 0.5 mg (2.70 µmol) of L-[d_8]phenylalanine. The bolus was injected into the tail vein of in each mouse at baseline. Because

Mice have greater metabolic activity compared to humans, this study used the maximum concentration possible for L-[$^{13}C_9$]tyrosine, namely 400 mg/L (2.1 mmol/L) which was the same concentration used in the human study.

From chemistry standpoint, stable isotopes are handled in the same way as native compounds. Therefore, it is likely that the differences urinary excretion of tyrosine and phenylalanine tracers compared to native compounds are attributed to the difference in route and method of administration.

When comparing micromolar concentrations of stable isotopes within each group, there is a common trend of 2 to 3-fold increase in L-[d_7]tyrosine compared to L-[d_8]phenylalanine. A possible explanation is that most of the injected L-[d_8]phenylalanine is converted to L-[d_7]tyrosine which is then excreted in the urine. This applies to both WT and AKU off NTBC mice. In the AKU on NTBC mice, tyrosine degradation is blocked. Therefore, more L-[d_7]tyrosine and L-[$^{13}C_9$]tyrosine are excreted in the urine. These observations are consistent with changes in native tyrosine and phenylalanine urinary concentrations in this study and in the literature (Ranganath et al., 2022b).

3.4.3 Changes in the plasma concentrations of compounds in the tyrosine pathway

3.4.3.1 *Changes in the plasma concentrations of native compounds in the tyrosine pathway*

This study found that NTBC reduced HGA and increased tyrosine in plasma, but it had no effect on phenylalanine .Furthermore, it activated the HPLA/HPPA pathway as evidence by the increase in HPLA and HPPA concentrations. Lewis (2018) has demonstrated that after 20 weeks of NTBC 2mg/L, phenylalanine was significantly elevated in AKU mice on NTBC compared to control mice but not at doses of 0.125 or 0.5 mg/L; while at 40 weeks, there was no significant difference amongst treatment groups at doses of 2mg/L or lower (Lewis, 2018). In the current study, mice received 4mg/L for one week. NTBC achieves complete inhibition of HPPD after 4 hours of dosing (Lock et al., 2000) meaning that longer NTBC treatment duration was not necessary for the purpose of this study.

The findings in the current study are in keeping with what others found in human studies (Suwannarat et al., 2005, Introne et al., 2011, Ranganath et al., 2016, Milan et al., 2017, Davison et al., 2018b, Milan et al., 2019).

3.4.3.2 *Changes in the plasma concentrations of tyrosine and phenylalanine stable isotopes / and TTR enrichment curves*

Compared to AKU mice on NTBC, the AUC for L-[d_8]phenylalanine concentrations against time in each of the WT and AKU mice off NTBC groups is approximately five to six times larger. Nonetheless, the percentage

decrease in concentrations against time for L-[d_8]phenylalanine is not significantly different amongst the three groups.

As regards L-[$^{13}C_9$]tyrosine, AUC and percentage change of concentrations against time (5 to 60 min) demonstrate that L-[$^{13}C_9$]tyrosine undergoes minimal degradation in AKU mice on NTBC with a percentage change of 34% compared to 93% in AKU mice off NTBC and 95% in WT mice. Interestingly, L-[d_7]tyrosine undergoes reduced degradation in AKU mice on NTBC (77%) compared to untreated AKU (85%) and WT mice (89%). This is still more than what is observed for L-[$^{13}C_9$]tyrosine and it could be that L-[d_7]tyrosine resulting from L-[d_8]phenylalanine conversion is better handled and degraded compared to L-[$^{13}C_9$]tyrosine.

Despite the lack of statistically significant difference, tyrosine decay curve is a flat line in parallel to x- axis reflecting the minimal degradation of tyrosine following NTBC. There is an overlap of error bars in both WT and AKU mice off NTBC which is likely due the small sample size making the study underpowered to detect a significant difference. This is a good demonstration that one cannot rely completely on statistical tests to evaluate of the results from experiments with small sample size.

3.4.4 Changes in the tissue homogenates concentrations of compounds in the tyrosine pathway

3.4.4.1 *Changes in the tissue homogenates concentrations of native compounds in the tyrosine pathway*

Concentrations of compounds in tissue homogenates can be several hundred times larger than of plasma. This is likely due to the water content of tissues. In mice, water constitutes approximately 70% of body weight (Bailey et al., 1960, Durbin et al., 1992) with nearly 65% of the total body water is contained in the intracellular compartment while 35% exists in the extracellular space (Chapman et al., 2010). Average blood volume in the mouse is 7-8%. Notably, total body water in mice becomes reduced with age (Fox et al., 2006). This study confirms for the first time that tissues concentrations of the tyrosine pathway compounds generally mirror the plasma.

Phenylalanine: The brain homogenate concentration of phenylalanine in the AKU mice on NTBC is 70% of that in the AKU mice off NTBC and 20% less compared to WT. This is consistent with findings from NTBC treated HT-1 mice (van Ginkel et al., 2019). This is a concern in HT-1 treated patients, as low phenylalanine may have a role in the neurocognitive impairment documented in this group (van Ginkel et al., 2017, van Vliet et al., 2019). This may have important implications for treating younger AKU patients with NTBC because the potential hypertyrosinaemia related cognitive impairment. Tyrosine is competitively transported across BBB through the Large Neutral Amino Acid Transporter 1 (LAT1) (also responsible for transporting phenylalanine across BBB). It is likely that, in the context of hypertyrosinaemia, phenylalanine becomes less available to be transported across BBB in comparison to

tyrosine. Notably, restricting dietary phenylalanine and tyrosine in HT-1 has resulted in normalising the brain concentrations of Large neutral amino acids (van Ginkel et al., 2019). Phenylalanine concentrations in the brains in AKU mice on NTBC is significantly lower compared to AKU mice off NTBC (*P*= 0.006).

Tyrosine: Tissue homogenates concentrations of tyrosine are universally larger across all tissues compared to both WT and non-treated NTBC mice. In AKU mice on NTBC, tyrosine in brain tissues was 9-fold higher compared to wild type and 8-fold higher compared to AKU mice off NTBC. This is comparable to the 10-fold change in tyrosine concentrations reported by Davison et al. (2019b).

The ratio of homogenate to plasma concentrations of tyrosine ranges from 613 (brain homogenates) to 2040 (liver homogenates) in WT mice, 469 (bone homogenates) to 1220 (liver homogenates) in AKU mice on NTBC and 589 (bone homogenates) to 2100 (liver homogenates) in AKU mice off NTBC. This reflects the larger intracellular compartment containing tyrosine-a small molecule that is distributing freely in water.

Tyrosine concentrations and the magnitude of post NTBC tyrosinosis in the brain is approximately 50% less compared to peripheral tissues (liver, kidney, quadriceps muscle, plasma) in AKU mice, which can probably be explained by the presence of the blood brain barrier. A close analogy is glucose where CSF glucose is 60-80% of the circulating glucose. Specifically, tyrosine is competitively transported across the blood-brain barrier by the LAT1 transporter. This could possibly limit the rise in tyrosine levels compared to

peripheral tissues and will reduce the damaging effects of tyrosinaemia on the developing brain. Despite the apparent variability, brain tyrosine is nearly 8-fold higher in the AKU mice on NTBC compared to the AKU mice off NTBC. In contrast, tyrosine increases by nearly 5-fold following NTBC in liver homogenates.

HGA: As expected HGA is increased across all tissues in AKU mice off NTBC. Post NTBC data in tissues demonstrate that systemic and tissue HGA concentrations falls to levels compared to WT. It is well documented that NTBC reduces circulating HGA concentrations; and this study provided, for the first time, the experimental proof that HGA concentrations in tissues are also reduced to levels comparable with WT. HGD is expressed in the brain (Bernardini et al., 2015). But brain HGA concentrations in AKU mice off NTBC are not very high compared to other tissues. This could be explained by the presence of the blood brain barrier, or it could be simply that the measured HGA is reflective of the blood content in the homogenised tissues of the brain.

HGA is significantly higher in bone homogenates of AKU mice off NTBC. This could mediate the increased bone turnover leading to the bone loss seen in AKU patients (Ranganath et al., 2020a).

HPLA: When comparing tissue homogenates to plasma, HPLA appears abundant in the plasma and hardly in the tissues. This could be due to the efficient urinary excretion of this compound in the urine and the limited intracellular transport of this. In AKU mice on NTBC, HPLA was detected in the brain at concentration that is 14 times higher than what is in circulating in

the plasma. Notably, elevated HPLA has been reported in CSF on AKU mice on NTBC (Davison et al., 2022b).

NTBC concentrations were highest in the liver and the kidney. This is because the concentrations of the HPPD enzyme in the liver and the kidney are higher than other tissues (Lock et al., 2000).

Stable isotopes of tyrosine and phenylalanine: These behave in the same way as the native compounds. This is demonstrated clearly by the data in the current study. Urinary concentrations of both stable isotopes of tyrosine are increased in NTBC treated mice. The reason for this is the block in HPPD in AKU mice on NTBC and the enzyme defect HGA dioxygenase enzyme leading to accumulation of compounds above the level of metabolic block.

3.4.5 Protein turnover in mice

While there was no significant difference in protein breakdown among the three groups of mice, protein synthesis was significantly lower in WT mice which were older than mice in the other two groups. The age difference in mice leads to difference in rate of metabolism observed when comparing young mice to older mice. Previous studies have identified age as confounder for interpreting metabolic studies in mice (Korou et al., 2013). Mice in this study were not matched in terms of age or gender. In fact, the age of some mice in WT and AKU mice off NTBC is three times that of some of NTBC treated mice.

Despite the lack of statistical significance, the rate of phenylalanine hydroxylation to tyrosine in the AKU mice on NTBC was reduced to 63% compared to WT mice; and reduced to 42% compared to AKU mice that were

off NTBC. Not surprisingly, attempts to control NTBC induced hypertyrosinaemia by restricting dietary phenylalanine have proved ineffective (Hughes et al., 2020). This could be due to a product inhibition-like effect driven by tyrosinaemia; with the latter leading to much lesser degree of phenylalanine conversion to tyrosine.

3.4.6 Study limitations

Non- matching of the mice groups is an important limitation of this study. The three groups were not age-matched with age median values of 24.8, 8.5 and 21.1 weeks in WT, AKU on NTBC and AKU off NTBC mice, respectively. The weight median values were 30, 23 and 24 gram in WT, AKU on NTBC and AKU off NTBC mice, respectively. This was down to logistical reasons and the availability of mice at the time of conducting study. The age and weight differences in mice could be associated with differences in body fluid compartments and possibly differences in absolute tyrosine and other related compounds values. Nonetheless, the gene defect and the NTBC-induced HPPD defect are likely to be similar. A crossover study design would have overcome the variability in AKU mice age and weight. However, this was not possible because murine tissues were harvested at the end of each experiment.

Another drawback of this study was that two mice in the AKU off NTBC group were given NTBC 7 days earlier. Although, at the time of the experiment, there was no discernible chromatographic peaks in the NTBC transition windows, it was possible to measure NTBC in the liver homogenates of these two mice.

Nonetheless, the biochemical phenotype in these two mice was comparable to those who did not receive NTBC and the overall conclusion is not different. It was not surprising to find NTBC in the livers of these two mice. Lock et al. (2000) have found that radiolabelled NTBC was detected in the murine liver and kidney seven days after a single dose of 30 μmol/kg (equivalent to 9.88 mg/kg).

A further limitation was the limited sampling of urine in mice. Urine was only collected from two mice in the NTBC -treated AKU group, five mice in the WT group and six mice in the non-treated AKU group. This limits the value of statistical analysis in an already small sample size. *P* values were reported as part of the one-way ANOVA with Tukey's multiple comparison test. The usefulness of *P* value, in this context, particularly in relation to AKU mice on NTBC is very limited. To overcome this, the number of folds change in compounds and stable isotopes concentrations was used when comparing the AKU mice on NTBC with the other two groups. Another flaw in the urine study is the assumption of equal urine sample volume and the lack of urinary creatinine measurement which would have allowed for the normalising the metabolite excretion in the urine samples which were obtained. Due the small volume of the urine samples obtained it was not practical to obtain creatinine measurements as in humans. Urine collection was a random one at the end of the experiment and there was no control for food effects, water consumption, concentration differences in urine due to fluids consumed. A more robust approach would have been to catheterise the mice which would be too invasive and stressful for mice. Therefore, compounds concentrations

were expressed in micromole/L and it was assumed that urine samples volumes were comparable.

The small sample size has mandated prioritising the measurements of native compounds and tracers' concentrations. These measurements were carried out using two separate assays each requiring a dedicated volume. This is in addition to sample volume used in the pilot study required for developing the LC-MS/MS assay for measurement of tyrosine and phenylalanine stable isotopes in mice urine. Urine samples from mice were treated as precious samples and glass inserts were used to enable us to use the minimum sample volume possible. It is recommended that plasma and urinary creatinine measurements in AKU are performed using Jaffe method to overcome HGA interference seen in enzymatic measurements of creatinine (Curtis et al., 2014). In the Royal Liverpool hospital biochemistry laboratory, creatinine assay is carried using a Roche modular platform (Cobas-Roche Diagnostics, West Sussex, England). This assay requires a minimum volume of 10 μL (Roche, 2010). An LC-MS/MS assay, not available at the time of the study, would have been a good alternative overcoming both the sample volume requirement as well the potential HGA interference in creatinine assay.

Finally, there is huge variation in the urinary HPPA concentrations within the AKU on NTBC group. Biochemical analysis was carried out with and without dilution and similar results were found. This discrepancy is likely due to the small sample size of two in the AKU on NTBC group. Sampling urine from more mice in the AKU on NTBC group would have overcome this.

3.5 Chapter 3 conclusion

Through measurement of tissue homogenate concentrations of tyrosine, this study provides for the first time, the experimental proof for the magnitude of tyrosine systemic increase following NTBC therapy. This study also provides a comprehensive examination of changes in the tyrosine degradation pathway metabolites across various tissues. Furthermore, the stable isotopes methodology has been deployed in AKU mice allowing the assessment of protein turnover in mice using the pulse approach.

Future work should focus on impact of dietary restriction on phenylalanine and tyrosine in mice (Hughes et al., 2020) and other intervention to minimise effects of NTBC induced tyrosinaemia in addition to studying the long-term effects of NTBC on protein turnover.

Acknowledgement and declaration

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3.6 Mice Study Appendix

Table 39: Summary of L-[d_7]tyrosine concentrations in mice during the study.
NA: not assessed, ND: not detected

	L-[d_7]tyrosine concentrations (µmol/L)					
	BALB/c Wild type					
Time (min)	44.3	44.4	52.1	52.2	53.1	53.2
0	ND	ND	ND	ND	ND	ND
5	NA	NA	9.22	7.29	4.04	3.61
20	1.81	7.13	2.42	2.80	2.76	2.81
40	0.65	3.25	1.11	1.20	2.13	1.16
60	0.29	1.79	0.52	0.71	1.18	0.04
Concentrations-Ratio *60 to 5 min*	NA	NA	0.06	0.10	0.29	0.01
Concentrations-Ratio *60 to 20 min*	0.16	0.25	0.22	0.25	0.43	0.01
Concentration-% decrease *5 to 60 min*	NA	NA	94%	90%	71%	99%
Concentration-% decrease *20 to 60 min*	84%	75%	78%	75%	57%	99%
	BALB/c HGD-/- on NTBC					
Time (min)	54.1	57.1	57.2	57.3	56.1	56.2
0	ND	ND	ND	ND	ND	ND
5	NA	8.2	6.5	6.6	5.2	5.2
20	3.8	5.1	2.9	3.2	3.7	3.8
40	2.8	2.8	1.7	2.1	2.1	2.3
60	2.9	1.4	1.1	1.6	1.2	1.7
Concentrations-Ratio *60 to 5 min*	NA	0.2	0.2	0.2	0.2	0.3
Concentrations-Ratio *60 to 20 min*	0.8	0.3	0.4	0.5	0.3	0.4
Concentration-% decrease *5 to 60 min*	NA	83%	83%	76%	76%	67%
Concentration-% decrease *20 to 60 min*	23%	72%	62%	52%	67%	55%
	BALB/c HGD-/- off NTBC					
Time (min)	54.2	54.3	58.1	58.2	58.3	60.1
0	0	0	0	0	0	0
5	NA	NA	1.8	10.2	3.4	3.8
20	2.3	3.1	1.5	2.8	2.9	2.7
40	0.8	1.1	1.1	0.7	1.4	1.5
60	0.9	0.8	0.4	0.2	0.4	1.0
Concentrations-Ratio *60 to 5 min*	NA	NA	0.23	0.02	0.11	0.26
Concentrations-Ratio *60 to 20 min*	0.38	0.25	0.28	0.09	0.13	0.36
Concentration-% decrease *5 to 60 min*	NA	NA	77%	98%	89%	74%
Concentration-% decrease *20 to 60 min*	62%	75%	72%	91%	87%	64%

Table 40: Summary of L-[$^{13}C_9$]tyrosine concentrations in mice during the study.
NA: not assessed

	L-[$^{13}C_9$]tyrosine concentrations (µmol/L)					
	BALB/c Wild type					
Time (min)	44.3	44.4	52.1	52.2	53.1	53.2
0	ND	ND	ND	ND	ND	ND
5	NA	NA	16.8	17.5	137.2	108.1
20	3.5	13.5	4.7	6.1	102.1	32.0
40	1.3	7.1	2.0	2.6	40.7	5.6
60	0.8	3.6	0.8	1.3	13.2	0.1
Concentrations-Ratio *60 to 5 min*	NA	NA	0.0	0.1	0.1	0.0
Concentrations-Ratio *60 to 20 min*	0.2	0.3	0.2	0.2	0.1	0.0
Concentration-% decrease *5 to 60 min*	NA	NA	95%	93%	90%	100%
Concentration-% decrease *20 to 60 min*	77%	73%	83%	79%	87%	100%
	BALB/c HGD-/- on NTBC					
Time (min)	54.1	57.1	57.2	57.3	56.1	56.2
0	ND	ND	ND	ND	ND	ND
5	NA	31.5	33.9	34.8	27.5	26.9
20	27.5	25.1	23.1	24.7	27.1	27.7
40	23.4	22.7	19.5	21.8	22.7	23.7
60	26.9	19.0	18.5	18.8	19.0	24.2
Concentrations-Ratio *60 to 5 min*	NA	0.6	0.5	0.5	0.7	0.9
Concentrations-Ratio *60 to 20 min*	1.0	0.8	0.8	0.8	0.7	0.9
Concentration-% decrease *5 to 60 min*	NA	40%	45%	46%	31%	10%
Concentration-% decrease *20 to 60 min*	2%	24%	20%	24%	30%	13%
	BALB/c HGD-/- off NTBC					
Time (min)	54.2	54.3	58.1	58.2	58.3	60.1
0	ND	ND	ND	ND	ND	ND
5	NA	NA	138.1	18.6	269.7	21.7
20	7.2	9.7	34.0	5.9	132.3	15.3
40	2.5	3.9	12.8	1.3	44.0	8.9
60	1.1	3.0	5.6	0.5	2.3	4.5
Concentrations-Ratio *60 to 5 min*	NA	NA	0.04	0.03	0.01	0.21
Concentrations-Ratio *60 to 20 min*	0.16	0.31	0.16	0.09	0.02	0.30
Concentration-% decrease *5 to 60 min*	NA	NA	96%	97%	99%	79%
Concentration-% decrease *20 to 60 min*	84%	69%	84%	91%	98%	70%

Table 41: Summary of L-[d_8]phenylalanine concentrations in mice during the study.
NA: not assessed

	L-[d_8]phenylalanine concentrations (µmol/L)					
	BALB/c Wild type					
Time (min)	44.3	44.4	52.1	52.2	53.1	53.2
0	ND	ND	ND	ND	ND	ND
5	NA	NA	27.6	31.1	227.9	212.6
20	4.5	7.9	6.3	8.1	112.7	55.2
40	0.8	1.5	1.3	2.2	41.4	9.0
60	0.2	0.3	0.2	0.6	18.1	0.1
Concentrations-Ratio *60 to 5 min*	NA	NA	0.01	0.02	0.08	0.00
Concentrations-Ratio *60 to 20 min*	0.04	0.04	0.03	0.08	0.16	0.00
Concentration-% decrease *5 to 60 min*	NA	NA	99%	98%	92%	100%
Concentration-% decrease *20 to 60 min*	96%	96%	97%	92%	84%	100%
	BALB/c HGD-/- on NTBC					
Time (min)	54.1	57.1	57.2	57.3	56.1	56.2
0	ND	ND	ND	ND	ND	ND
5	NA	22.2	19.6	26.9	24.2	22.1
20	6.0	8.3	2.7	4.1	17.3	5.3
40	1.1	1.6	0.5	1.1	2.0	1.0
60	0.6	0.3	0.2	0.3	0.9	0.3
Concentrations-Ratio *60 to 5 min*	NA	0.01	0.01	0.01	0.04	0.02
Concentrations-Ratio *60 to 20 min*	0.09	0.03	0.07	0.08	0.05	0.06
Concentration-% decrease *5 to 60 min*	NA	99%	99%	99%	96%	98%
Concentration-% decrease *20 to 60 min*	91%	97%	93%	92%	95%	94%
	BALB/c HGD-/- off NTBC					
Time (min)	54.2	54.3	58.1	58.2	58.3	60.1
0	ND	ND	ND	ND	ND	ND
5	NA	NA	225.7	25.6	307.1	29.2
20	4.7	5.0	61.0	6.8	133.4	16.8
40	0.8	0.7	19.7	0.8	48.3	7.5
60	0.2	0.3	8.5	0.2	3.8	3.4
Concentrations-Ratio *60 to 5 min*	NA	NA	0.04	0.01	0.01	0.12
Concentrations-Ratio *60 to 20 min*	0.04	0.05	0.14	0.02	0.03	0.20
Concentration-% decrease *5 to 60 min*	NA	NA	96%	99%	99%	88%
Concentration-% decrease *20 to 60 min*	96%	95%	86%	98%	97%	80%

4.0 INVESTIGATING THE EFFECTS OF NTBC ON THE TYROSINE DEGRADATION PATHWAY IN HUMANS

4.1 Introduction:

In October 2020, the European Medicine Agency (EMA) approved NTBC for the treatment of AKU adults following the positive clinical outcomes of SONIA 2 (EMA, 2020). As outline previously in this thesis, NTBC causes tyrosinaemia in AKU patients. This occasionally results in tyrosine keratopathy which spontaneously resolves upon discontinuation of NTBC. Other tyrosinaemia related adverse effects include dermal toxicity (Meissner et al., 2008, Stewart et al., 2014), vitiligo (Ranganath et al., 2021a), cataract (Ahmad et al., 2022), and impairment of neurocognitive function in HT-1 children (Macsai et al., 2001, Thimm et al., 2012, Bendadi et al., 2014, van Ginkel et al., 2016b, García et al., 2017, van Vliet et al., 2019). Clarifying the extent of post NTBC-hypertyrosinaemia is needed to allow better understanding and management of the iatrogenic tyrosinaemia.

The previous chapter has outlined the case for assessing the magnitude of NTBC-induced tyrosinosis in AKU mice and has demonstrated the significant rise in tyrosine concentrations in murine tissues, namely femur bone, brain, heart, kidney, liver, and quadriceps muscle. Quantification of the flux of tyrosine metabolites has revealed that concentrations of serum tyrosine metabolites significantly increase, in a dose-dependent fashion, following NTBC therapy (Milan et al., 2019, Ranganath et al., 2022a). It is speculated that the tyrosine increase is due to the NTBC diverting the tyrosine away from the benzoquinone acetic acid (BQA) pathway (Figure 1). One way to confirm

this hypothesis is to measure the concentrations of BQA in AKU patients before and after NTBC. Unfortunately, there is no commercially available assay to measure BQA and it would be very challenging to establish one as it is a very unstable compound that gets oxidised quickly. An alternative way to accurately quantify the magnitude of tyrosine changes post-NTBC would be the direct measurement of tyrosine and related compounds. Stable isotopes would be very useful here. Using phenylalanine and tyrosine stable isotopes, this study investigated the tyrosine degradation pathway in AKU patients before and after treatment with NTBC. This should provide direct evidence for changes in tyrosine compounds with NTBC use. The inclusion of a healthy control group aided the comparison of tyrosine pools and degradation pathway in the state of disease in AKU against the state of health in control subjects. The study also looked at the flux of tyrosine metabolites across the tyrosine pathway and any changes in this pathway were be compared to the healthy control group. This is the first-time pathway analysis has been addressed in AKU patients both before and after NTBC.

The conversion of phenylalanine to tyrosine in healthy subjects is estimated to be ~16% (Clarke and Bier, 1982). However, this has not been studied before in AKU patients and it is not known how much of the tyrosine originates from phenylalanine in AKU patients. This would be very useful to know as the only source of phenylalanine is diet while tyrosine can be obtained through diet and from the conversion of phenylalanine to tyrosine. Further confirmation of this could potentially guide the dietary advice in AKU patient who are starting NTBC treatment.

Assessing tissue tyrosine concentrations in humans would be impractical. Therefore, compartmental modelling was deployed to estimate the intracellular and extracellular pool size of tyrosine in healthy volunteers and in AKU patients before and after NTBC. This study has also explored the wider impact of NTBC on the tyrosine pathway compounds in human serum and urine. Furthermore, it evaluated the decay of tyrosine and phenylalanine following pulse injection of L-[13C9]tyrosine and L-[d8]phenylalanine. Finally, it assessed the effects of NTBC on protein turnover and phenylalanine hydroxylation in AKU patients following NTBC.

4.2 Materials and methods:

4.2.1 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 5N 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, 1500xg, 4 ^{o}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, 1500xg, 4 ^{o}C) and supernatant was taken. All samples were frozen and stored at -80 ^{o}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., 2014, 2015, 2022).

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, the existing LC-MS/MS methods (Hughes et al., 2014, Hughes et al., 2015) were modified to enable measurements of serum and urinary concentrations of

L-[$^{13}C_9$]tyrosine, L-[d_8]phenylalanine and L-[d_7]tyrosine. The assay was validated in accordance with international guidelines as outlined in Chapter 2.

4.2.2 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 volunteers database. AKU patients were recruited from the National AKU centre. 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 analyser DC-430MA). All participants completed the study. The flow of participants in this study is shown in Figure 45. The characteristics of the study subjects are shown in Table 42.

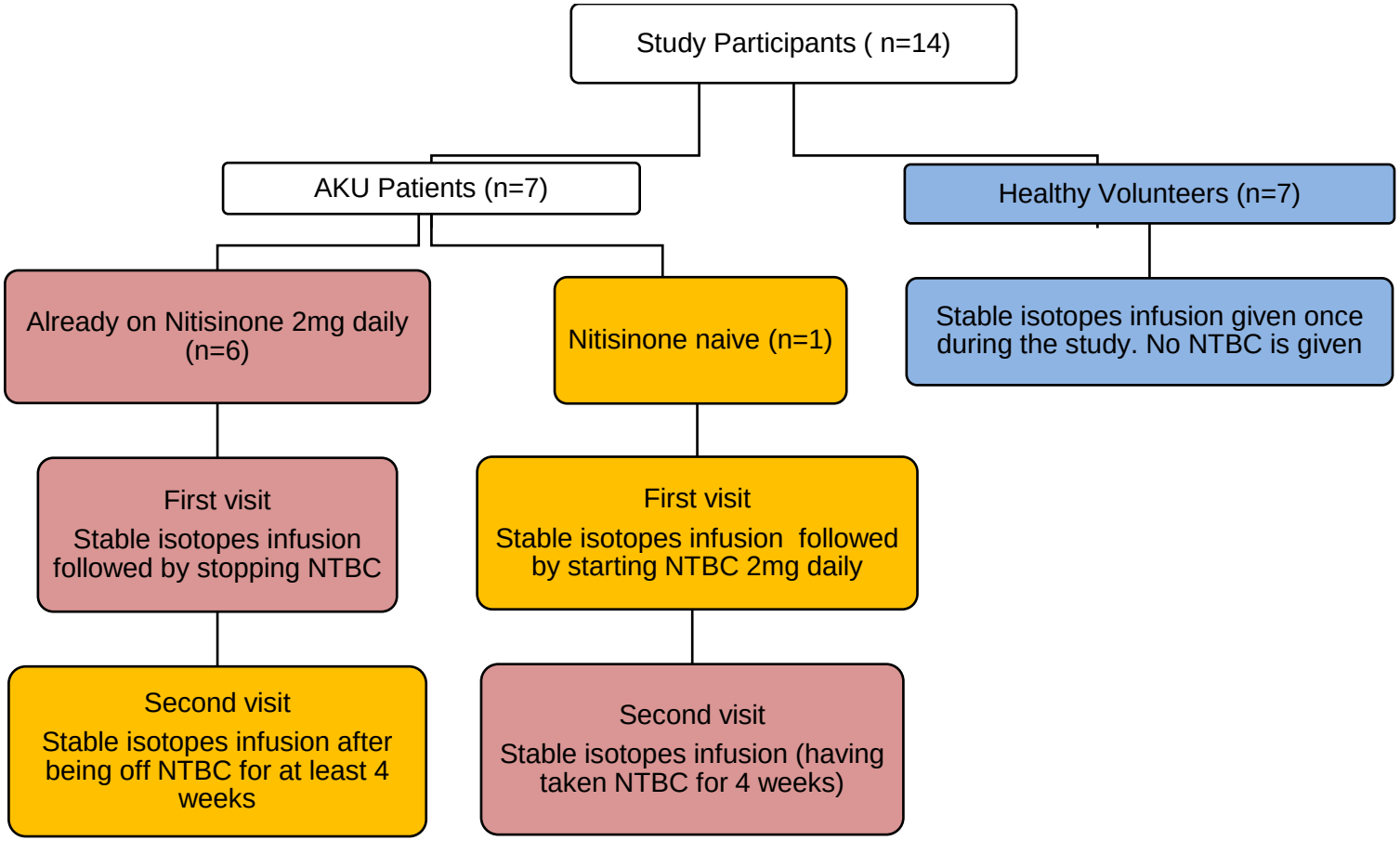

Figure 45: The flow of participants in the human study.

Table 42: The characteristics of the study subjects.
BW: body weight, TBW: total body water, FFM: fat free mass, FFM (%BW): FFM expressed as percentage of body weight, BMI: body mass index. Data represent mean± SD.†Paired t-test was used for comparison of data between AKU on and AKU off NTBC groups, ‡Unpaired t test was used for comparison of data between HV and AKU off NTBC groups, ††the three groups were compared using one way ANOVA (p= 0.742), ‡‡ the three groups were compared using Kruskal-Wallis test (p= 0.242), p<0.05 is considered statistically significant.

Clinical parameters	**AKU on NTBC (n=7)**	**AKU off NTBC (n=7)**	***p†***	**Healthy volunteers (n=7)**	***p‡***
Sex (male/ female)	3/4	3/4	NA	7/0	NA
Age (years)	47 ±14	47 ±14	NA	32 ± 6	0.027
Race/ethnicity	6 Caucasians 1 Asian	6 Caucasians 1 Asian	NA	7 Caucasians	NA
Body composition					
BW (kg)	73.7 ± 19.1	72.6± 19.0	0.013	77.4 ± 10.5	0.569
TBW (kg)	37.1 ± 10.1	36.3 ± 9.4	0.099	43.5 ± 3.7	0.086
FFM (kg)	52.7 ± 12.6	52.4 ± 12.7	0.045	63.9 ± 5.2	0.045
FFM (%BW)	72 ± 4	72 ± 4	0.172	83 ± 5	0.001
BMI ($kg.m^{-2}$)	26.4 ± 5.7	26.0 ± 5.6	0.015	24.4 ± 3.3	0.513
L-[d_8]phenylalanine dose (µmol)	272.7± 22.0	280.7± 17.4	††	277.1 ± 18.1	††
L-[$^{13}C_9$]tyrosine dose (µmol)	100.4 ± 3.2	99.2 ± 5.3	‡‡	97.9 ± 3.6	‡‡

4.2.2.1 *Inclusion criteria*

4.2.2.1.1 Inclusion criteria for healthy volunteers

Subjects were required to satisfy all the following criteria at the screening visit, unless otherwise stated:

1. Subjects were:
    - healthy males
    - of any ethnic origin
    - between 18 and 60 years of age, inclusive
2. Subjects had a
    - body mass index (BMI) between 18.0 and 30.0 kg/m^2, inclusive
    - body weight between 50 and 100 kg, inclusive
3. Subjects were in good health, as determined by:
    - medical history
    - physical examination
    - vital signs assessment
    - 12-lead electrocardiogram (ECG)
    - clinical laboratory evaluations
4. Signed written informed consent given to participate in the study and to abide by the study restrictions.

4.2.2.1.2 Inclusion criteria for AKU patients

A patient must fulfil the following criteria to be included in the study. These were adapted from SONIA 2 study (Ranganath et al., 2020d):

1. Diagnosis of AKU.

2. Not currently on NTBC. If already taking NTBC, he/she should be willing to stop NTBC for 4 weeks before the first baseline tracers' infusion.

3. Age ≥18years.

4. Stable weight for at least a month prior to the study first visit.

5. Willing and able to visit the investigational site for study visits.

6. Signed written informed consent given.

4.2.2.2 *Exclusion criteria*

4.2.2.2.1 Exclusion criteria for healthy volunteers

Subjects were excluded from the study if they satisfy any of the following criteria at the screening visit, unless otherwise stated:

1. Subjects who have donated:

1.1. blood in the 3 months prior to screening.

1.2. plasma in the 7 days prior to screening.

1.3. platelets in the 6 weeks prior to screening.

2. Subjects who:

2.1. consume more than 28 units of alcohol per week if male.

2.2. have a significant history of alcoholism or drug/chemical abuse, as determined by the Investigator. Note: 1 unit of alcohol equals ½ pint (285 mL) of beer or lager, 1 glass (125 mL) of wine or 1⁄6 gill (25 mL) of spirits

3. Subjects who were unwilling to abstain from alcohol for 48 hours prior to study visits.

4. Subjects who were unwilling to abstain from exercise more strenuous than walking prior to study visits.

5. Subjects who were current smokers.

6.Subjects who have an average consumption of more than 5 cups of caffeinated beverages (tea/coffee/cocoa/cola) per day.

7. Subjects who have received:

7.1. any drug, herbal remedy, or vitamin/mineral supplement which, in the opinion of the principal investigator, precludes their participation in the study.

8. Subjects who have an abnormality in heart rate, blood pressure or temperature prior to first tracers' infusion that in the opinion of the Investigator increases the risk of participating in the study.

9. Subjects who have an abnormality in the 12-lead ECG that in the opinion of the Investigator increases the risk of participating in the study

10. Subjects who are still participating in another clinical study (e.g., attending follow-up visits) or who have participated in a clinical study involving administration of an investigational drug (new chemical entity) in the 3 months (or 5 half-lives, whichever is longer) prior to first dose administration.

11. Subjects who have a significant history of drug allergy, as determined by the Investigator.

12. Subjects who have any clinically significant abnormal physical examination finding.

13. Subjects who have any clinically significant allergic condition (excluding non-active hay fever), as determined by the Investigator.

14. Subjects who have, or who have a history of, any clinically significant neurological, gastrointestinal, renal, hepatic, cardiovascular, psychiatric, respiratory, metabolic, endocrine, haematological, or other major disorder, as determined by the Investigator.

15. Subjects who have had a clinically significant illness within 4 weeks prior to first dose administration, as determined by the Investigator.

16.Subjects who have any clinically significant abnormal laboratory safety findings at screening and prior to first dose administration, as determined by the Investigator (one repeat assessment is acceptable).

17. Subjects who have previously taken part in or withdrawn from this study.

18. Subjects who, in the opinion of the Investigator, should not participate in this study.

4.2.2.2.2 Exclusion criteria for AKU patients:

At the time of planning this study, 58 AKU patients were under the care of the NAC with information available on all the AKU patients receiving NTBC and those who are not on NTBC. AKU patients were recruited for this study from these two groups. Patients were approached by the AKU society. Once they agreed, eligibility was checked using the NAC medical records and if required the patients' own GP, metabolic physician or treating hospital doctor.

The presence of any of the following excluded a patient from inclusion in the study. These were adapted from SONIA 2 study (Ranganath et al., 2020d):

1. Treatment with NTBC within 4 weeks of first tracer injection.

2. Participation in another clinical study within 3 months of study first visit.

3. Known allergy to NTBC or any of the constituents of the investigational product.

4. Female patient of child-bearing potential not using a reliable method of contraception.

5. Currently pregnant or lactating.

6. Current malignancy.

7. Uncontrolled hypertension (blood pressure greater than 180 mmHg systolic or greater than 95 mmHg diastolic).

8. Unstable cardiovascular disease.

9. Serum potassium < 3.0 mmol/L.

10. eGFR < 60 mL/min

11. ALT > 3 x upper limit of normal.

12. Haemoglobin < 100 g/L.

13. Platelets < 100 x 109/L.

14. Total white blood count < 3.0 x 109/L or neutrophil count < 1.5 x 109/L.

15. History of alcohol or drug abuse.

16. Psychiatric or somatic illness that interferes with compliance or communication with health care personnel.

17. Foreseeable inability to cooperate with given instructions or study procedures.

18. Any significant weight loss as judged by the investigator

19. History of heart failure or symptomatic aortic stenosis

20. Any other medical condition which in the opinion of the investigator makes the patient unsuitable for inclusion.

4.2.3 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.

4.2.4 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, and 120 min.

4.2.4.1 *Stable isotopes dose justification*

This study used 50mL injection of normal saline containing 20 mg of L-[$^{13}C_9$]tyrosine and 50 mg of L-[d_8]phenylalanine. Each was given over 5 minutes. This fixed dose approach (as opposed to a patient weight-based

approach for both phenylalanine and tyrosine tracers) has been used previously (van Eijk et al., 2007, Mason et al., 2017).

In previous studies, summarised in Table 43, Table 44 and, Table 45, a primed continuous infusion approach was used. The priming doses in these studies represent a small fraction of the total given to study participants who received further continuous infusion for a duration ranging from two hours (van Eijk et al., 2007) up to 24 hr in the case of Neonatal Intensive care unit (NICU) babies in Roberts et al. (1998) study. This study used what can be considered a priming dose.

The doses used in this study are comparable or lower than what have been given in previous studies. The doses of tracers used in this study is also much lower than the 0.05 gram/kg used in the case of labelled leucine given to healthy volunteers using the “flooding dose” method to study the protein synthesis in muscles (Garlick et al., 1997). This is detailed further below:

4.2.4.1.1 Tyrosine tracer dose justification

The choice of the tyrosine tracer dose was based on previous published studies (Table 44 and Table 45) , particularly Mason et al. (2017). The proposed dose is lower than the tyrosine priming dose given in most of the previous studies (Table 43).

Tyrosine solubility in water was also considered. Water solubility of L-[$^{13}C_9$]tyrosine is 0.479 g/L at 25 °C, so 100 mg tyrosine need to be dissolved in a minimum of 210 mL. Practically, using a maximum injection volume of 50mls of 0.9 % saline, this means that only 20mg (105 µmol) of tyrosine can be given.

Table 43: Summary of tyrosine and phenylalanine tracers weight-based doses used in previous studies.

PICU: Paediatric Intensive Care Unit, CF: Cystic Fibrosis, NICU: Neonatal Intensive Care Unit. *Represents the combined dose of both tracers given. Tyr: tyrosine, Phe: phenylalanine.

Study	Tyrosine tracer	Tyr-Bolus Dose	Phenylalanine tracer	Phe-Bolus Dose	Human participant group	Duration of priming
Clarke and Bier 1982	L-[1-^{13}C] Tyr	2.7 µmol/kg	L-[ring-$^{2}H_5$]Phe	No bolus dose given	6 healthy adult males	Not stated
Thompson et al 1989	L-[$^{2}H_2$] Tyr L-[-$^{2}H_4$] Tyr	1.8 µmol/kg	L-[ring-$^{2}H_5$]Phe	2.9 µmol/kg	6 healthy adults	Not stated
Cortiella et al 1992	L-[1-^{13}C] Tyr L-[$^{2}H_2$] Tyr	5.4 µmol/kg*	L-[1-^{13}C]Phe	3.1 µmol/kg	5 healthy adult males	2 minutes
Marchini et al 1993	^{13}C-Tyr $^{2}H_2$-Tyr $^{2}H_4$-Tyr	4.3 µmol/kg*	^{13}C-Phe $^{2}H_5$-Phe	5.8 µmol/kg*	5 healthy adult males	2 minutes
Castillo et al 1994	[3,3,-$^{2}H_2$] Tyr L-[1-^{13}C] Tyr	6.1 µmol/kg*	L-[1-^{13}C]Phe	5.4 µmol/kg	6 new-borns and five infants (all admitted to NICU and had sepsis)	Not stated
Sanchez et al 1995	^{13}C-Tyr [3,3,$^{2}H_2$] Tyr	5.5 µmol/kg*	L-[1-^{13}C]Phe	4.5 µmol/kg	12 healthy adult males	
Clark et al 1996	[d_2] Tyr [d_4] Tyr	2.5 µmol/kg	[d_5]Phe	4 µmol/kg	9 premature infants	5 minutes
Roberts et al 1998	[3,3,-$^{2}H_2$] Tyre	3.6 µmol/kg	L-[1-^{13}C]Phe	15.6 µmol/kg	Sixteen parenterally fed infants in NICU	15 minutes
Roberts et al 2001	[3,3,-$^{2}H_2$] Tyr	3.6 µmol/kg	L-[1-^{13}C]Phe	15.6 µmol/kg	13 parenterally fed infants in NICU	15 minutes
De-Betue et al 2011	L-[ring-$^{2}H_2$] Tyr L-[ring-$^{2}H_4$] Tyr	2.5 µmol/kg*	L-[ring-$^{2}H_5$]Phe	4.4 µmol/kg	18 critically ill infants admitted to PICU	Not stated
Meesters et al 2009	L-[ring-$^{2}H_2$] Tyr L-[ring-$^{2}H_4$] Tyr	1.47 µmol/kg*	L-[ring-$^{2}H_5$]Phe	3.65 µmol/kg	5 critically ill patients with septic shock	Not stated
Moran et al 2001	[^{15}N] Tyr, [$^{2}H_4$] Tyr	2.8* µmol/kg	L-[^{15}N]Phe	3.6 µmol/kg	12 clinically stable adult CF patients and 12 matched healthy volunteers	Not stated

Table 44: Summary of tyrosine and phenylalanine tracers fixed doses used in previous studies.
†A fixed dose was given to all participants. Tyr: tyrosine, Phe: phenylalanine

Study	**Tyrosine tracer**	**Tyr-Bolus Dose**	**Phenylalanine tracer**	**Phe-Bolus Dose**	**Human participant group**	**Duration of priming** (volume used if stated)
(van Eijk et al., 2007)	L-[ring-$^{2}H_{2}$] Tyr	71.2 µmol†	L-[ring-$^{2}H_{5}$]Phe	164.3 µmol†	8 healthy male volunteers	duration not stated (45 mL)
(Mason et al., 2017)	L-[ring-$^{2}H_{4}$] Tyr	20 µmol†	L-[ring-$^{13}C_{6}$]Phe	296 µmol†	11 healthy subjects	duration not stated (8 mL)
This study (Khedr et al., 2020)	L-[$^{13}C_{9}$] Tyr	105 µmol†	L-[d_{8}]Phe	289 µmol†	10 healthy subjects and 10 AKU patients	5 minutes (50mL)

Table 45: A comparison of the total bolus/ prime doses given in previous studies with the current study.

*A fixed dose was given. For illustration purpose only, the equivalent weight adjusted dose calculated here from the fixed dose for a presumed 50 kg subject.

Study	**Tyrosine tracer Bolus Dose** (µmol/kg)	**Total tyrosine bolus dose for a 50 Kg participant** (µmol)	**Phenylalanine tracer Bolus Dose** (µmol/kg)	**Total Phenylalanine bolus dose for a 50 Kg participant** (µmol)
Clarke and Bier 1982	2.7 µmol/kg	135	No bolus dose given	-
Thompson et al (1989)	1.8 µmol/kg	90	2.9 µmol/kg	145
Cortiella et al 1992	5.4 µmol/kg	270	3.1 µmol/kg	155
Marchini et al 1993	4.3 µmol/kg	215	5.8 µmol/kg	290
Castillo et al 1994	6.1 µmol/kg	305	5.4 µmol/kg	270
Sanchez et al 1995	5.5 µmol/kg	275	4.5 µmol/kg	225
Clark et al 1996	2.5 µmol/kg	125	4 µmol/kg	200
Roberts et al 1998	3.6 µmol/kg	180	15.6 µmol/kg	780
Roberts et al 2001	3.6 µmol/kg	180	15.6 µmol/kg	780
De-Betue et al 2011	2.5 µmol/kg	125	4.4 µmol/kg	220
Meesters et al 2009	1.47 µmol/kg	73.5	3.65 µmol/kg	182.5
Moran et al 2001	2.8* µmol/kg	140	3.6 µmol/kg	180
This study	2.1* µmol/kg	105	3.8* µmol/kg	289 µmol

4.2.4.1.2 Phenylalanine tracer dose justification

Mason et al. (2017) have used 296 µmol of the phenylalanine tracer in their bolus injection study. This study is using a comparable 289 µmol (or 50mg) of the phenylalanine tracer. To illustrate how small the doses of the tracers used here are, the paragraphs below show examples of the phenylalanine tracer doses:

Wolfe and Chinkes (2004) suggested a phenylalanine tracer infusion of 0.05 µmol/kg/min (3 µmol/kg/hr) with a prime dose of 3 µmol/kg. The molecular weight for L-[d_8]phenylalanine is 173 kg/mol so dosage was 0.5 mg/kg for the prime dose and 0.5 mg/kg/hr for the infusion. Total required dose for a 50 Kg patient would be:

$$(50*0.50)+(50*4*0.50)=25+100=125 \text{ mg}$$

Tuvdendorj et al. (2014) compared the primed constant infusion with the bolus injection. The total dose used for the former was 14 µmol/kg (2.5mg/kg). For the bolus injection 15 µmol/kg (2.6mg/kg) was required. For a 50 kg patient, the total dose required would be 125 mg using the constant infusion which comparable to the 130 mg used in the bolus injection approach for the phenylalanine. In both scenarios, the dose given is roughly three times the dose proposed here.

4.2.4.2 *Justification for the bolus injection approach*

This study has adopted the pulse approach. The use of stable isotopes in metabolic research has predominantly adopted a primed constant infusion approach (Clarke and Bier, 1982, Wilkinson, 2018) which allows for reaching

a ‘steady state’. Recently, there has been a shift towards a bolus injection approach as it is more convenient for patients, less labour intensive and cheaper. Studies have looked at the bolus injection approach in animals (Zhang et al., 2002) and humans (Tang et al., 2007, Tuvdendorj et al., 2014, Mason et al., 2017). For the determination of the pool size, it is still possible to use a single bolus injection study design (Wolfe and Chinkes, 2004). In this study, the tracer infusion protocol is based on the work of Mason et al (2017).

4.2.4.3 *Justification for the choice of phenylalanine and tyrosine tracers*

Given that the in-house assay for tyrosine uses deuterated tyrosine and phenylalanine as internal standards, L-[$^{13}C_9$]tyrosine (Figure 46) was chosen as it would enable tracking of its metabolites.

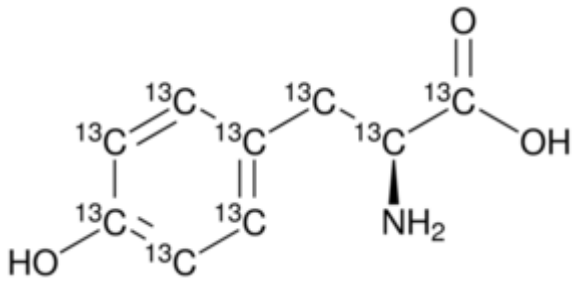

Figure 46: L-[$^{13}C_9$]tyrosine.

In addition, L-[d_8]phenylalanine (Figure 47) was chosen to assess the hydroxylation of phenylalanine to tyrosine. L-[d_8]phenylalanine is converted to L[d_7]tyrosine which is distinguishable from L-[$^{13}C_9$]tyrosine using mass spectrometric analysis.

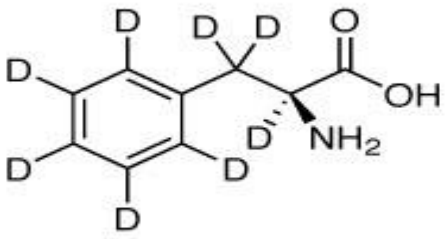

Figure 47: L-[d_8]phenylalanine

4.2.4.4 *Preparation of tyrosine and phenylalanine tracers prior to administration*

These were prepared according to good manufacturing practice (GMP) standards at the Royal Liverpool Hospital Radiopharmacy Department. Tracers were prepared fresh for each participant under sterile conditions in a 50 mL syringe which contained 20 mg of L-[$^{13}C_9$]tyrosine and 50 mg of L-[d_8]phenylalanine. Aliquots were tested to confirm the solution sterility as well as the concentration of both tracers (see chapter 2 for aqueous assay).

4.2.5 NTBC therapy

After completing the isotopes infusion in the first visit, AKU patients were given 2 mg NTBC to take daily for at least 4 weeks. At the time of carrying out the study, AKU patients attending the national AKU centre were treated with 2 mg of NTBC daily. This was an off license use that was allowed by NHS England Specialised Services and has been used since 2012. The daily dose of 2 mg is effective in reducing the production of HGA and is tolerated well by AKU patients (Introne et al., 2011). This is echoed in the NAC experience

(Ranganath et al., 2018). In the SONIA 1 trial 32 patients were treated with doses of NTBC ranging from 2 mg to 8 mg for a period of 4 weeks with no safety concerns emerging over this short period (Ranganath et al., 2016).

The half-life of NTBC is up to 86 hours (EMA 2015). This equals to 3.6 days; it is accepted that five half-lives are required to clear the drug after it is stopped (18 days in this case). Therefore, patients already on NTBC were asked to stop it temporarily for 4 weeks after their first tracers' injection.

Although HGA concentrations rose after stopping NTBC, it was very unlikely to have any noticeable effect over the one-month period. AKU patients have raised HGA since birth but they do not develop overt clinical features before the third decade of life (Ranganath and Cox, 2011). Damage from HGA happens because of the ochronosis which takes years and decades to accumulate.

4.2.6 Data analysis

4.2.6.1 *Data preparation*

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.

4.2.6.2 *Quantification of the flux of tyrosine pathway compounds*

This has been deployed before to further understand the ochronosis pathway and fate of HGA in AKU patients (Milan et al., 2019, Ranganath et al., 2022a). In this study this analysis was used to underscore the changes in the tyrosine pathway following NTBC therapy.

4.2.6.2.1 Sum TBW compounds

This represents the sum of the amounts of metabolites in total body water. Sum TBW compounds include s-phenylalanine, s-tyrosine, s-HGA, s-HPPA and s-HPLA.

4.2.6.2.2 Sum urine compounds

This represents the amounts of metabolites excreted in the 2-hour urine. Sum urine compounds include u-phenylalanine, u-tyrosine, u-HGA, u-HPPA and u-HPLA.

4.2.6.2.3 Sum all

This represents the sum of the amounts of metabolites in total body water (Sum TBW compounds) and the amounts of metabolites excreted in the 2-hour urine (Sum urine compounds). Since FFM was measured for each participant using bioelectric impedance analysis, it was possible to adjust Sum all for FFM.

4.2.6.3 *Non-compartmental Analysis*

This study adopted sum of two exponentials to describe the kinetics of the tracers in the two phases of distribution and utilization. The area under the curve (AUC) was calculated by integrating the TTR-time curve from time 0 to infinity. Since the TTR data was already normalized by the amount of pulsed tracer and the body weight, the population mean of the whole-body production (WBP) is equal to the inverse of the AUC and can be calculated as 1/AUC, whereas the population standard deviation of the WBP was calculated by equation (1) as suggested by statistical principle formulas.

Equation 1: $(1/ (\text{AUC mean})) * \text{SQRT} ((\text{AUC Std})^2+(\text{AUC mean})^2)$

4.2.6.4 *Compartmental modelling*

A two compartmental model consisting of an extracellular pool and an intracellular pool, as sketched in Figure 48, was used. The extracellular pool (EC) represents plasma and fluid around or between cells, whereas the intracellular pool (IC) represents the fluids inside the organs' cells. In this model, the production and disposal of the substrates only occur in the intracellular pool.

The system in Figure 48 can be mathematically described with a set of two first-order differential equations (Cobelli et al., 2007, Mason et al., 2017, Cobelli and Carson, 2019). Since the IC pool was not accessible and the measurements were taken from only EC pool, we summarized the equations of the compartmental model into a single second-order differential equation. The solution of this equation in time domain was a sum of two-exponential

functions, which resulted in the similar curve as the non-compartmental analysis.

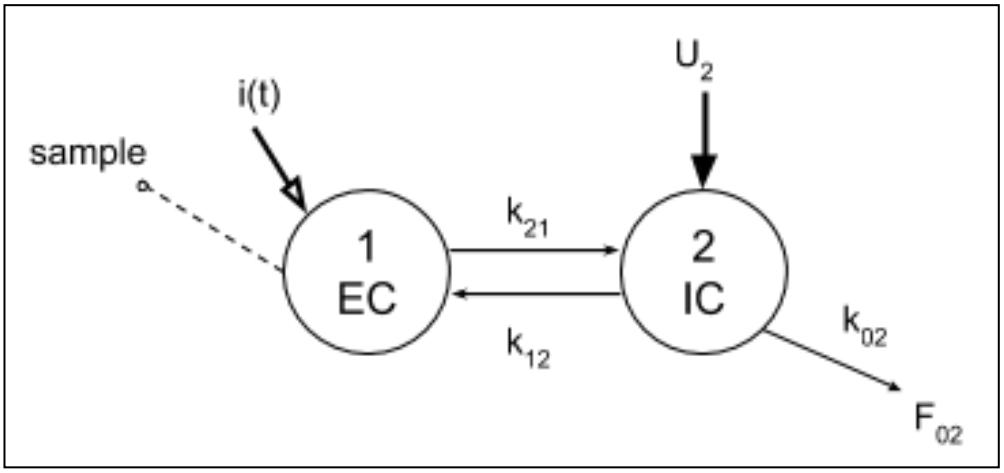

Figure 48: Schematics of the compartmental model.
EC is the extracellular pool, IC is the intracellular pool i(t) represents the input, which was a pulse infusion in the present study. K_{12} and K_{21} are the tracer transport rate between compartments 1 (EC) and 2 (IC). U_2 and F_{02} are the rate of production and disposal, which occurs in intracellular pool.

The compartmental parameters of the tracer were computed by equations 2, 3, 4 and were later used in equations 5, 6, 7 and 8 to estimate the trace parameters (Cobelli et al., 2007, Mason et al., 2017). Tracee parameters include the size of the extracellular and intracellular pools in addition to the bidirectional fluxes between the two pools as well as the production rate into the intracellular pool.

Equation 2: $$k_{21} = \frac{a \times k_1 + b \times k_2}{a + b}$$

Equation 3: $$k_{12} = \frac{a \times b \times (k_1 - k_2)^2}{(a + b)(a \times k_1 + b \times k_2)}$$

Equation 4: $$k_{02} = \frac{b \times k_1 + a \times k_2}{a + b} - k_{12}$$

Equation 5: $$F_{21} = F_{12} \Rightarrow k_{21} \times Q_1 = k_{12} \times Q_2$$

Equation 6: $$Q_1 = Q_{EC} = \frac{1}{a + b} \; and \; Q_2 = Q_{IC} = \frac{k_{21}}{k_{12}} \times Q_1$$

Equation 7: $$F_{12} = k_{12} \times Q_2$$

Equation 8: $$F_{02} = U_2 = k_{02} \times Q_2$$

4.2.6.5 *Protein turnover calculations*

AUCs were calculated from Prism.

- Whole body production of phenylalanine (WBP PHE)= 1/ Total Peak Area of L-[d_8]phenylalanine (PHE_8)
- SE- WBP PHE= (1/ total Peak Area of PHE_8) * SQRT (Std Error)^2+(Total Peak Area of PHE_8)^2
- Whole body production of tyrosine (WBP TYR)= 1/ Total Peak Area of L-[$^{13}C_9$]tyrosine (TYR_9)
- SE- WBP TYR= (1/ Total Peak Area of TYR_9) * SQRT (Std Error)^2+(Total Peak Area of TYR_9)^2
- Phenylalanine hydroxylation to tyrosine (PHE>TYR) (µmol/kg ffm/min) = WBP TYR*[(AUC-TYR7)/(AUC-PHE8)]
- PHE>TYR (µmol/kg ffm/hr) = PHE>TYR (µmol/kg ffm/min) *60
- We have assumed that, in the post-absorptive state:
    - Protein breakdown (PB) = Protein synthesis (PS)+ (PHE>TYR)
    - Whole-body Protein Breakdown (µmol/kg ffm/min) = WBP PHE
    - Whole-body Protein Synthesis (µmol/kg ffm/min) = WBP PHE-[PHE>TYR (µmol/kg ffm/min)]

4.2.7 Power and sample size calculation

4.2.7.1 *Healthy control sample size*

We aimed to recruit 10 male healthy volunteers. While no formal statistical assessment has been carried out, this choice of gender and the low number of participating healthy control subjects is common in studies of amino acids metabolism. This sample size is relatively comparable to previous studies who

investigated amino acids metabolism. The sample size used in some of these studies is presented below:

- Roberts et al. (1998): 6 male adults aged 20-43
- James et al. (1976): 6 males aged 31-64
- Jones et al. (1978): 11 males aged 43± 10
- Clarke and Bier (1982): 6 male aged 21-23
- Thompson et al. (1989): 6 adults (1 female and 5 males), aged 25 – 49
- Cortiella et al. (1992): 6 male adults aged 20-22
- Tessari et al. (1999): 6 adults (4 males and 2 females): aged 39±5years

4.2.7.2 *AKU patients' samples size*

The aim has been to recruit 10 AKU patients from the NAC. AKU is an ultra-rare metabolic disease with an estimated prevalence of 1 in 250,000 to 1,000,000 people (Phornphutkul et al., 2002). Therefore, classic sample size calculations would be inappropriate in the context of this rare disease.

In the UK, at the time of conducting the study, there were 58 AKU patients who attended the NAC. All but eight received NTBC. The sample size was chosen on what can be realistically achieved in term of subject recruitment and was comparable to other studies investigating amino acid metabolism. The characteristics of the study subjects are shown in Table 4.1.

4.2.8 Study approval

Human research complied with the Declaration of Helsinki. The human protocol was approved by the IRB of the Royal Liverpool University hospital (RD&I reference: 5327), Northwest - Liverpool Central Research Ethics

Committee and Health Research Authority (REC reference: 17/NW/0032). All patients and healthy volunteers provided written informed consent before participation in the study. The Medicines and Healthcare products Regulatory Agency in the United Kingdom (MHRA) has confirmed that the study is not a clinical trial of an investigational Medicinal Product as defined by the EU directive 2001/20/EC.

4.2.9 Statistics

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.

4.3 Results

4.3.1 Effects of NTBC on the urinary concentrations of native compounds in the tyrosine pathway in humans

We measured the 2-hour urinary excretion of phenylalanine (u-Phe), urinary tyrosine (u-Tyr), urinary 4-hydroxyphenyllactic acid (u-HPLA), urinary 4-hydroxyphenylpyruvic acid (u-HPPA) and urinary homogentisic acid (u-HGA). The use of NTBC in AKU increased the concentration of u-Tyr 6 fold (P = 0.014), u-HPLA 31 fold (P = 0.004) and u-HPPA 18 fold (P = 0.007). In contrast, it reduced the concentration of u-HGA 6 fold (P = 0.002). No effect was noted on u-Phe. This is summarised in Figure 49 and Table 46.

Table 46: Summary of the NTBC effects on the two-hour urinary excretion of different urinary compounds in the tyrosine pathway.

[u.Phe]: 2h-urinary excretion of phenylalanine, [u.TYR]: 2h-urinary excretion of urinary tyrosine, [u.HGA]: 2h-urinary excretion of urinary homogentisic acid, [u.HPPA]: 2h-urinary excretion of urinary hydroxyphenylpyruvic acid, [u.HPLA]: 2h-urinary excretion of urinary hydroxyphenyllactic acid. †Paired t-test comparison of data in the AKU on NTBC group against AKU off NTBC group, ‡ Unpaired t-test comparison of data in the HV group against AKU off NTBC group.

	AKU on NTBC			AKU off NTBC				HV			
Parameters (µmol/2h)	n	Mean	SD	n	Mean	SD	*p*†	n	Mean	SD	*p* ‡
[u-Phe]	7	6.1 ±	4.2	7	6.7 ±	4.6	0.587	7	4.1 ±	1.9	0.193
[u-Tyr]	7	165 ±	106	7	28 ±	17	0.014	7	7.4 ±	5.2	0.009
[u-HGA]	7	274.9 ±	246.6	7	1644.0 ±	764.3	0.002	7	0.0 ±	0.0	NA
[u-HPLA]	7	2010 ±	1098	7	64 ±	77	0.004	7	0.0 ±	0.0	NA
[u-HPPA]	7	2316.0 ±	1422.0	7	130.0 ±	106.8	0.007	7	0.0 ±	0.0	NA

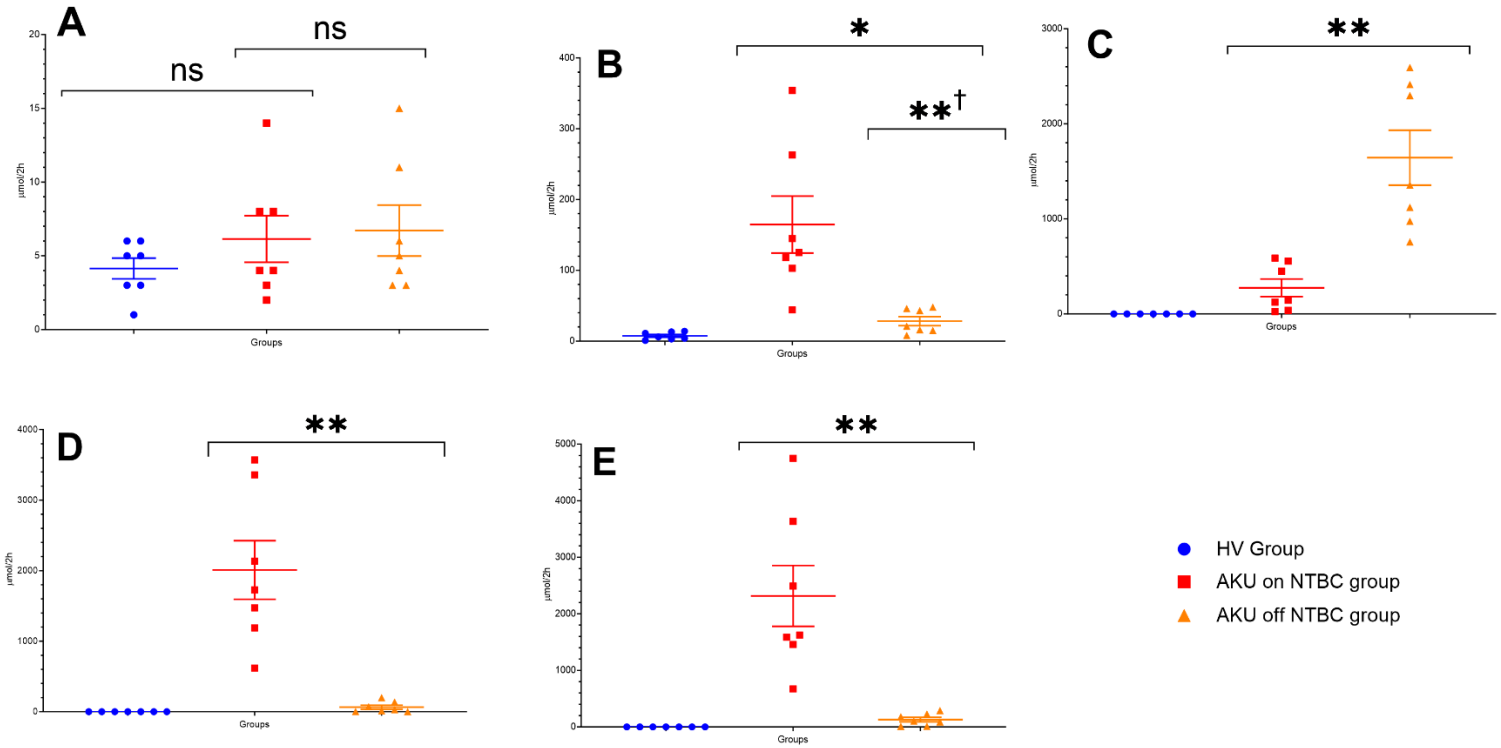

Figure 49: Effects of NTBC on the different urinary compounds in the tyrosine pathway in the study groups.
(A) phenylalanine, (B) tyrosine, (C): homogentisic acid, (D): hydroxyphenyllactic acid, (E): hydroxyphenylpyruvic acid, HV: healthy volunteers. AKU off NTBC group subjects were off NTBC for at least four weeks. 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. ns: $P \geq 0.05$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

4.3.2 Effects of NTBC on the serum concentrations of native 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 ($P < .001$). 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 7-folds($P < 0.001$). No effect was noted on s-Phe. This is summarised in Figure 50 and Table 47.

Table 47: Summary of the NTBC effects on the different serum compounds in the tyrosine pathway.
N: total number of measurements undertaken in all subjects per group, [s-Phe]: serum phenylalanine concentration, [s-Tyr]: serum tyrosine concentration, [s-HGA]: serum homogentisic acid concentration, [s-HPPA]: serum hydroxyphenylpyruvic acid concentration, [s-HPLA]: serum hydroxyphenyllactic acid concentration. †Paired t-test comparison of data in the AKU on NTBC group against AKU off NTBC group, ‡Unpaired t-test comparison of data in the HV group against AKU off NTBC group.

	AKU on NTBC				AKU off NTBC					HV				
Parameters (µmol/L)	**N**	**Mean**		**SD**	**N**	**Mean**		**SD**	***p*†**	**N**	**Mean**		**SD**	***p*‡**
[s-Phe]	83	50.1	±	2.4	81	50.1	±	3.3	0.991	84	60.7	±	2.5	<0.001
[s-Tyr]	83	712.7	±	25.2	80	117.8	±	8.1	<0.001	83	54.3	±	2.5	<0.001
[s-HGA]	84	3.5	±	0.2	84	26.6	±	2.3	<0.001	84	0.6	±	0.3	<0.001
[s-HPLA]	84	47.5	±	3.1	84	3.2	±	0.3	<0.001	84	0.9	±	0.2	<0.001
[s-HPPA]	84	33.4	±	1.4	84	0.0	±	0.0	NA	84	0.0	±	0.0	NA

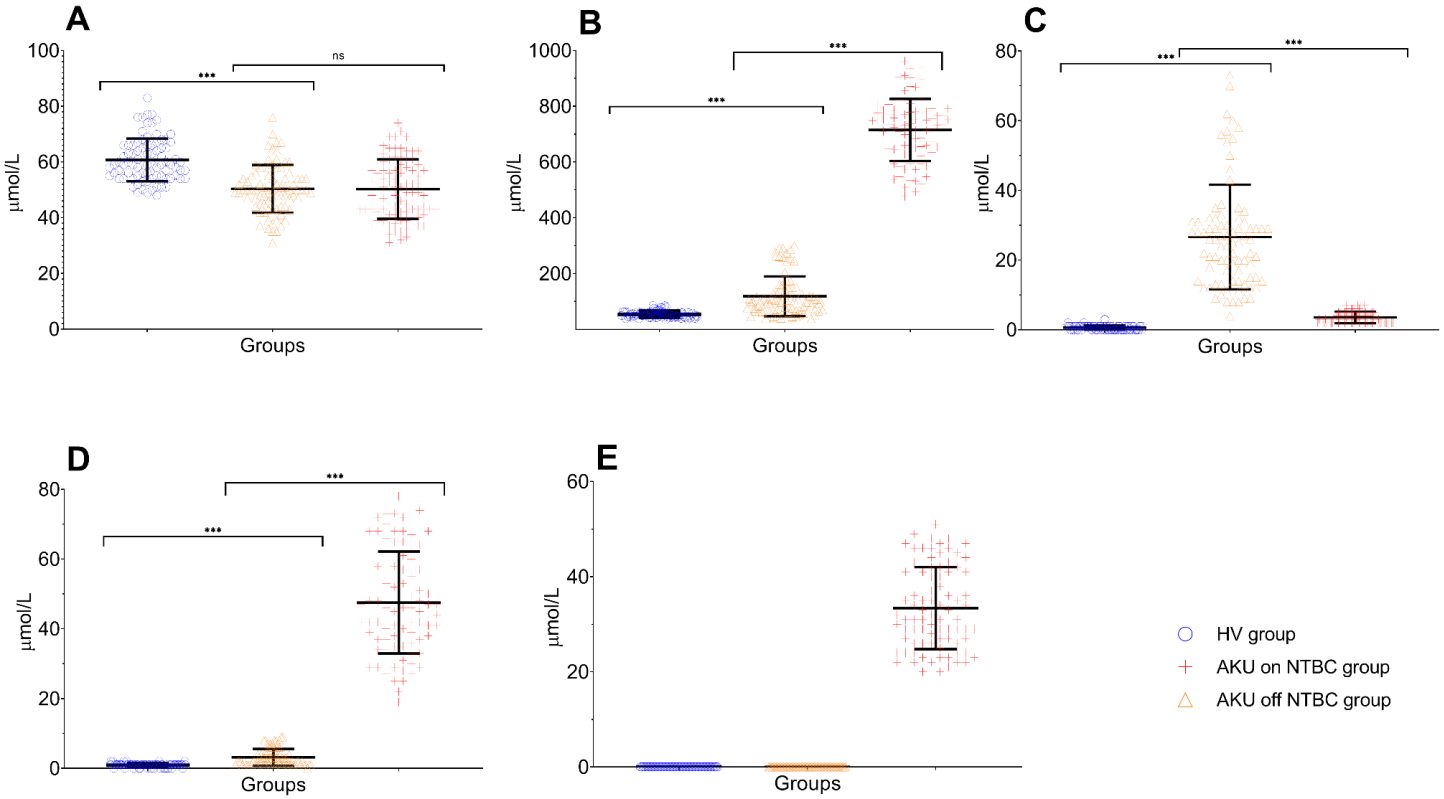

Figure 50: NTBC effects on the different serum compounds in the tyrosine pathway in the study groups.
(A) phenylalanine, (B) tyrosine, (C): homogentisic acid, (D): hydroxyphenyllactic acid, (E): hydroxyphenylpyruvic acid, HV: healthy volunteers. Line and error bars are mean ± SD. Comparison of data in 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. *** $P < 0.001$.

4.3.3 Quantification of flux of tyrosine pathway compounds:

The tyrosine pathway compounds are phenylalanine, tyrosine, HGA, HPPA, and HPLA. This study compared the amounts of these compounds pre and post NTBC.

4.3.3.1 *Comparison of sum total body water compounds (µmol)*

There is approximately a 6-fold difference in the Sum of total body water compounds (Sum TBW compounds), when comparing the AKU on NTBC group against HV and nearly 5-fold difference when compared to the AKU off NTBC groups. This is illustrated in Figure 51 and Table 48.

Table 48: Comparison of Sum total body water compounds.
HGA: homogentisic acid, HPPA: hydroxyphenylpyruvic acid, HPLA: hydroxyphenyllactic acid. TBW: Total Body Water. HV: Healthy volunteers, NA: not applicable, ND: not determined, †Difference is in comparison to HV, ††Difference is in comparison to AKU off NTBC group.

Estimated amounts of compounds in total body water (µmol)	HV	AKU 0ff NTBC	AKU on NTBC
Phenylalanine (µmol)	2639 ± 328	1862 ± 656	1913 ± 836
Tyrosine (µmol)	2381 ± 583	3775 ± 1687	26575 ± 8405
HGA (µmol)	27 ± 14	1056 ± 811	144 ± 98
HPLA (µmol)	38 ± 13	102 ± 63	1815 ± 842
HPPA (µmol)	ND	ND	1293 ± 613
Sum TBW Compounds (µmol)	5085 ± 854	6795 ± 1520	31739 ± 10527
Number of folds change compared to HV	NA	1.3	6.2
Number of folds change compared to AKU off NTBC	NA	NA	4.7
Difference in Sum total body water compounds (µmol) compared to HV	NA	1710 †	24945 ††

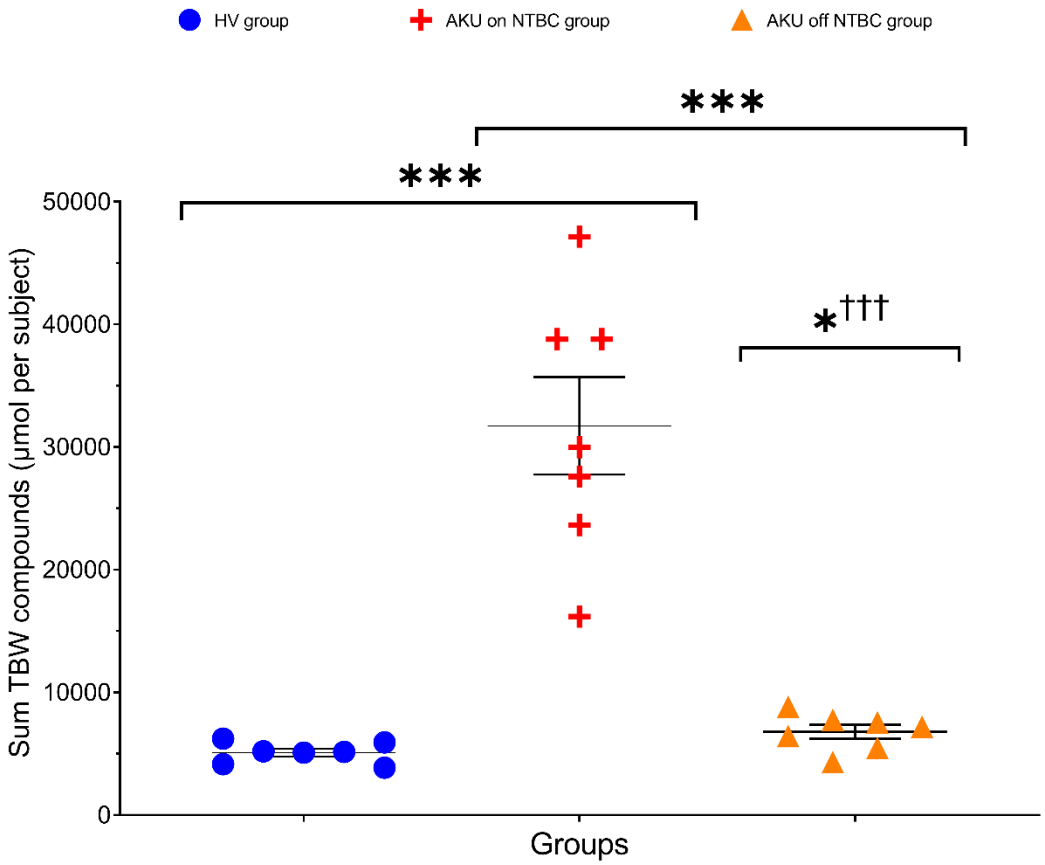

Figure 51: Comparison of Sum of Total Body Water (TBW) compounds across the study groups.

Paired t-test comparison of data in the AKU on NTBC group against AKU off NTBC group, unpaired t-test comparison of data in the HV group against AKU off NTBC group. *P <0.05, ***P< 0.001. †††Comparison of HV and AKU off NTBC groups.

4.3.3.2 *Comparison of sum 2-hour urine compounds (µmol)*

There is a 156-fold difference in the Sum of 2-hour urine compounds (Sum Urine compounds), when comparing the AKU off NTBC

group against HV, and nearly a 400-fold difference when comparing the AKU on NTBC group against the healthy volunteers group. This is illustrated in Table 49 and Figure 52.

Table 49: Comparison of Sum 2-hour urine compounds (µmol) across the study groups.
HGA: homogentisic acid, HPPA: hydroxyphenylpyruvic acid, HPLA: hydroxyphenyllactic acid. TBW: Total Body Water. HV: Healthy volunteers, NA: not applicable, ND: not determined.

Measured compounds in 2-hour urine collection			
Measured compounds in 2-hour urine collection	**HV**	**AKU 0ff NTBC**	**AKU on NTBC**
Age (years)	32 ± 6	47 ± 14	47 ± 14
Creatinine (mmol/2h)	1.3 ± 0.3	0.9 ± 0.3	1.3 ± 0.5
Phenylalanine (µmol/2h)	4 ± 2	7 ± 5	6 ± 4
Tyrosine (µmol/2h)	7 ± 5	28 ± 17	165 ± 106
HGA (µmol/2h)	ND	1644 ± 764	275 ± 247
HPLA (µmol/2h)	ND	64 ± 77	2010 ± 1098
HPPA (µmol/2h)	ND	130 ± 107	2317 ± 1422
Sum Urine Compounds (µmol/2h)	12 ± 7	1872 ± 746	4772 ± 2834
Number of folds change compared to HV	NA	156	398
Number of folds change compared to AKU off NTBC	NA	NA	2.5
Sum Urine Compounds /Creatinine ratio	9 ± 5	2158 ± 455	3458± 1026

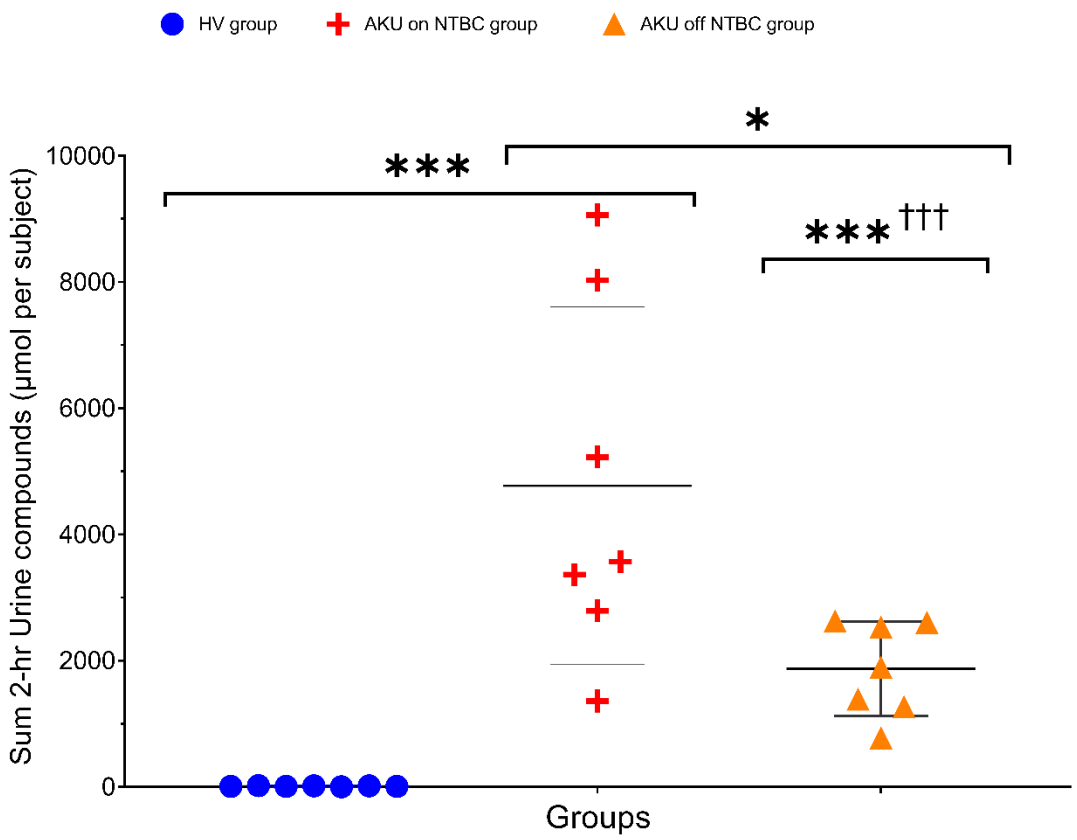

Figure 52: Comparison of Sum 2-hour Urine compounds (µmol) across the study groups.
Comparison of data in the AKU on NTBC group against AKU off NTBC group was by paired two-tailed t-test. Comparison of data in the HV group against AKU off NTBC group was by unpaired two-tailed t-test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

4.3.3.3 *Comparison of sum of all compounds (µmol) across the study groups*

As these compounds are small molecules that freely distribute in TBW, it is possible to estimate the amount (µmol) of TBW metabolites through multiplying TBW by the concentrations of these circulating metabolites (Madsen et al., 1977). There is a marked difference between sum of all compounds (TBW and urine), when comparing the AKU groups (on and off NTBC) against HV group. In the AKU off NTBC group, the sum of all compounds is 1.7-fold larger compared to the HV group (P<0.001). NTBC therapy in the AKU group increases the Sum of all compounds to 7-fold compared to the HV group (P<0.001) and 4-fold compared to the AKU off NTBC group (P<0.001). This is illustrated in Table 50,Table 51,Table 52, and Figure 53.

Table 50: Comparison of Sum of all compounds across the study groups.

HGA: homogentisic acid, HPPA: hydroxyphenylpyruvic acid, HPLA: hydroxyphenyllactic acid. TBW: Total Body Water. HV: Healthy volunteers, NA: not applicable, ND: not determined (below the lower limit of measurements of the assay).

	HV	**AKU 0ff NTBC**	**AKU on NTBC**
Age (years)	32 ± 6	47 ± 14	47 ± 14
Total Body Water (kg)	43.5 ± 3.7	36.3 ± 9.4	37.1 ± 10.1
Sum Urine Compounds (µmol/2h)	12 ± 7	1872 ± 746	4772 ± 2834
Sum TBW Compounds (µmol)	5085 ± 854	6795 ± 1520	31739 ± 10527
Sum of all (urine and TBW) compounds (µmol)			
Sum all compounds (µmol)	5097 ± 859	8667 ± 1737	36511 ± 13125

Table 51: Comparison of the sum of all compounds in AKU group on and off NTBC.

Summated Calculations	AKU on NTBC compared to AKU off NTBC
Difference in Sum all compounds (µmol) = (Sum all compounds on NTBC)- (Sum all compounds off NTBC)	27845 ± 12063
Ratio: Sum all compounds off NTBC / Sum all compounds on NTBC	0.3 ± 0.1
Ratio: (difference in Sum all compounds)/Sum all metabolite off NTBC	3.2 ± 1.1
Difference in Sum all metabolite as a percentage of Sum all compounds off NTBC (%)	317 ± 110

Table 52: Comparison of the sum of all compounds in AKU off NTBC and the HV group.
HV: healthy volunteers.

Summated Calculations	**HV compared to AKU off NTBC**
HV-AKU off NTBC Difference in Sum all compounds (µmol) = (Sum all compounds off NTBC)- (Sum all compounds HV)	3570 ± 1737
Ratio: Sum all compounds in HV / Sum all compounds in AKU off NTBC	0.61 ± 0.15
Ratio: (difference in Sum all compounds)/Sum all metabolite HV	0.70 ± 0.34
Difference in Sum all metabolite as a percentage of Sum all compounds HV (%)	70 ± 34

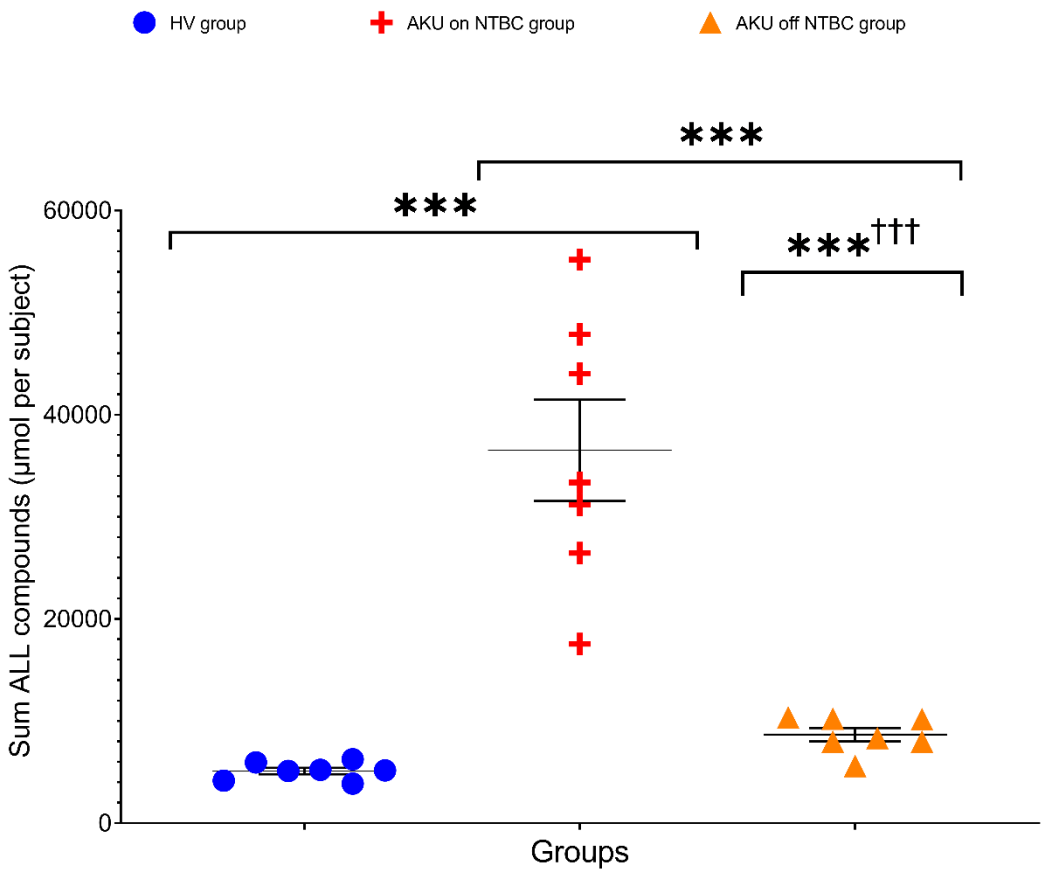

Figure 53: Comparison of Sum of all compounds across the study groups.
Comparison of data in the AKU on NTBC group against AKU off NTBC group was by paired two-tailed t-test. Comparison of data in the HV group against AKU off NTBC group was by unpaired two-tailed *t-test*. ***$P<$ 0.001. †††Comparison of HV and AKU off NTBC groups.

4.3.3.4 *Comparison of sum of all compounds adjusted for fat free mass (µmol/kg ffm)*

There is a stark difference between sum of all compounds (TBW and urine) adjusted for FFM, when comparing the AKU groups (on and off NTBC) against HV group. In untreated AKU patients, the sum of FFM adjusted- all compounds is twice the amount of that in HV group (115% increase, $P<0.001$). NTBC therapy in the AKU group increases the Sum of all compounds to 8.5 folds of that in the HV group (752% increase, $P<0.001$). This is illustrated in Figure 54 and Table 53.

Table 53: Comparison of Sum of all compounds adjusted to fat free mass (FFM) (µmol/kg ffm) across the study groups.
†Comparison of HV group against AKU on NTBC using paired *t*-test, ††Comparison of AKU group on NTBC against HV using unpaired *t*-test,††† Comparison of HV group against AKU off NTBC using unpaired *t*-test.

	HV group	AKU on NTBC group	AKU off NTBC group
n	7	7	7
Mean (µmol/kg ffm)	79.6	678	171
SD (µmol/kg ffm)	9.45	129	43.9
SEM	3.57	48.7	16.6
P	<0.001†	<0.001††	<0.001†††
Ratio (in comparison to HV group)	NA	8.5	2.1
Absolute difference (in comparison to HV group)	NA	598	91
% Difference compared to HV group	NA	752	115

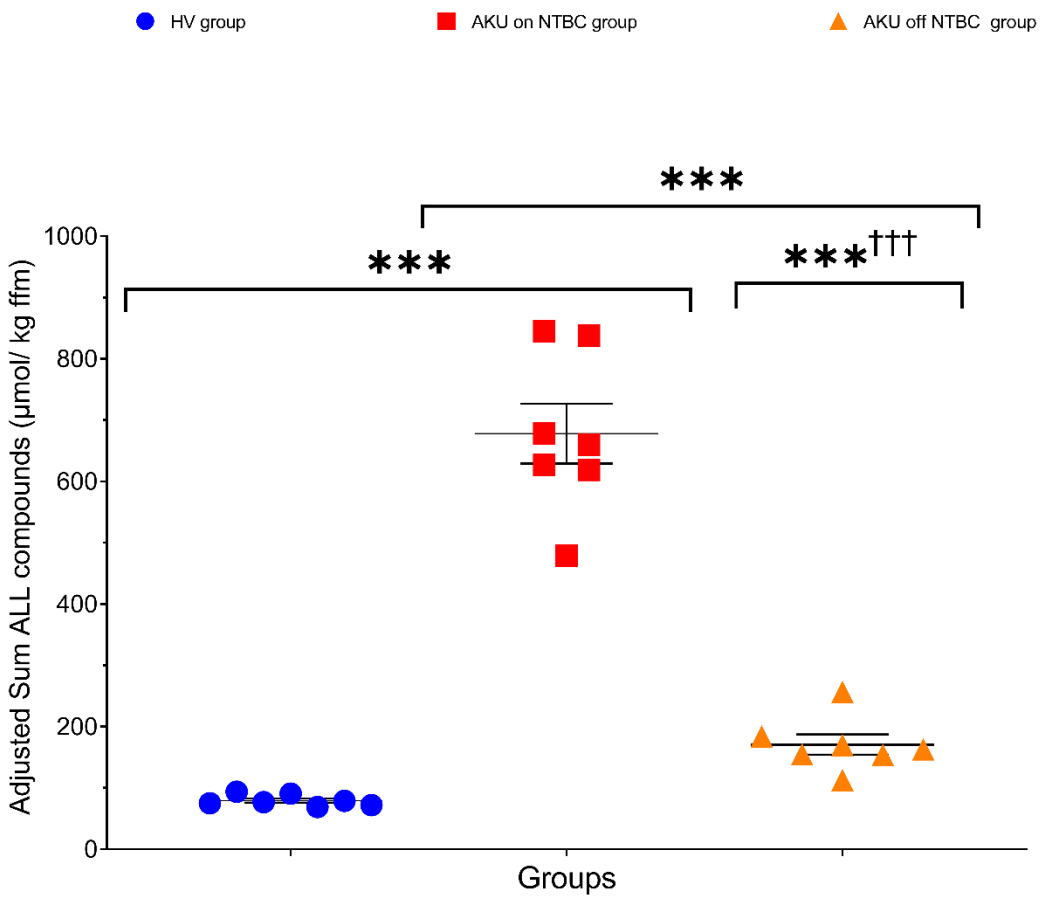

Figure 54: Comparison of Sum of all compounds adjusted to FFM (µmol/kg ffm) across the study groups.

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*< 0.001. †††Comparison of HV and AKU off NTBC groups.

4.3.4 Effects of NTBC on tyrosine and phenylalanine decay curves in humans

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 54. Kruskal-Wallis test was used to compare AUC for L-[$^{13}C_9$]tyrosine in the three groups.

In the AKU on NTBC group, AUC for L-[$^{13}C_9$]tyrosine was significantly lower compared to the AKU off NTBC group (0.19 + 0.01 vs 0.70 + 0.05 minute; *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 + 0.05 vs 0.88 + 0.02 minute; *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 (Figure 55, panels A and B). In contrast, phenylalanine degradation is not affected (Figure 55, panel C).

Table 54: A summary of average parameters in the two-exponential model.
Tyr: tyrosine, Phe: phenylalanine.

Variable (unit of measurement)	Values (per group)											
	L-[d_8]-Phe: TTR1= A1*exp(-a1*x)+ A2*exp(-a2*x)											
	HV				AKU on NTBC				AKU off NTBC			
	Mean		SE	(%CV)	Mean		SE	(%CV)	Mean		SE	(%CV)
A1 (adimensional)	0.023	±	0.002	10	0.032	±	0.001	4	0.024	±	0.001	4
a1 (min^{-1})	0.183	±	0.027	15	0.159	±	0.010	6	0.151	±	0.012	8
A2 (adimensional)	0.010	±	0.001	11	0.008	±	0.001	9	0.008	±	0.001	9
a2 (min^{-1})	0.029	±	0.003	10	0.025	±	0.002	8	0.026	±	0.002	7
	L-[$^{13}C_9$]-Tyr: TTR2= B1*exp(-b1*x)+ B2*exp(-b2*x)											
	HV				AKU on NTBC				AKU off NTBC			
	Mean		SE	(%CV)	Mean		SE	(%CV)	Mean		SE	(%CV)
B1 (adimensional)	0.029	±	0.001	5	0.001	±	0.000	14	0.018	±	0.004	21
b1 (min^{-1})	0.105	±	0.009	8	0.077	±	0.025	33	0.104	±	0.027	26
B2 (adimensional)	0.014	±	0.001	7	0.002	±	0.000	10	0.011	±	0.001	13
b2 (min^{-1})	0.017	±	0.001	5	0.004	±	0.001	31	0.014	±	0.002	11
	L-[d_7]-Tyr: TTR3= C1*exp(-c1*x)+ C2*exp(-c2*x)											
	HV				AKU on NTBC				AKU off NTBC			
	Mean		SE	(%CV)	Mean		SE	(%CV)	Mean		SE	(%CV)
C1 (adimensional)	0.348	±	0.049	14	0.007	±	0.016	236	0.160	±	0.226	141
c1 (min^{-1})	0.107	±	0.035	33	0.030	±	0.074	242	0.046	±	0.076	163
C2 (adimensional)	0.304	±	0.058	19	0.007	±	0.018	257	0.106	±	0.253	239
c2 (min^{-1})	0.016	±	0.003	20	0.003	±	0.019	583	0.009	±	0.022	260

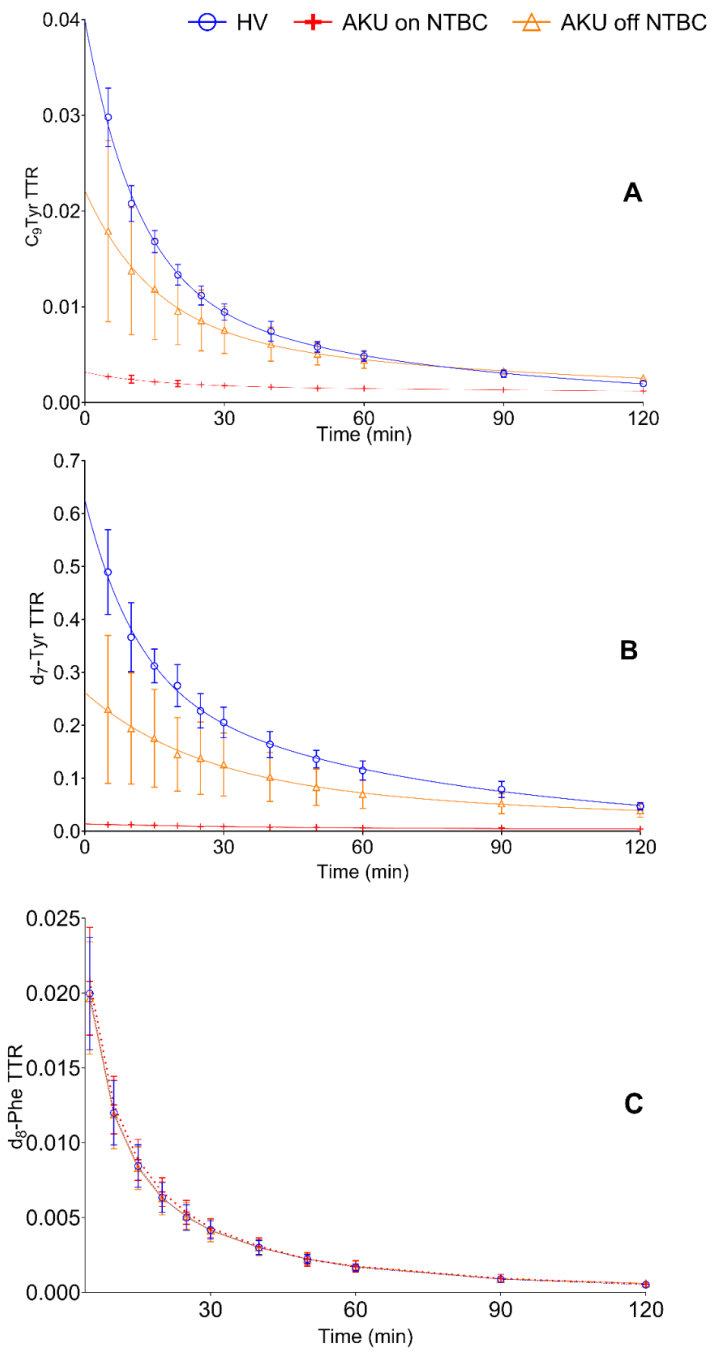

Figure 55: Comparison of tyrosine and phenylalanine enrichment versus time following a bolus injection of 105 µmol of L-[$^{13}C_9$]tyrosine and 270 µmol of L-[d_8]phenylalanine in each of the three groups. A: comparison of L-[$^{13}C_9$]tyrosine enrichment versus time. C_9TyrTTR is [L-[$^{13}C_9$]tyrosine]/[median 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 [d8]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.

4.3.5 Effects of NTBC on tyrosine pool size in NTBC treated AKU patients

Comparison of the tyrosine-estimated pool size across the three groups has demonstrated that the tyrosine extracellular (EC) pool size in NTBC-treated AKU patients is nearly 5-fold larger than that of AKU patients who did not receive NTBC ($P < 0.001$) and 13-fold larger compared to healthy volunteers ($P < 0.001$). Furthermore, the tyrosine intracellular (IC) pool size in NTBC-treated AKU patients is almost 3-fold larger than that of AKU patients who did not receive NTBC ($P< 0.001$) and 4-fold larger compared to healthy volunteers ($P < 0.001$).

Compared to healthy volunteers, the tyrosine EC pool size in AKU patients off NTBC is 2.7-fold larger ($P = 0.044$). Furthermore, the tyrosine IC pool size in AKU patients off NTBC is 1.4-fold larger when compared to healthy volunteers ($P = 0.027$). This is summarised in Figure 56 and Table 55.

Tyrosine is a small molecule that distributes freely in the intra- and extra- fluid compartment. In an adult male, 2/3 of total body water is found intracellularly while the remaining 1/3 is found in the extra-cellular space (i.e., intracellular water in double the size of the extracellular water). Therefore, it can be postulated that IC pool size is twice that of EC pool size in NTBC treated patients. Surprisingly, tyrosine IC and EC pools size are comparable in the NTBC treated AKU group. This is likely because intracellular compartment cannot be sampled, and assumptions have to be made when carrying out compartmental modelling. Some of the assumptions may not hold true in practical sense.

Table 55: Estimated tyrosine pools in the study groups.
EC (Q1): tyrosine Extracellular Pool. IC (Q2): tyrosine Intracellular Pool. †Unpaired *t*-test comparison of data in AKU on NTBC group against healthy volunteers (HV). ††Paired *t*-test comparison of data in the AKU on NTBC group against AKU off NTBC group, ‡Unpaired *t*-test comparison of data in the HV group against AKU off NTBC group.

	AKU on NTBC				*p*†	AKU off NTBC				*p*††	HV				*p* ‡
Parameters (µmol/kg ffm)	n	Mean		SD		n	Mean		SD		n	Mean		SD	
Tyrosine-EC (Q1)	7	312.5	±	50.5	<0.001	7	63.8	±	46.9	<0.001	7	23.8	±	3.1	0.044
Tyrosine-IC (Q2)	7	353.6	±	60.6	<0.001	7	123.7	±	32.6	<0.001	7	85.8	±	23.1	0.027

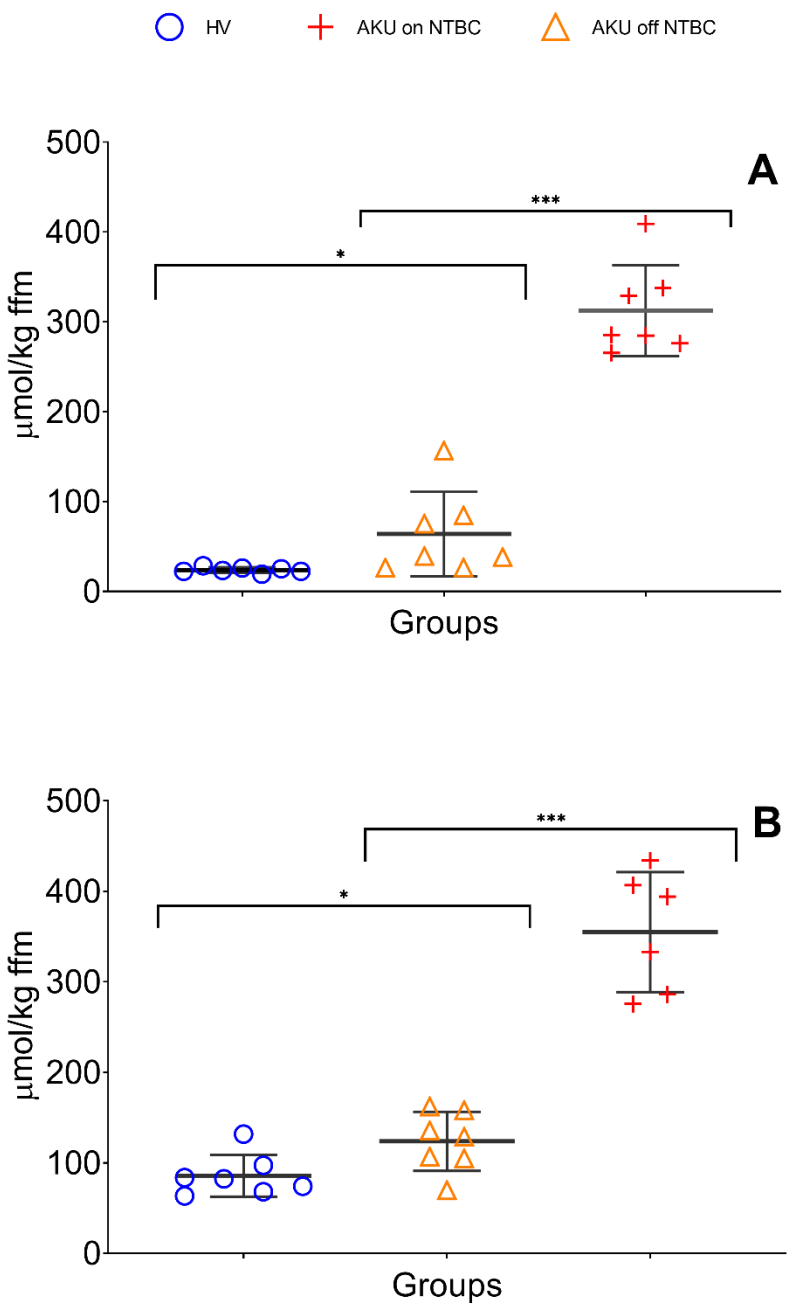

Figure 56: Estimated tyrosine pool size across the study groups.
A: estimated pool size of the extracellular tyrosine, B: estimated pool size of the intracellular tyrosine HV: healthy volunteers group, AKU on NTBC: AKU patients on NTBC group, AKU off NTBC: AKU patients off NTBC group. Line and error 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. * $P < 0.05$, *** $P < 0.001$.

Relationship between various variables and Extracellular (EC)- tyrosine pool size in humans

This study examined the relationship between the EC-tyrosine pool size and several variables in attempt to elucidate factors that can potentially contribute to changes in the EC-tyrosine pool size. Simple linear regression was used to plot extracellular tyrosine pool size values in each group against each of the following six variables: age, total body water (TBW), body weight (BW), body mass index (BMI), fat free mass (FFM) and fat free mass as a percentage of body weight (FFM%). Table 56 summarises equations and goodness of fit as expressed by R^2 for each of the linear regressions.

Overall, there is a trend of positive relationships between each of the above variables and EC-tyrosine pool. Statistically, only the relationship to TBW in the AKU off NTBC group is found to be significant ($P < 0.05$). This can be explained by the small sample size to which classical statistical tests should be interpreted with caution.

The effect of gender on EC-tyrosine in the context of the above six variable have not been statistically assessed due to the small numbers in each gender group (4 males and 3 females). But data from both genders are separately displayed in Figure 58 and clearly demonstrate that gender has no clear effect on the EC-tyrosine pool size when the above six variable are considered.

Table 56: Parameters of the linear regressions of extracellular tyrosine pool size against various variables.
* $P < 0.05$, ns: not significant.

		Tyrosine Extracellular Pool Size (EC= Y)		
Variable (X)	**Parameter**	**HV group**	**AKU on NTBC group**	**AKU off NTBC group**
AGE	Equation	Y = 0.1043*X + 20.50	Y = -0.8998*X + 354.4	Y = -1.181*X + 118.8
	Goodness of Fit (R^2)	0.05	0.06	0.13
	P value	ns	ns	ns
Total Body Water (TBW)	Equation	Y = 0.3719*X + 7.640	Y = -1.969*X + 384.8	Y = -3.741*X + 201.3
	Goodness of Fit (R^2)	0.20	0.14	0.60
	P value	ns	ns	*
Body Weight (BW)	Equation	Y = 0.1419*X + 12.83	Y = -1.049*X + 389.2	Y = -1.776*X + 193.8
	Goodness of Fit (R^2)	0.23	0.16	0.52
	P value	ns	ns	ns
Body Mass Index (BMI)	Equation	Y = 0.5911*X + 9.402	Y = -5.130*X + 447.1	Y = -5.308*X + 203.1
	Goodness of Fit (R^2)	0.41	0.33	0.41
	P value	ns	ns	ns
Fat Free Mass (FFM)	Equation	Y = 0.3008*X + 4.572	Y = -1.241*X + 377.7	Y = -2.708*X + 206.1
	Goodness of Fit (R^2)	0.26	0.10	0.53
	ns	ns	ns	ns
FFM as a percentage of body weight (FFM%)	Equation	Y = -0.2288*X + 42.83	Y = 6.631*X - 165.8	Y = 2.114*X - 88.64
	Goodness of Fit (R^2)	0.16	0.27	0.03
	P value	ns	ns	ns

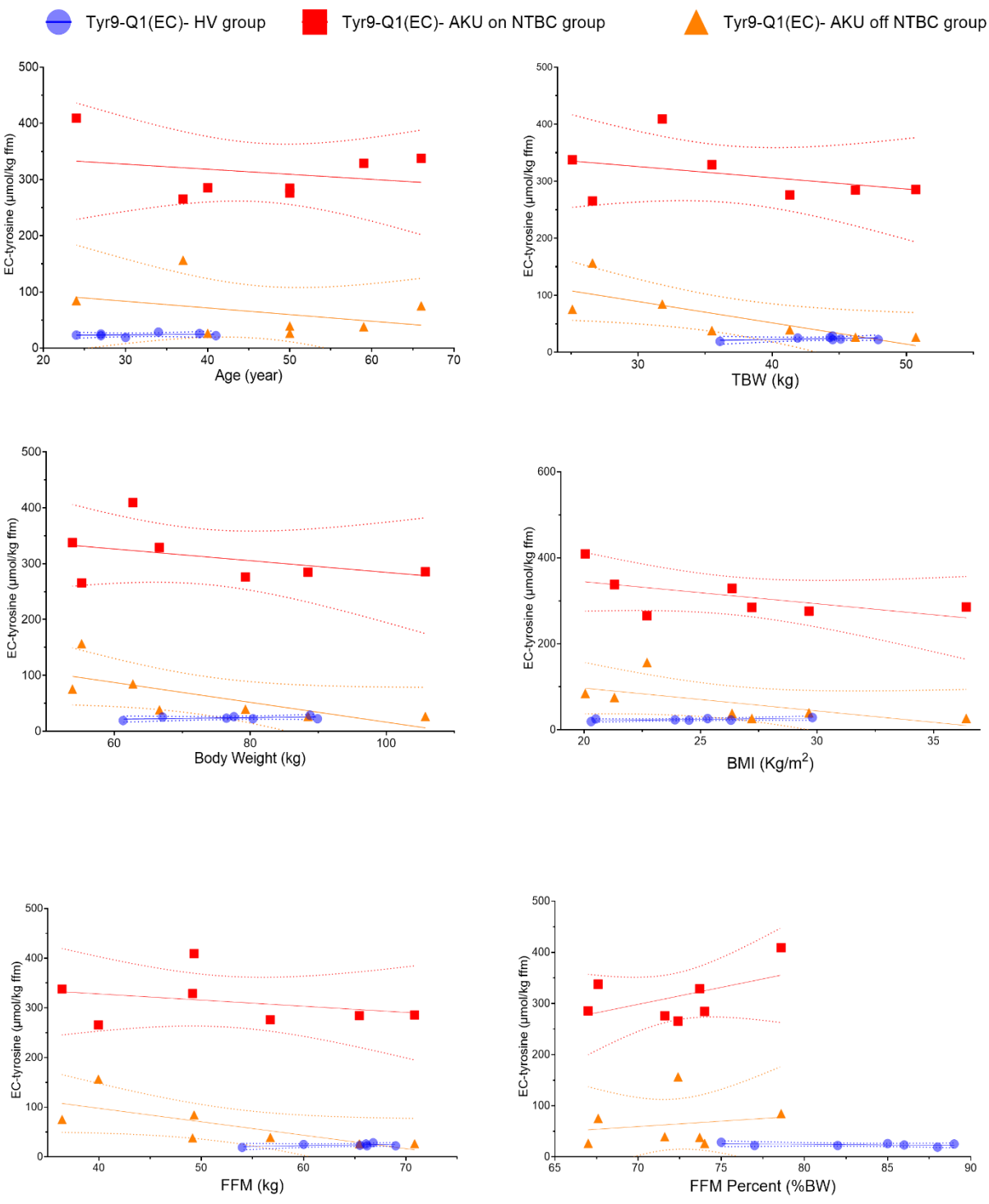

Figure 57: The relationship between extracellular tyrosine pool size (Tyr9-Q1 (EC)) and various variables.
TBW: Total Body Water, BW: Body Weight, BMI: Body Mass index, FFM: Fat Free Mass, FFM precent: Fat Free Mass as a percentage of body weight.

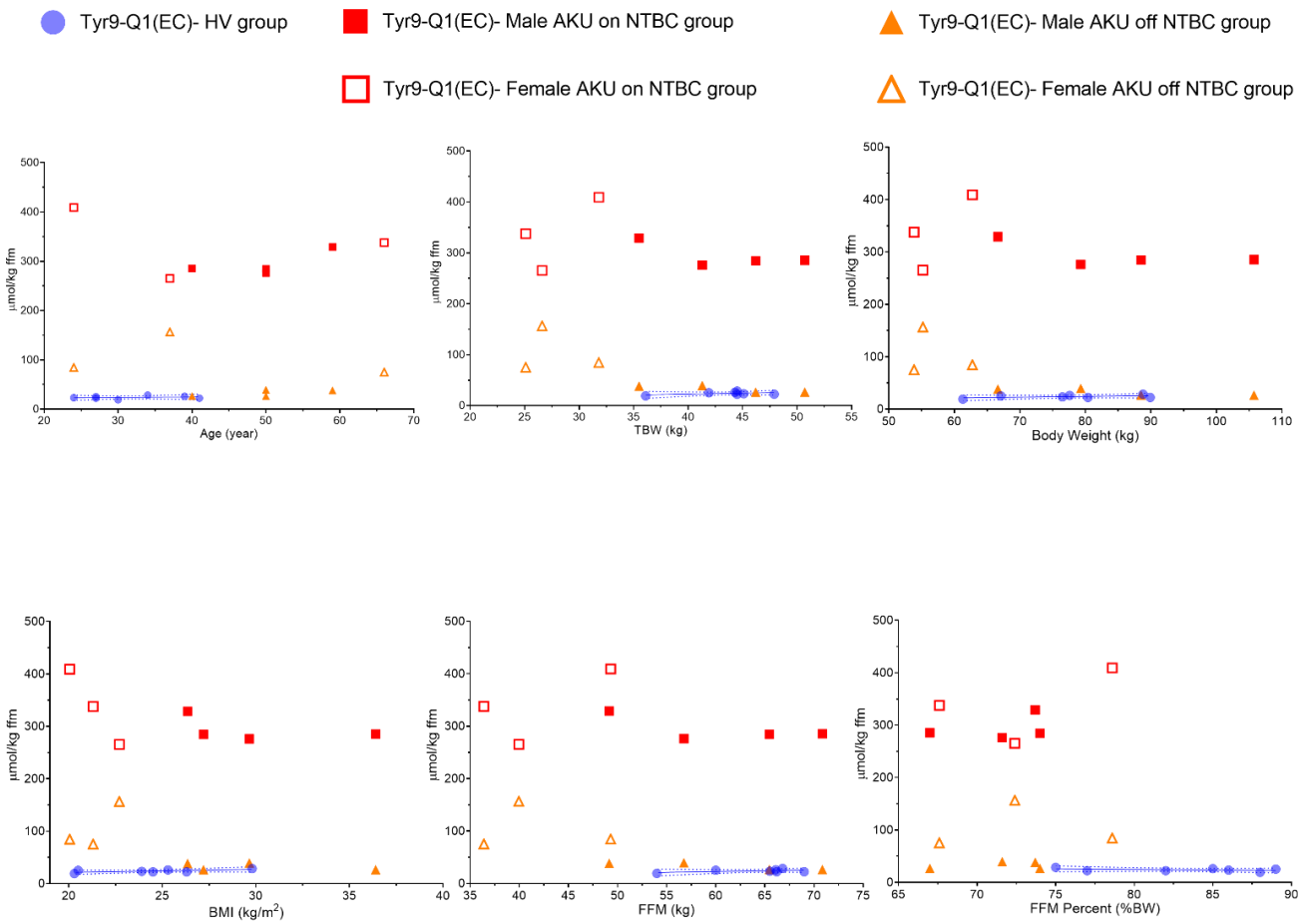

Figure 58: The relationship between extracellular tyrosine pool size (Tyr9-Q1 (EC)) and various variables- males versus females.
TBW: Total Body Water, BW: Body Weight, BMI: Body Mass index, FFM: Fat Free Mass, FFM precent: Fat Free Mass as a percentage of body weight.

4.3.7 Relationship between various variables and intracellular (IC)-tyrosine pool size in humans

The study examined the relationship between the IC-tyrosine pool size and several variables in attempt to elucidate factors that can potentially contribute to changes in the IC-tyrosine pool size. Simple linear regression was used to plot extracellular tyrosine pool size values in each group against each of the following six variables: age, TBW, BW, BMI, FFM and FFM%. Table 57 summarises equations and goodness of fit as expressed by R^2 for each of the linear regressions.

Overall, there is no clear relationship between each of the above variables and IC-tyrosine pool. This can be explained by the small sample in which classical statistical tests should be interpreted with caution.

The effect of gender on IC-tyrosine in the context of the above six variable have not been statistically assessed due to the small sample number, but data from both genders are displayed separately in Figure 60. No firm conclusions can be made regarding the effect of gender on the IC-tyrosine pool size when the above six variable are considered.

Table 57: Parameters of the linear regressions of intracellular tyrosine pool size against various variables.
* $P < 0.05$, ns: not significant.

		Tyrosine Intracellular Pool Size (IC= Y)		
Variable (X)	**Parameter**	**HV group**	**AKU on NTBC group**	**AKU off NTBC group**
AGE	Equation	Y = -1.553*X + 135.0	Y = 0.2509*X + 341.9	Y = -1.083*X + 174.2
	Goodness of Fit (R^2)	0.19	0.003	0.22
	P value	ns	ns	ns
Total Body Water (TBW)	Equation	Y = 0.3368*X + 71.14	Y = 0.6648*X + 329.2	Y = -0.9027*X + 156.9
	Goodness of Fit (R^2)	0.003	0.01	0.07
	P value	ns	ns	ns
Body Weight (BW)	Equation	Y = -0.6454*X + 135.7	Y = -0.07445*X + 359.1	Y = -0.3766*X + 151.3
	Goodness of Fit (R^2)	0.09	0.001	0.05
	P value	ns	ns	ns
Body Mass Index (BMI)	Equation	Y = -3.136*X + 162.2	Y = -2.322*X + 414.6	Y = -0.06615*X + 125.4
	Goodness of Fit (R^2)	0.20	0.05	0.0001
	P value	ns	ns	ns
Fat Free Mass (FFM)	Equation	Y = -0.3379*X + 107.4	Y = 0.5275*X + 325.9	Y = -0.6748*X + 159.2
	Goodness of Fit (R^2)	0.01	0.01	0.07
	ns	ns	ns	ns
FFM as a percentage of body weight (FFM%)	Equation	Y = 2.009*X - 81.26	Y = 7.393*X - 179.7	Y = -0.1118*X + 131.8
	Goodness of Fit (R^2)	0.22	0.24	0.0002
	P value	ns	ns	ns

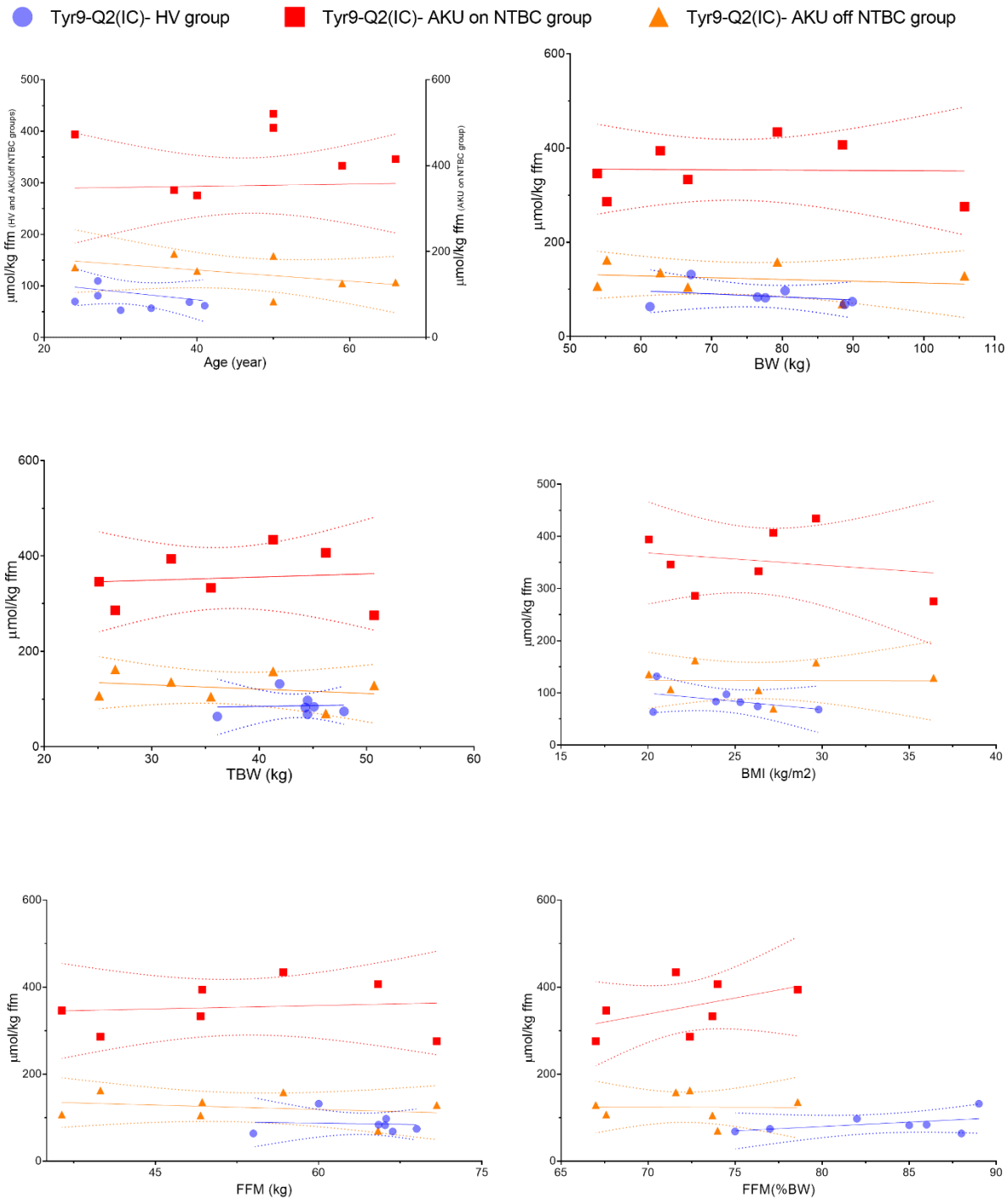

Figure 59: The relationship between intracellular tyrosine pool size (Tyr9-Q2 (IC)) and various variables.
TBW: Total Body Water, BW: Body Weight, BMI: Body Mass index, FFM: Fat Free Mass, FFM precent: Fat Free Mass as a percentage of body weight.

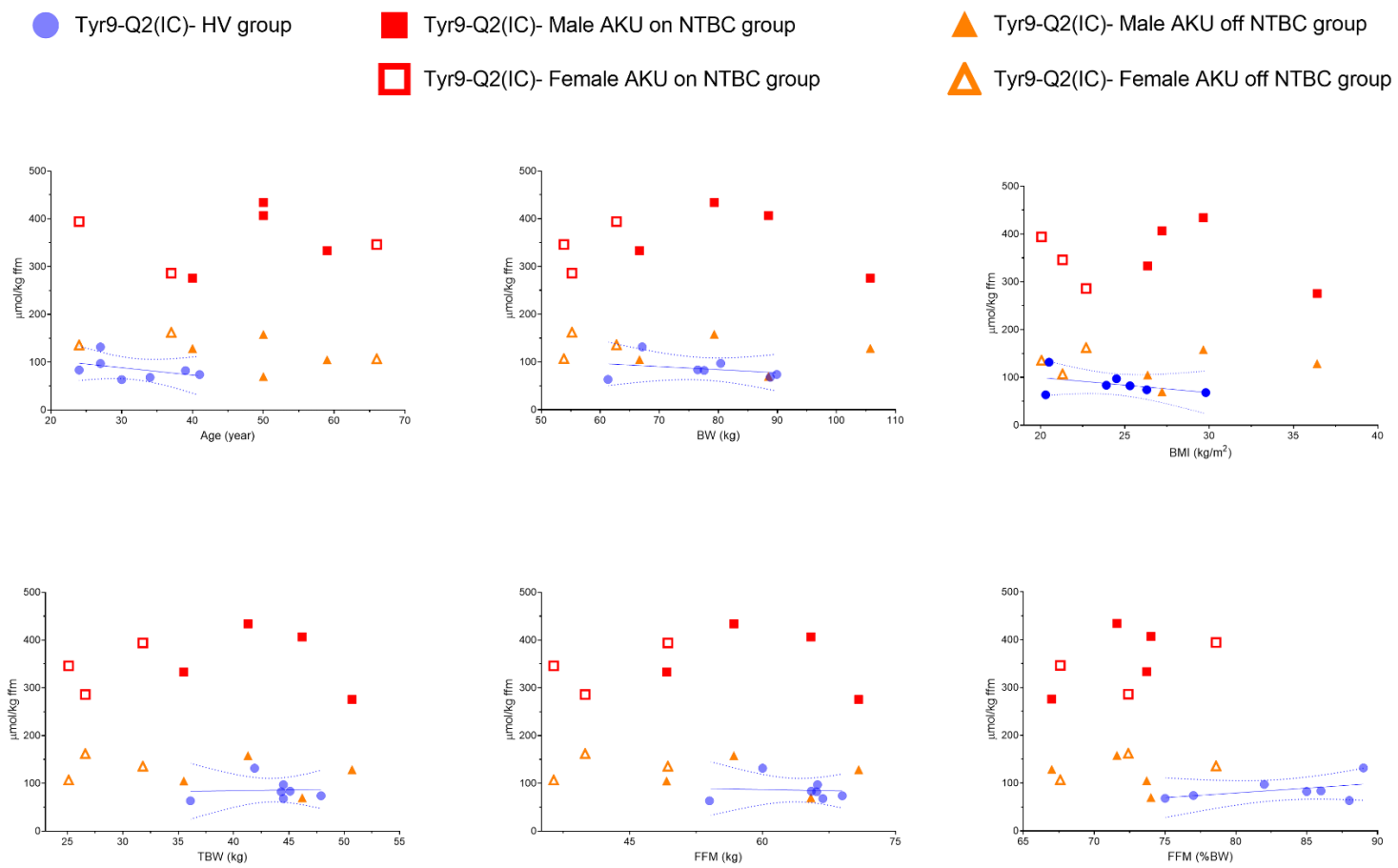

Figure 60: The relationship between intracellular tyrosine pool size (Tyr9-Q2 (IC)) and various variables- males versus females.
TBW: Total Body Water, BW: Body Weight, BMI: Body Mass index, FFM: Fat Free Mass, FFM precent: Fat Free Mass as a percentage of body weight.

4.3.8 Effects of NTBC on whole-body protein turnover in the post-absorptive state in the study groups

There is no difference in protein synthesis or breakdown among the AKU and healthy subjects. However, NTBC appears to reduce phenylalanine hydroxylation significantly in AKU when compared to AKU off NTBC (P = 0.016), and when compared to healthy volunteers (P <0.0001). Notably, conversion in healthy volunteers and AKU off NTBC is comparable. This is summarised in Figure 61 and Table 58. It is likely that the elevated concentrations of tyrosine cause negative feedback on phenylalanine hydroxylase, leading to reduction in phenylalanine hydroxylation. This has been reported before and considered as a pathway adaptation to minimise formation of tyrosine from phenylalanine in NTBC treated AKU patients (Ranganath et al., 2022d).

In the following sections, six variables were examined in attempt to elucidate factors that can potentially contribute to changes in phenylalanine hydroxylation to tyrosine as well as protein synthesis/ breakdown. These variables were: age, TBW, BW, BMI, FFM and FFM%. Furthermore, measurements obtained from males and females were displayed separately to evaluate if gender has any impact.

Table 58: Estimation of average phenylalanine hydroxylation, protein synthesis and breakdown.
WB-PB: Whole Body Protein Breakdown, WB-PS: Whole Body Protein Synthesis, Phe→Tyr: phenylalanine hydroxylation to tyrosine. †Unpaired *t*-test comparison of data in the HV group against AKU on NTBC group. ††Paired *t*-test comparison of data in the AKU on NTBC group against AKU off NTBC group. ‡ Unpaired *t*-test comparison of data in the HV group against AKU off NTBC group.

Parameters (µmol/kg ffm/hr)	AKU on NTBC				*p*†	AKU off NTBC				*p*††		HV				*p* ‡
	n	Mean		SD		n	Mean		SD			n	Mean		SD	
WB-PB	7	143.2	±	20.1	0.675	7	150.4	±	27.5	0.527		7	149.3	±	22.8	0.941
Phe→Tyr	7	2.3	±	1.0	<0.0001	7	7.9	±	2.6	0.001		7	10.8	±	1.7	0.032
WB-PS	7	140.9	±	19.4	0.747	7	142.4	±	25.8	0.881		7	138.6	±	21.6	0.767

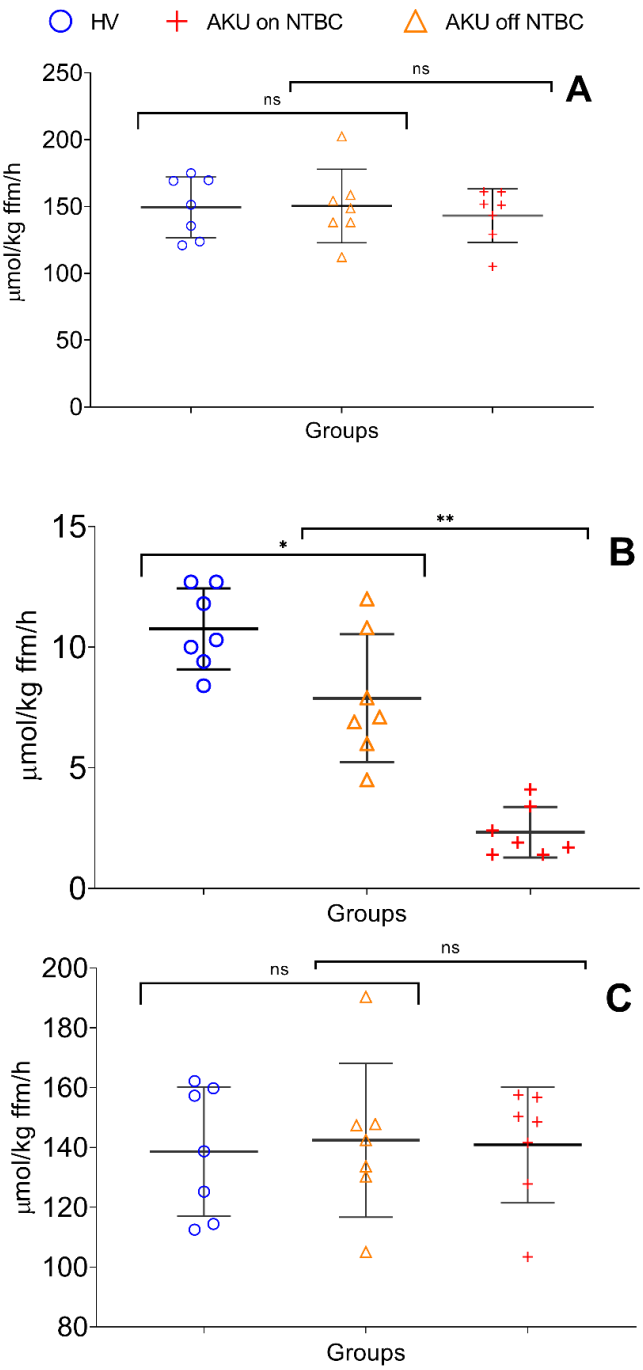

Figure 61: Representation of the phenylalanine hydroxylation to tyrosine, whole-body protein synthesis and breakdown in the study groups.
A: whole-body protein breakdown, B: phenylalanine hydroxylation to tyrosine (reflecting net balance), C: whole-body protein synthesis protein. HV: healthy volunteers, 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. ns: $P \geq 0.05$, * $P < 0.05$, ** $P < 0.01$.

4.3.8.1 *Relationship between various variables and whole-body protein breakdown in the study groups*

The study examined the relationship between the whole-body protein breakdown (WB-PB) and several variables in attempt to elucidate factors that can potentially contribute to changes in WB-PB. Simple linear regression was used to plot WB-PB values in each group against each of the following six variables: age, TBW, BW, BMI, FFM and FFM% (Figure 62). Table 59 summarises equations and goodness of fit as expressed by R^2 for each of the linear regressions.

Only in healthy volunteers, WB-PB showed a weak negative relationship with age (R^2 0.71, P<0.5). This was not seen in AKU patients regardless of NTBC therapy. In the AKU off NTBC group, WB-PB showed a weak negative relationship with TBW (R^2 0.61, P<0.5) and FFM (R^2 0.69, P<0.5). In contrast, there was no statistically significant relationship between WB-PB and each of the following variables, BMI, BW and FFM% in any of the three groups. Overall, there is no consistent relationship between each of the above variables and WB-PB. This can be a result of the small sample size in which classical statistical tests should be interpreted with caution.

To examine the impact of gender on the WB-PB, data from both genders were displayed separately against the six variables (Figure 63). Statistical testing was not carried out due to the small sample number. Gender appears to have no effect although no firm conclusions can be made regarding the effect of gender on WB-PB when the above six variable are considered.

Robert et al. (1984) found that age did not affect protein turnover rate when the latter is expressed per unit of TBW or muscle mass. In contrast, Pannemans et al. (1995) observed that protein turnover is reduced with aging in both males and females when they consumed the recommended protein intake-even after correcting for body composition. Furthermore, age and female gender were found to be associated with lower protein synthesis and breakdown although these differences are insignificant when protein turnover parameters are adjusted for fat free mass (Morais et al., 2006).

Table 59: Parameters of the linear regressions of whole-body protein breakdown (WB-PB) against various variables.
* *P* < 0.05, ns: not significant.

		whole-body protein breakdown (WBPB= Y)		
Variable (X)	**Parameter**	**HV group**	**AKU on NTBC group**	**AKU off NTBC group**
AGE	Equation	Y = -2.968*X + 243.5	Y = -0.2008*X + 152.6	Y = 1.295*X + 90.06
	Goodness of Fit (R^2)	0.71	0.02	0.44
	P value	*	ns	ns
Total Body Water (TBW)	Equation	Y = -3.775*X + 313.4	Y = -1.309*X + 191.3	Y = -2.198*X + 231.1
	Goodness of Fit (R^2)	0.38	0.408	0.61
	P value	ns	ns	*
Body Weight (BW)	Equation	Y = -1.467*X + 262.8	Y = -0.5362*X + 182.4	Y = -1.120*X + 232.3
	Goodness of Fit (R^2)	0.46	0.26	0.60
	P value	ns	ns	ns
Body Mass Index (BMI)	Equation	Y = -4.714*X + 264.2	Y = -1.093*X + 171.9	Y = -3.142*X + 232.8
	Goodness of Fit (R^2)	0.47	0.09	0.41
	P value	ns	ns	ns
Fat Free Mass (FFM)	Equation	Y = -2.832*X + 330.4	Y = -0.9868*X + 195.1	Y = -1.813*X + 245.6
	Goodness of Fit (R^2)	0.41	0.39	0.69
	ns	ns	ns	*
FFM as a percentage of body weight (FFM%)	Equation	Y = 2.465*X - 55.59	Y = -1.314*X + 238.0	Y = -1.331*X + 246.4
	Goodness of Fit (R^2)	0.3416	0.06794	0.03702
	P value	ns	ns	ns

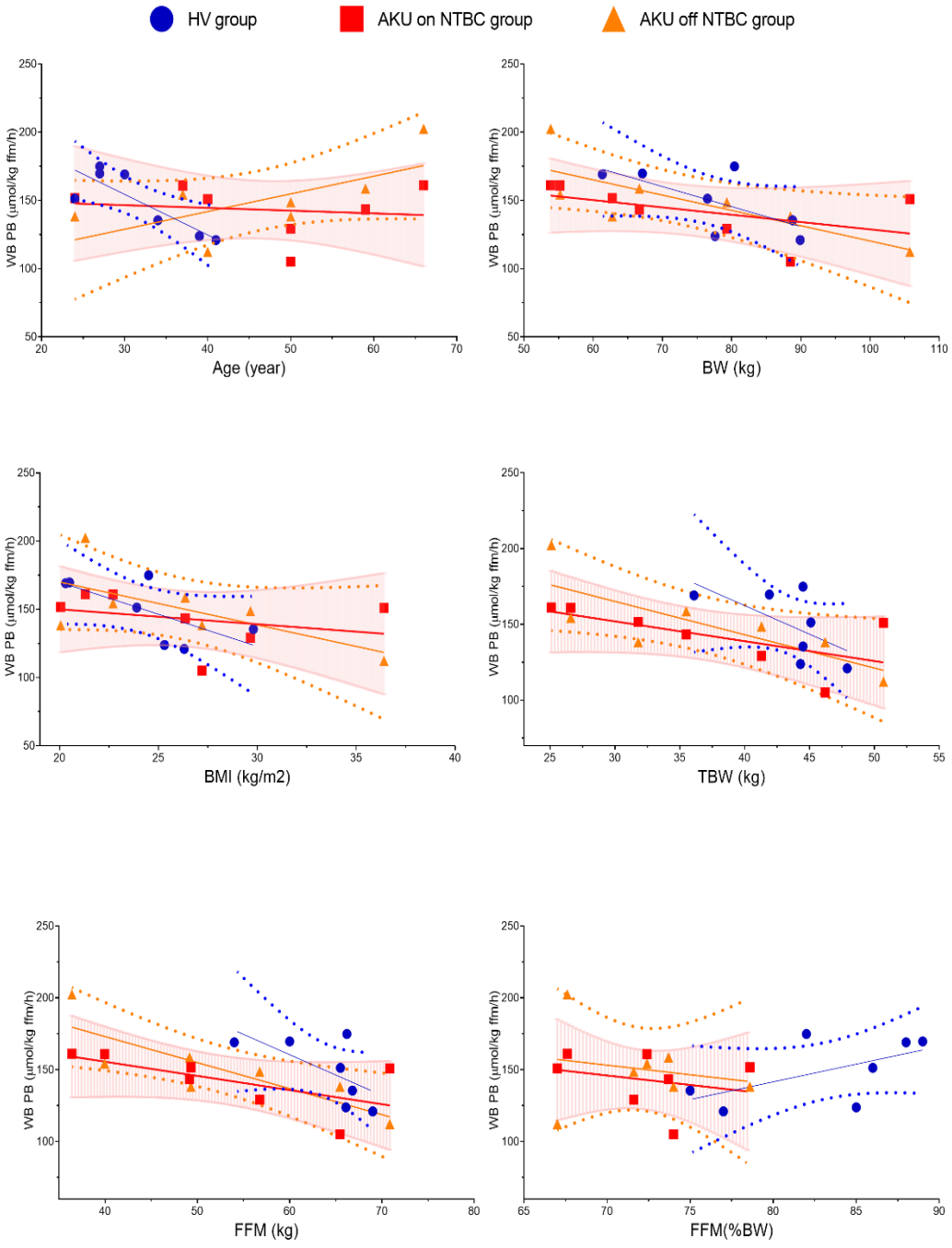

Figure 62: The relationship between Whole-Body Protein Breakdown (WB-PB) and various variables.
TBW: Total Body Water, BW: Body Weight, BMI: Body Mass index, FFM: Fat Free Mass, FFM precent: Fat Free Mass as a percentage of body weight.

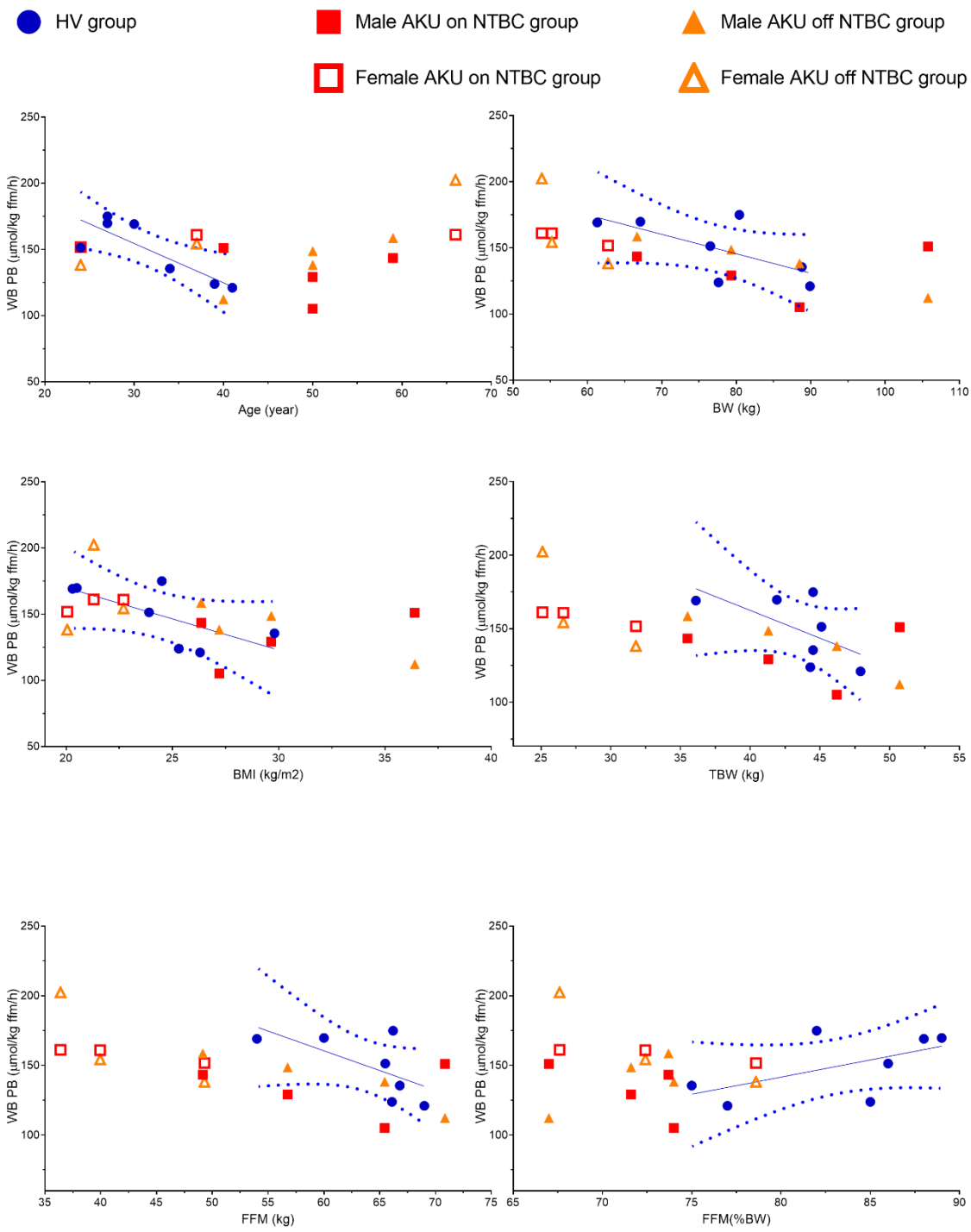

Figure 63: The relationship between Whole-Body Protein Breakdown (WB-PB) and various variables.- males versus females.
TBW: Total Body Water, BW: Body Weight, BMI: Body Mass index, FFM: Fat Free Mass, FFM precent: Fat Free Mass as a percentage of body weight.

4.3.8.2 *Effects of NTBC on whole-body protein synthesis in the study groups*

The study examined the relationship between the whole-body protein breakdown (WB-PB) and several variables in attempt to elucidate factors that can potentially contribute to changes in WB-PB. Simple linear regression was used to plot WB-PB values in each group against each of the following six variables: age, TBW, BW, BMI, FFM and FFM%. Table 60 summarises equations and goodness of fit as expressed by R^2 for each of the linear regressions which are represented in Figure 64.

In AKU off NTBC group, WB-PS showed a weak negative relationship with TBW (R^2 0.65, P<0.5), with BW (R^2 0.64, P<0.5) and with FFM (R^2 0.73, P<0.5). In contrast, there was no statistically significant relationship between WB-PS and age, BMI or FFM% in the AKU off NTBC group. Similarly, there was no statistically significant relationship between WB-PS and each of the six variables in healthy volunteers or AKU on NTBC patients. It is difficult to explain the presence/lack of relationship with the above variables within the AKU group (on and off NTBC) and firm conclusion cannot be drawn, given the context of small sample size in which classical statistical tests should be interpreted with caution.

To examine the impact of gender on the WB-PS, data from both genders were displayed separately against the six variables (Figure 65). Statistical testing was not carried out due to the small sample number. Gender appears to have no effect although no firm conclusions can be made regarding the effect of gender on WB-PS when the above six variable are considered.

Table 60: Parameters of the linear regressions of whole-body protein synthesis (WB-PS) against various variables.
* $P < 0.05$, ns: not significant.

		whole-body protein synthesis (WBPS= Y)		
Variable (X)	**Parameter**	**HV group**	**AKU on NTBC group**	**AKU off NTBC group**
AGE	Equation	Y = -2.758*X + 226.0	Y = -0.2128*X + 150.8	Y = 1.128*X + 89.93
	Goodness of Fit (R^2)	0.68	0.02	0.38
	P value	ns	ns	ns
Total Body Water (TBW)	Equation	Y = -3.617*X + 295.8	Y = -1.247*X + 186.7	Y = -2.124*X + 220.5
	Goodness of Fit (R^2)	0.385	0.395	0.65
	P value	ns	ns	*
Body Weight (BW)	Equation	Y = -1.410*X + 247.7	Y = -0.5113*X + 178.3	Y = -1.084*X + 221.7
	Goodness of Fit (R^2)	0.47	0.25	0.64
	P value	ns	ns	*
Body Mass Index (BMI)	Equation	Y = -4.567*X + 249.9	Y = -1.043*X + 168.2	Y = -3.097*X + 223.7
	Goodness of Fit (R^2)	0.49	0.09	0.46
	P value	ns	ns	ns
Fat Free Mass (FFM)	Equation	Y = -2.736*X + 313.5	Y = -0.9377*X + 190.1	Y = -1.737*X + 233.7
	Goodness of Fit (R^2)	0.43	0.37	0.73
	ns	ns	ns	*
FFM as a percentage of body weight (FFM%)	Equation	Y = 2.361*X - 57.70	Y = -1.180*X + 226.0	Y = -0.9969*X + 214.3
	Goodness of Fit (R^2)	0.35	0.06	0.02
	P value	ns	ns	ns

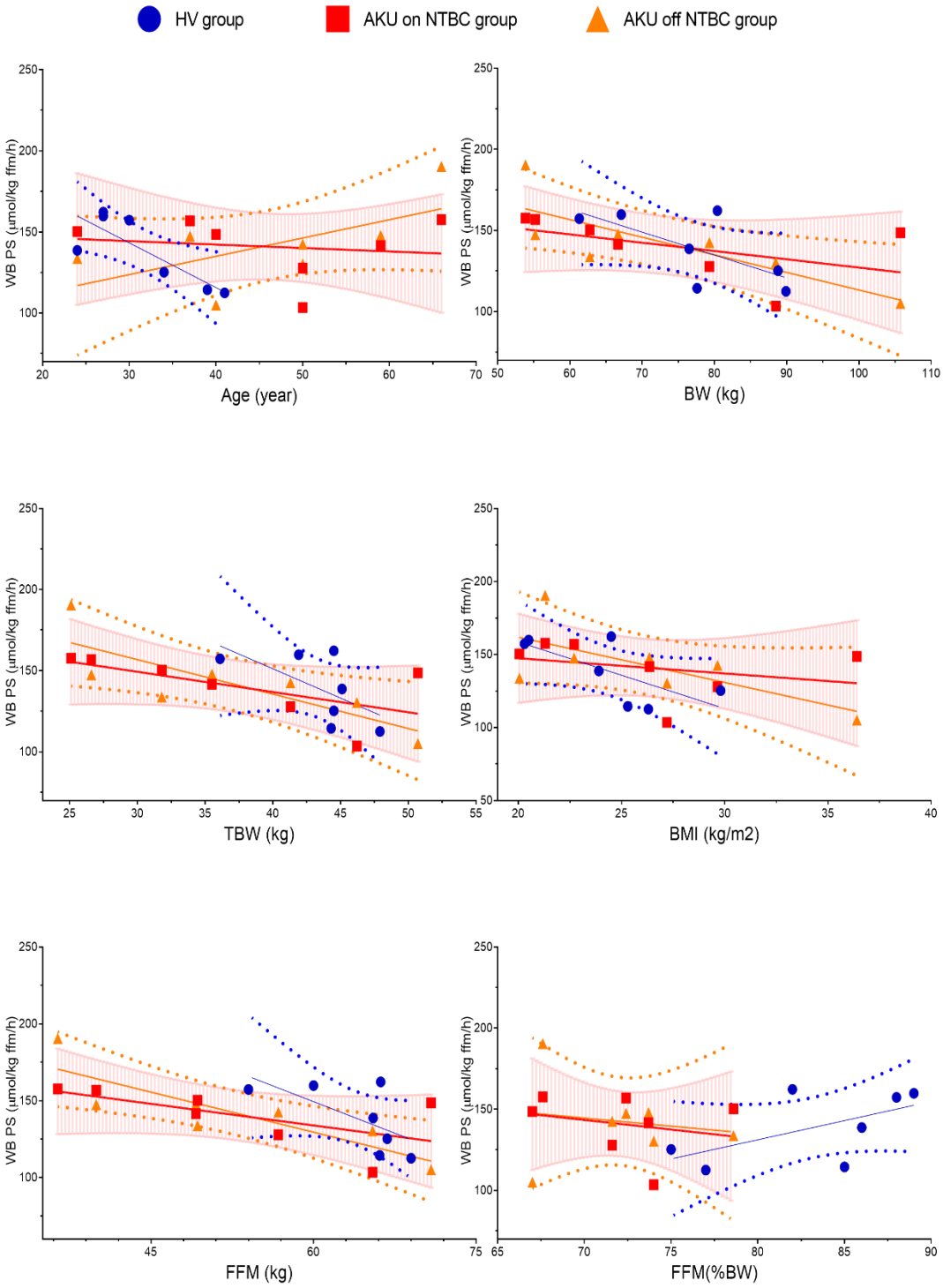

Figure 64: The relationship between Whole-Body Protein Synthesis (WB-PS) and various variables.
TBW: Total Body Water, BW: Body Weight, BMI: Body Mass index, FFM: Fat Free Mass, FFM precent: Fat Free Mass as a percentage of body weight.

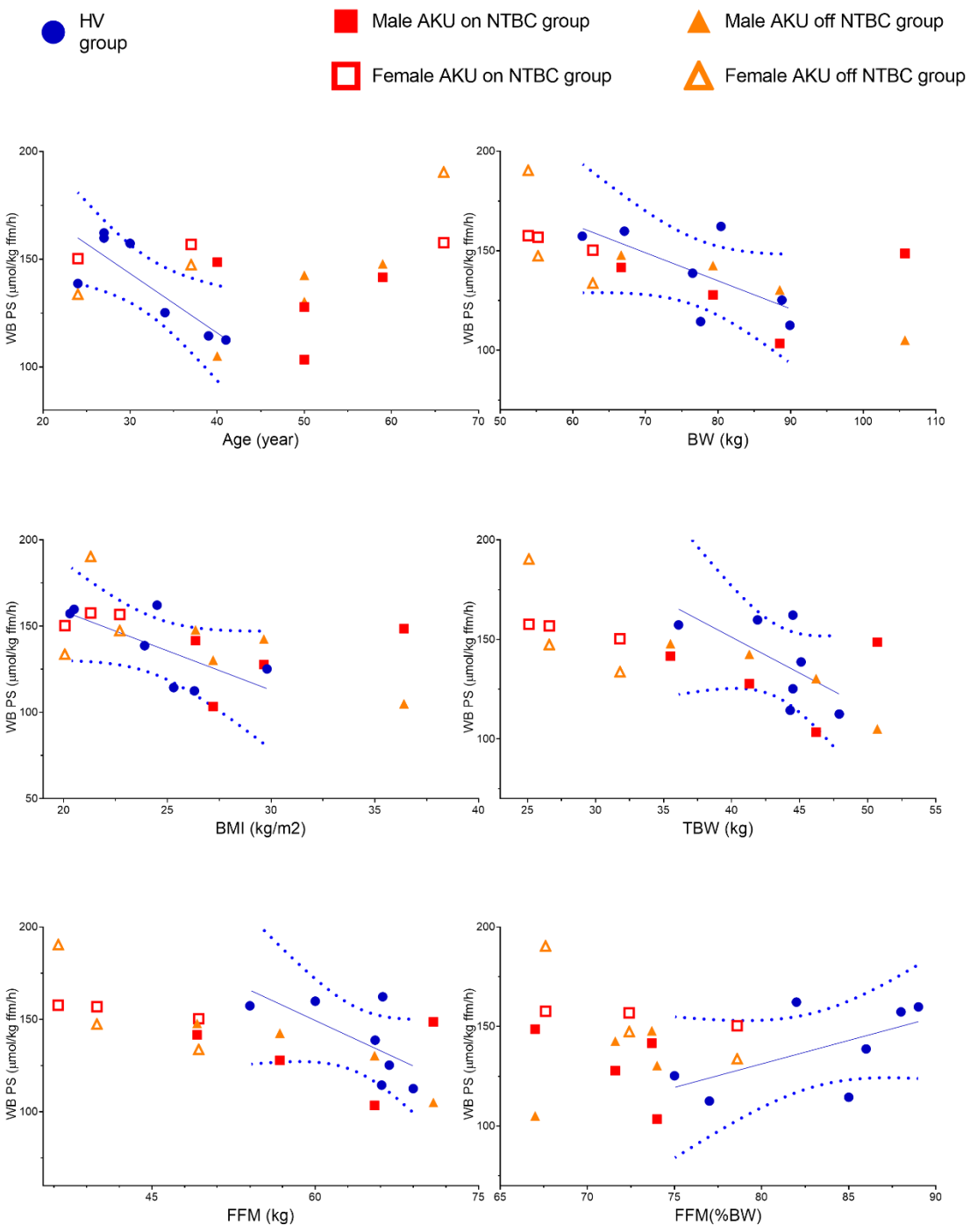

Figure 65: The relationship between Whole-Body Protein Synthesis (WB-PS) and various variables.- males versus females.
TBW: Total Body Water, BW: Body Weight, BMI: Body Mass index, FFM: Fat Free Mass, FFM precent: Fat Free Mass as a percentage of body weight.

4.3.8.3 *Effects of NTBC on phenylalanine hydroxylation to tyrosine in the study groups*

The study examined the relationship between phenylalanine hydroxylation to tyrosine (Phe→Tyr) and several variables in attempt to elucidate factors that can potentially contribute to changes in phenylalanine hydroxylation to tyrosine. Simple linear regression was used to plot Phe→Tyr values in each group against each of the following six variables: age, TBW, BW, BMI, FFM and FFM%. Table 61 summarises equations and goodness of fit as expressed by R^2 for each of the linear regressions which are represented visually in Figure 66.

In healthy volunteers, Phe→Tyr showed a weak negative relationship with age (R^2 0.72, P<0.5). Surprisingly, there was a modest positive relationship with age in the AKU off NTBC group (R^2 0.80, P<0.01). In contrast, there was no statistically significant relationship between Phe→Tyr and the remainder of the variables in health volunteers or AKU off NTBC groups. Similarly, there was no statistically significant relationship between Phe→Tyr and each of the six variables.

The effect of gender on Phe→Tyr in the context of the above six variable have not been statistically assessed due to the small sample size, but data from both genders are displayed separately in Figure 67. No firm conclusions can be made regarding the effect of gender on phenylalanine hydroxylation to tyrosine when the above six variable are considered.

Table 61: Parameters of the linear regressions of phenylalanine hydroxylation to tyrosine (Phe→Tyr) against various variables.

* *P* < 0.05, ***P* <0.01, ns: not significant.

		phenylalanine hydroxylation to tyrosine (Phe→Tyr = Y)		
Variable (X)	**Parameter**	**HV group**	**AKU on NTBC group**	**AKU off NTBC group**
AGE	Equation	Y = -0.2195*X + 17.72	Y = 0.01141*X + 1.797	Y= 0.1673*X + 0.09257
	Goodness of Fit (R^2)	0.72	0.02	0.80
	P value	*	ns	**
Total Body Water (TBW)	Equation	Y = -0.1633*X + 17.86	Y = -0.06059*X + 4.555	Y = -0.07765*X + 10.74
	Goodness of Fit (R^2)	0.13	0.32	0.08
	P value	ns	ns	ns
Body Weight (BW)	Equation	Y = -0.06027*X + 15.42	Y = -0.02433*X + 4.108	Y = -0.03779*X + 10.65
	Goodness of Fit (R^2)	0.14	0.20	0.07
	P value	ns	ns	ns
Body Mass Index (BMI)	Equation	0.10	0.06	0.01
	Goodness of Fit (R^2)	Y = -0.1559*X + 14.56	Y = -0.04662*X + 3.552	Y = -0.05212*X + 9.253
	P value	ns	ns	ns
Fat Free Mass (FFM)	Equation	Y = -0.1006*X + 17.19	Y = -0.04773*X + 4.837	Y = -0.07812*X + 11.99
	Goodness of Fit (R^2)	0.10	0.33	0.14
	ns	ns	ns	ns
FFM as a percentage of body weight (FFM%)	Equation	Y = 0.1123*X + 1.417	Y = -0.1274*X + 11.52	Y = -0.3281*X + 31.55
	Goodness of Fit (R^2)	0.13	0.23	0.24
	P value	ns	ns	ns

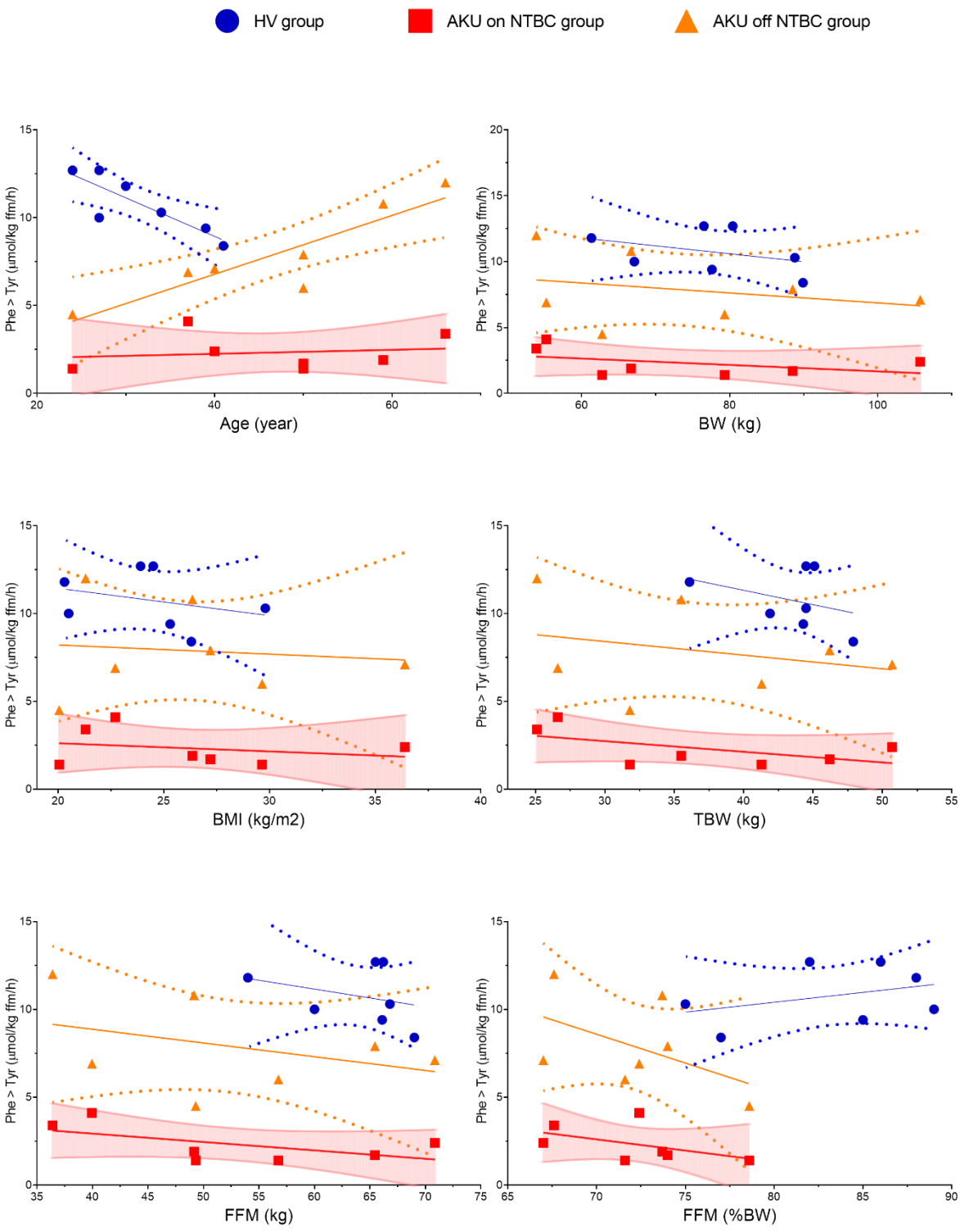

Figure 66: The relationship between phenylalanine hydroxylation to tyrosine (Phe→Tyr) and various variables.
TBW: Total Body Water, BW: Body Weight, BMI: Body Mass index, FFM: Fat Free Mass, FFM precent: Fat Free Mass as a percentage of body weight.

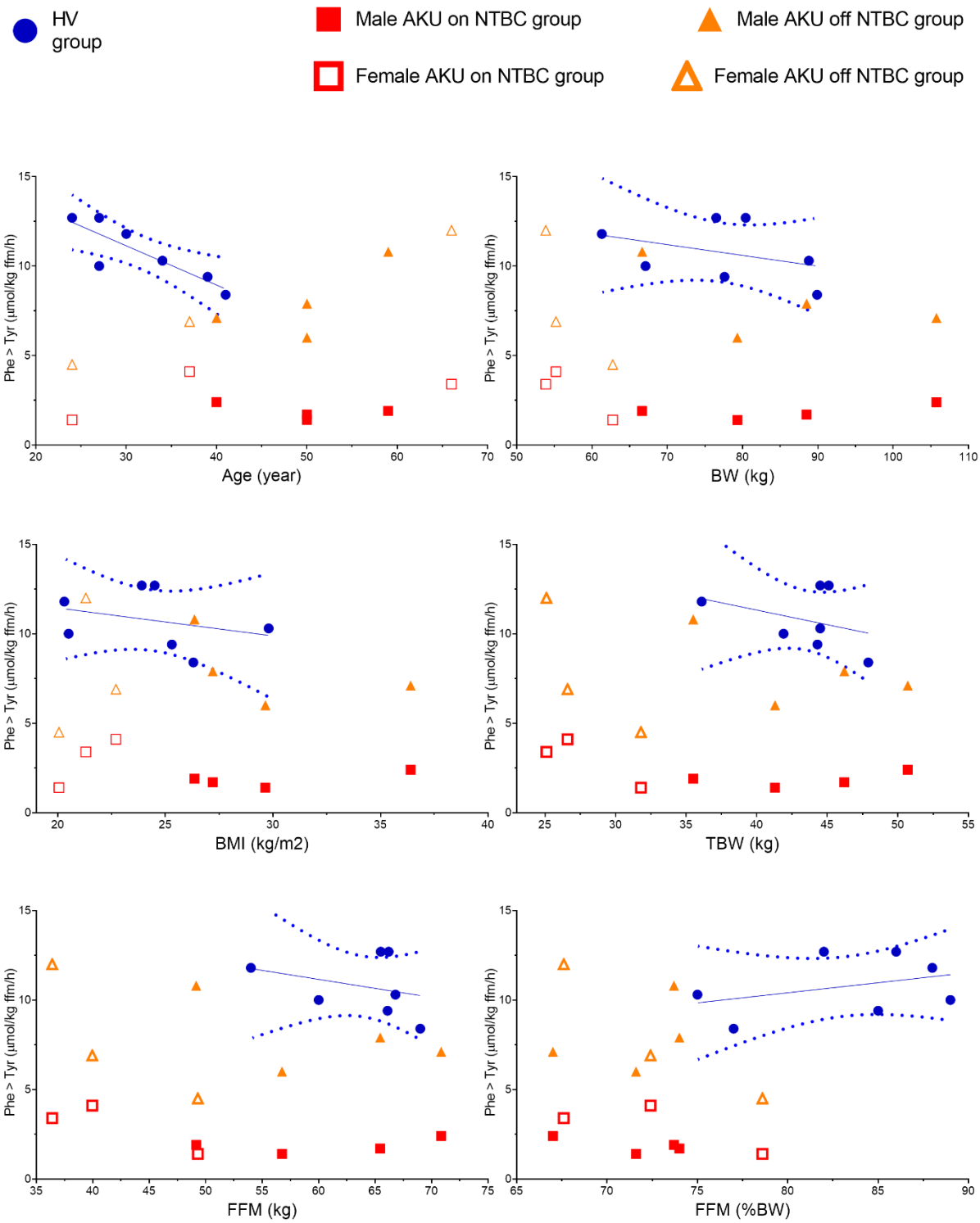

Figure 67: The relationship between phenylalanine hydroxylation to tyrosine (Phe→Tyr) and various variables- Males versus Females.
TBW: Total Body Water, BW: Body Weight, BMI: Body Mass index, FFM: Fat Free Mass, FFM precent: Fat Free Mass as a percentage of body weight.

4.4 DISCUSSION

NTBC induced hypertyrosinaemia has been observed for over two decades in HT-1 patients (McKiernan, 2013) and more recently in AKU patients (Introne et al., 2011, Ranganath et al., 2016). While this is expected from the action of NTBC, this study presents, for the first time, the experimental evidence for the reduction in the degradation of tyrosine and magnitude of hypertyrosinaemia.

Effects of NTBC on tyrosine compounds: this study found that NTBC-induced HPPD inhibition causes increases in tyrosine and tyrosine compounds 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 HT-1 (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, Ranganath et al., 2022c), 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 HT-3 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).

The concentrations of serum tyrosine, phenylalanine, HGA and HPLA are all significantly higher in the non-treated AKU group when compared to healthy volunteers. In this study, AKU patients and healthy volunteers 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 compounds 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 negative feedback (product inhibition- like effect). This leads to an increase in the compounds 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, or that HPPA is converted very efficiently to HPLA. This is supported by observations that HPPA and HPLA were undetected before starting NTBC in AKU (Milan et al., 2019).

The conversion of phenylalanine to tyrosine is reduced in patients receiving NTBC compared to healthy volunteers and AKU patients off NTBC. It is likely that the elevated concentrations of tyrosine cause negative feedback on phenylalanine hydroxylase. This has been considered as a pathway adaptation to minimise formation of tyrosine from phenylalanine (Ranganath et al., 2022d). Notably, conversion in healthy volunteers and AKU off NTBC is comparable.

Quantification of EC and IC-tyrosine pool sizes: Estimation of EC-tyrosine pools clearly demonstrated several-fold increase in size following NTBC. This is already demonstrated in the tyrosine decay curve and is to be expected. Accumulated tyrosine is partially metabolised to HPPA and HPLA. Probably, HPPA, HPLA and tyrosine are increased more proportionately and diffuse out, but HPPA and HPLA, like HGA, are excreted in the urine. This is unlike tyrosine which is reabsorbed. This is consistent with findings from the animal study documented in the previous chapter.

Compared to healthy volunteers, the EC-tyrosine pool size in AKU patients off NTBC is 2.7-fold 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). It is possible that NTBC did not completely worn off causing the difference in pool size between smaller than what would be expected.

Similarly, estimations of the Tyrosine IC pools confirms that NTBC leads to increase in tyrosine IC pool size in AKU patients. This is in keeping with findings from animal study (previous chapter) as well as what would be expected pathophysiologically. In general, It is likely that increased extracellular amino acid concentrations lead to increased intracellular amino acid concentrations through transmembrane equilibrium and concentrative transport (Hyde et al., 2003). Water content of the intracellular compartment is double that of the extracellular one (Mohiuddin., 2022). Therefore, one could expect the IC pool size to be twice that of the EC tyrosine pool size.

Surprisingly, the two pools are almost comparable. The discrepancy noted here could be because compartmental modelling relies on assumptions, which do not hold completely true in physiological sense. An ideal solution would have been to sample the IC pools, but it is impractical for obvious reasons.

There are variations of the degree at which tyrosine degradation is reduced. This could be due to the difference in FFM and the use of fixed dose tracers which lead to different mg/kg tracer load and TTR.

When compared as groups, the L-[$^{13}C_9$]tyrosine decay curves for healthy volunteers and AKU of NTBC almost overlap. This is not surprising as Tyrosine is partially metabolised to HGA which in turn is converted to ochronotic pigments. In healthy volunteers, Tyrosine is broken down to fumarate and acetoacetate (as outlined in Figure 1).

Effects of age, TBW, BW, BMI, FFM and FFM% on the tyrosine pool size: This study assessed the effect of these six variables on the tyrosine pool size (both IC and EC). There was a weak relationship between tyrosine EC pool and TBW in AKU patients when off NTBC but not in when on NTBC. Furthermore, there was no clear relationship between any of the above variables and IC-tyrosine pool. Similarly, gender does not appear to affect EC or IC tyrosine pool sizes. It can be concluded that these variables do not affect tyrosine pool. This needs further confirmation using larger sample size.

Use of stable isotopes to study the impact of NTBC on tyrosine degradation: This study has adopted the pulse approach. The use of stable isotopes in metabolic research has predominantly adopted a primed constant infusion approach (Wilkinson, 2018) which allows for reaching a 'steady state'. For the determination of the pool size, it is still possible to use a single bolus injection study design (Wolfe and Chinkes, 2004). Recently, there has been a shift towards a bolus injection approach as it is more convenient for patients, less labour intensive and cheaper. Studies have looked at the bolus injection approach in animals (Zhang et al., 2002) and humans (Tang et al., 2007, Tuvdendorj et al., 2014, Mason et al., 2017). The tracer infusion protocol in this study is based on the work of Mason et al. (2017).

Phenylalanine hydroxylation to tyrosine is reduced in NTBC treated patients. This could be due to negative feedback from increased tyrosine and would limit the flux of tyrosine down its degradation pathway. This has implications in terms of dietary manipulation as restricting phenylalanine alone in NTBC treated patients will not help in reducing the burden of hypertyrosinaemia. This view is supported by results from mice experiments (Milan et al., 2017, Hughes et al., 2020). A review of the literature, as regards phenylalanine hydroxylation, reveals a heterogeneity in the studies performed in terms of participants demographics, stable isotopes used and infusion protocols (Clarke and Bier, 1982, Thompson et al., 1989, Cortiella et al., 1992, Marchini et al., 1993, Mason et al., 2017) (summarised in Table 3). This work has not been done before in AKU, but the within AKU subject comparison in addition to the inclusion of a healthy volunteers group helped to reduce potential confounders and ensure consistency within the study.

Protein turnover: Data from the NAC suggest that AKU patients are in a state of protein energy malnutrition at presentation before starting NTBC (Judd et al., 2020). Following NTBC therapy, low protein diet results in loss of muscle mass (Ranganath et al., 2022b). Since managing NTBC-induced hypertyrosinaemia involves a degree of dietary restriction of protein, it is of interest to assess protein synthesis and breakdown in this group of patients.

Although this study did not intend to assess protein synthesis and breakdown, its design and use of stable isotopes has enabled the estimation of both. Historically continuous infusions of tracers were required. However, recent studies showed that pulse methods can be used reliably to study protein metabolism (Engelen et al., 2019). In this study, protein synthesis (PS) and breakdown (PB) were not affected by NTBC use. This is surprising as AKU is a chronic multisystem disease and leads to significant morbidity due to aggressive arthropathy in later life. One would expect increased protein turnover. However, a longer study duration would have been required to assess this further. It would have been interesting to compare AKU subjects' PS and PB data at baseline and then after a longer period of treatment with NTBC. Protein synthesis and breakdown has not been studied before in AKU and this study is a first step in addressing the gap in the scientific evidence in this important area.

Given the lack of previous similar studies in AKU, other examples of chronic disease were sought for comparison. Engelen et al (Engelen et al., 2000) found increased PS and PB in patients with severe and stable COPD. This is

similar to findings in other chronic diseases such as HIV (Macallan et al., 1995) and cancer (Jeevanandam et al., 1984, Melville et al., 1990). However, in Rheumatoid arthritis there is an increased protein breakdown (Rall et al., 1996). Further studies are required to clarify the long-term impact of NTBC on protein turnover in AKU.

Study limitations: This 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. The study 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/m2. This heterogeneity could explain the wide distribution of data points observed in the different urinary compounds measured in the AKU on NTBC group (Figure 52). Moreover, the SD values for the enrichment of tyrosine stable isotopes were quite high in the AKU off NTBC group (Figure 55). 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.

When studying protein turnover, the four-week period between the two isotopes infusions for each AKU patients was too short for the results to be conclusive. Ideally, isotopes infusions should be done at baseline and then after six to twelve months of NTBC therapy to assess changes in protein turnover. This can be supplemented by an AKUSSI assessment to ensure that disease severity has not changed in the interim. Furthermore, diet was not controlled amongst study participants across the three groups. This means participants have had varied protein intake which would have affected protein turnover dynamics (Garlick et al., 1991). Of note, one patient was off NTBC when enrolled the other were already on it. This limits the study of NTBC on protein turnover as ideally patient should be off NTBC then re-assessed on NTBC. Realistically, this would have been unethical from research perspective and impractical to do as part of clinical service.

4.5 Chapter 4 conclusion

In summary, this study provides, for the first time, the experimental evidence for the extent of NTBC-related acquired tyrosinosis. The term ‘acquired tyrosinosis’ is proposed for use instead of ‘hypertyrosinaemia’ to reflect the magnitude of tyrosine increases with NTBC use.

NTBC appears to reduce phenylalanine hydroxylation to tyrosine but has no impact on protein turnover in AKU in the short term. No firm conclusion can be drawn regarding the effect of age, TBW, BW, BMI, FFM and FFM% on protein breakdown and synthesis in AKU patients treated with NTBC. Equally, phenylalanine hydroxylation to tyrosine does not seem to be affected by these factors too. However, further studies are required to establish long term effects within the context of protein restricted diet.

Further research is required to assess its wider impact on organs 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. Having a national centre to look after this cohort of patients represents the ideal environment for tracking long term side effects of NTBC therapy while close collaboration with academic institutions, pharmaceutical companies and patient societies paves the way forward for resolving potential challenges related to complications from NTBC therapy.

Acknowledgements

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5.0 CONCLUDING REMARKS

This thesis described the investigations of the effects of NTBC on the tyrosine degradation pathway in AKU affected mice and human. For the first time in AKU, stable isotopes were deployed to enable further understanding of NTBC on the tyrosine pool size.

Post NTBC hypertyrosinaemia is expected and has been extensively described previously. NTBC reduces the urinary excretion of HGA and increases the excretion of tyrosine, HPPA and HPLA without overall change in urinary metabolites. Surprisingly, there was a significant dose dependent increase in serum tyrosine, HPPA and HPLA (Milan et al., 2019). It is speculated that the tyrosine increase is due to the NTBC diverting the tyrosine away from the benzoquinone acetic acid (BQA) pathway (figure1). One way to confirm this hypothesis is to measure the concentrations of BQA in AKU patients before and after NTBC. Unfortunately, there is no commercially available assay to measure BQA and it would be very challenging to establish one as it is a very unstable compound that get oxidised quickly. An alternative way to accurately quantify the magnitude of tyrosine changes post-NTBC would be the direct measurement of tyrosine and related metabolites.

Using phenylalanine and tyrosine stable isotopes, this study has investigated the tyrosine degradation pathway in AKU patients before and after NTBC therapy. This has provided direct evidence for changes in tyrosine metabolites with NTBC use. The robustness of these findings is further strengthened by the inclusion of a healthy control group for further comparison.

As isotopes studies were not done in AKU before, it was important to answer two important questions. The first question was which tyrosine and phenylalanine isotopes to use. The second one was whether to use pulse or continuous infusion approach. The choice of stable isotopes had to consider the compatibility with analytical method for measuring these tracers. Hughes et al. (2014) have developed an LC-MSMS method for measurement of urinary HGA and native tyrosine and phenylalanine. They also developed an LC-MSMS method for the measurement of serum HGA, NTBC, native tyrosine and phenylalanine (Hughes et al., 2015). Therefore, it made sense to use the same analytical platform and modify the existing methods to measure both phenylalanine and tyrosine tracers used in the study. The use of internal standards in the LC-MSMS assay in reducing errors arising from ion suppression or enhancement. The choice of tyrosine and phenylalanine tracers for this study had to take into consideration the internal standards used in the two methods used to measure native compounds. The isotopes used as internal standards were L-[d_4]Tyrosine (after initially using L-[d_2]tyrosine),L-[d_5]phenylalanine and L-[$^{13}C_6$]HGA (Hughes, 2021). It has also dictated the choice of internal standards for the assay of tyrosine and phenylalanine stable isotopes. One crucial factor was the stability. The loss deuterium in internal standards in LC-MSMS assay is well recognised (Davison et al., 2013) and for this reason it was decided to use L-[ring-$^{13}C_6$]tyrosine (99%) and L-[ring-$^{13}C_6$]phenylalanine (99%) as internal standards for the tracers assay.

The second question was whether to use pulse or continuous infusion approach. Historically, studies deployed tyrosine and phenylalanine tracers to

develop further understanding of protein metabolism, hydroxylation of phenylalanine, establishing the amino acids requirements in health and disease state. In contrast, this study intended to use these tracers for a completely different objective- to assess tyrosine pool size before and after NTBC. The continuous infusion approach to achieve a steady state of equilibrium/ steady state. Recent work have demonstrated that pulse approach is equally valid, reliable, less expensive, and more convenient to patients (Mason et al., 2017).

Previous LC-MSMS methods were labour intensive and required some form of extraction. It was possible to modify the existing methods and adopted for the measurements of tyrosine and phenylalanine stable isotopes chosen for this study. Namely, L-[d_7]tyrosine and L-[d_8]phenylalanine and L-[$^{13}C_9$]tyrosine. Using a simple ”dilute and shoot” approach, it was possible to carry measurement of these tracers in both serum and urine of mice and human.

Analytical study: Previous LC-MSMS methods for the measurement of tyrosine and phenylalanine stable isotopes were labour intensive and required some form of extraction. In this study, it was possible to modify the existing LC-MSMS methods for the measurements of tyrosine and phenylalanine stable isotopes chosen for this study. Namely, L-[d_7]tyrosine and L-[d_8]phenylalanine and L-[$^{13}C_9$]tyrosine. Using a simple” dilute and shoot” approach, it was possible to measure these tracers in murine tissue homogenates as well as serum and urine of mice and human. This new LC-MS/MS method proves that measurement of phenylalanine and tyrosine

isotopes can be made simpler and easier, and it should pave the way for a more emphatic role of LC-MC/MC in stable isotopes methodology.

Mice study: This study has demonstrated the wider impact of HPPD blockade and provided, for the first-time tissue level data as well as the experimental evidence for tyrosinosis. The term tyrosinosis is more accurate and reflective of the scale of tyrosine increase not just in blood but also in tissues. It has also demonstrated that brain tyrosinosis causes reduced phenylalanine levels. Availability of amino acids in the brain is controlled by the Large neutral amino acids transporter (LAT) which is expressed at the brain capillaries at the blood brain barrier (BBB) (Boado et al., 1999). The reduction in phenylalanine concentrations is likely due to the tyrosine dominating the competitive transport across BBB in NTBC treated mice. NTBC caused an almost 8-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 hypertyrosinaemia 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., 2019c).

Human study: This study provided, for the first time, the experimental evidence for increase in tyrosine pool size post NTBC therapy in AKU patients. The flux of compounds across the tyrosine degradation pathway has also been examined and showed massive increase in the amount of these compounds post TNBC. These findings are consistent with previous studies (Milan et al., 2019, Ranganath et al., 2022a).

This study found that of NTBC on protein turnover in AKU is not affected by NTBC use. This is surprising as AKU is a chronic multisystem disease and leads to significant morbidity due to aggressive arthropathy in later life. One would expect increased protein turnover. However, a longer study duration would have been required to assess this further. It would have been interesting to compare AKU subjects' PS and PB data at baseline and then after a longer period of treatment with NTBC. Protein synthesis and breakdown has not been studied before in AKU and this study is a first step in addressing the gap in the scientific evidence in this important area.

The estimation of phenylalanine hydroxylation to tyrosine in the healthy volunteers in this study is consistent with the literature (Clarke and Bier, 1982, Thompson et al., 1989, Cortiella et al., 1992). This is an added strengthen to the comparison of phenylalanine hydroxylation to tyrosine in AKU patients. Furthermore, it is a further validation for the use of stable isotopes pulse technique in studying human metabolism. Previous studies have mostly deployed continuous infusions. This pulse method is cheaper, less labour intensive, and more convenient for study participants.

Since 1932, the term 'tyrosinosis' has been used to described various metabolic disorders in which elevated blood tyrosine is a common feature

(Medes, 1932, Halvorsen et al., 1966, Gentz et al., 1967, Halvorsen, 1967, Kogut et al., 1967, Fairney et al., 1968, Wadman et al., 1968, Harries et al., 1969, Pickering and Bower, 1972, Zaleski and Hill, 1973). After establishing the biochemical and genetic basis, the term was dropped in favour of tyrosinaemia (HT-1, HT-2, HT-3)(Scott, 2006, Chen, 2017).

The findings of this study make a strong case for the use of the term ‘tyrosinosis’ in the context of NTBC therapy to emphasise the fact that increases in tyrosine are not restricted to the blood and that it extends to the tissues too. It could be argued that the term "tyrosinosis" might be misleading because it was originally linked to inherited metabolic conditions, focusing on their genetic component. Therefore, a more precise term would be “acquired tyrosinosis” which will help retain the emphasis on tissue tyrosine increase while ensuring that the reader is very clear that this is not an inherited/genetic metabolic condition.

Future work: over the last decade, NTBC has been established as a disease modifying therapy for AKU in adults. Having a national centre to provide care for adult AKU patients has been invaluable in long term monitoring of disease and consequences of therapy (Ranganath et al., 2021a, Davison et al., 2022a, Ahmad et al., 2022). Future research should focus on establishing adjuvant interventions to reduce hypertyrosinaemia. In current clinical practice, protein restriction is deployed as tool to limit tyrosinaemia with the level of protein restriction is tightened in patients with higher tyrosine concentrations (Ranganath et al., 2022d). On a practical level, this can be very challenging for adult patients who habitually are used to unrestricted protein consumption

for at least two decades of their life. Similar challenges in complying with long term dietary restriction have been reported in patients with phenylketonuria (PKU) (Walter et al., 2002, Jurecki et al., 2017).

Recombinant phenylalanine ammonia lyase (PAL) catalyses the conversion of phenylalanine to ammonia and trans-cinnamic acid and has been approved for the use in PKU (FDA, 2018b, EMA, 2019). Work has been done on utilising Tyrosine ammonia-lyase (TAL, EC 4.3.1.23) that convert of L-Tyr into p-coumaric acid (Hendrikse et al., 2020). If approved, TAL could relieve AKU patients from the protein restriction.

Since the biochemical defect is present since birth, it makes sense to start the treatment earlier in life. In the context of on a non-lethal condition (i.e., AKU), there is a trade-off between exposing children to risks of NTBC induced tyrosinaemia and the subclinical ochronosis. One particular concern is the neurological effects of tyrosinaemia on the growing brain. Specifically, the effect of large amino acids transport across blood-brain barrier. Further understanding of long-term consequences of tyrosinaemia in children is required to enable safe use of NTBC. The ongoing paediatric SOFIA study should provide the evidence for early ochronosis in children and help decide on the appropriate age threshold for starting NTBC.

Gene therapy: FDA has recently approved gene therapy for adults with haemophilia B (FDA, 2022).Studies are carried out using gene therapy in inherited metabolic diseases such as Fabry (Therapeutics, 2019). Work is under way to investigate the possibility of gene therapy as a cure in AKU. Funding and developing the right technology are some of the challenges that

need to be overcome. In the meantime, NTBC will remain relatively cheap and safe disease modifying therapy for AKU.

6.0 REFERENCES:

Abumrad, N. N., Rabin, D., Diamond, M. P. & Lacy, W. W. 1981. Use of a heated superficial hand vein as an alternative site for the measurement of amino acid concentrations and for the study of glucose and alanine kinetics in man. Metabolism: Clinical and Experimental, 30, 936-940.

Ahmad, M. S. Z., Ahmed, M., Khedr, M., Borgia, A., Madden, A., Ranganath, L. R. & Kaye, S. 2022. Association of alkaptonuria and low dose nitisinone therapy with cataract formation in a large cohort of patients. JIMD Rep, 63, 351-360. doi: 10.1002/jmd2.12288.

Al-Sbou, M. & Mwafi, N. 2012. Nine cases of Alkaptonuria in one family in southern Jordan. Rheumatology International, 32, 621-625.

Al-Sbou, M., Mwafi, N. & Lubad, M. A. 2012. Identification of forty cases with alkaptonuria in one village in Jordan. Rheumatology International, 32, 3737-3740.

Al-Shagahin, H., Mwafi, N., Khasawneh, M., Al Zubi, K. & Alsbou, M. 2019. Ear, nose, and throat manifestations of alkaptonuria patients from Jordan. Indian Journal of Otology, 25, 109-113.

Alajoulin, O. A., Alsbou, M. S., Ja'afreh, S. O. & Kalbouneh, H. M. 2015. Spontaneous Achilles tendon rupture in alkaptonuria. Saudi Med J, 36, 1486-9.

Ascher, D. B., Spiga, O., Sekelska, M., Pires, D. E. V., Bernini, A., Tiezzi, M., Kralovicova, J., Borovska, I., Soltysova, A., Olsson, B., Galderisi, S., Cicaloni, V., Ranganath, L., Santucci, A. & Zatkova, A. 2019. Homogentisate 1,2-dioxygenase (HGD) gene variants, their analysis and genotype–phenotype correlations in the largest cohort of patients with AKU. European Journal of Human Genetics.

Avadhanula, S., Introne, W. J., Auh, S., Soldin, S. J., Stolze, B., Regier, D., Ciccone, C., Hannah-Shmouni, F., Filie, A. C., Burman, K. D. & Klubo-Gwiezdzinska, J. 2020. Assessment of Thyroid Function in Patients With Alkaptonuria. JAMA Netw Open, 3, e201357.

Bachmanov, A. A., Reed, D. R., Beauchamp, G. K. & Tordoff, M. G. 2002. Food intake, water intake, and drinking spout side preference of 28 mouse strains. Behavior genetics, 32, 435-443.

Bailey, C., Kitts, W. & Wood, A. 1960. Changes in the gross chemical composition of the mouse during growth in relation to the assessment of physiological age. Canadian Journal of Animal Science, 40, 143-155.

Bendadi, F., de Koning, T. J., Visser, G., Prinsen, H. C. M. T., de Sain, M. G. M., Verhoeven-Duif, N., Sinnema, G., van Spronsen, F. J. & van Hasselt, P. M. 2014. Impaired Cognitive Functioning in Patients with Tyrosinaemia Type I Receiving Nitisinone. The Journal of Pediatrics, 164, 398-401.

Bernardini, G., Laschi, M., Geminiani, M., Braconi, D., Vannuccini, E., Lupetti, P., Manetti, F., Millucci, L. & Santucci, A. 2015. Homogentisate 1,2 dioxygenase is expressed in brain: implications in alkaptonuria. Journal of Inherited Metabolic Disease, 38, 807-814.

Beysen, C., Angel, T. E., Hellerstein, M. K. & Turner, S. M. 2019. Isotopic Tracers for the Measurement of Metabolic Flux Rates. In: KRENTZ, A. J., WEYER, C. & HOMPESCH, M. (eds.) Translational Research Methods in Diabetes, Obesity, and Nonalcoholic Fatty Liver Disease: A Focus on Early Phase Clinical Drug Development. Cham: Springer International Publishing.

Boado, R. J., Li, J. Y., Nagaya, M., Zhang, C. & Pardridge, W. M. 1999. Selective expression of the large neutral amino acid transporter at the blood-brain barrier. Proc Natl Acad Sci U S A, 96, 12079-84.

Boedeker, C. H. D. 1859. Über das alcapton: ein bietrag zur frage: welche stoffe des harns können kupferreduction bewirken? Zeitschrift für Rationelle Medicin, 7, 130-45.

Boirie, Y., Albright, R., Bigelow, M. & Nair, K. S. 2004. Impairment of phenylalanine conversion to tyrosine in end-stage renal disease causing tyrosine deficiency. Kidney Int, 66, 591-6.

Cant, H. E. O., Chatzidaki, I., Olsson, B., Rudebeck, M., Arnoux, J.-B., Imrich, R., Eddowes, L. A. & Ranganath, L. R. 2022. Improving the clinical accuracy and flexibility of the Alkaptonuria severity score index. JIMD Reports, 63, 361-370.

Castillo, L., Yu, Y. M., Marchini, J. S., Chapman, T. E., Sanchez, M., Young, V. R. & Burke, J. F. 1994. Phenylalanine and tyrosine kinetics in critically ill children with sepsis. Pediatr Res, 35, 580-8.

Chapman, M. E., Hu, L., Plato, C. F. & Kohan, D. E. 2010. Bioimpedance spectroscopy for the estimation of body fluid volumes in mice. Am J Physiol Renal Physiol, 299, F280-3.

Chen, H. 2017. Tyrosinaemias. Atlas of genetic diagnosis and counseling.

Chévez Barrios, P. & Font, R. L. 2004. Pigmented conjunctival lesions as initial manifestation of ochronosis. Arch Ophthalmol, 122, 1060-3.

CHMP. 2011. Guideline on bioanalytical method validation. Guideline on bioanalytical method validation [Online]. Available: https://www.ema.europa.eu/en/documents/scientific-guideline/guideline-bioanalytical-method-validation_en.pdf.

Clark, S. E., Karn, C. A., Ahlrichs, J. A., Wang, J., Leitch, C. A., Leichty, E. A. & Denne, S. C. 1997. Acute changes in leucine and phenylalanine kinetics produced by parenteral nutrition in premature infants. Pediatr Res, 41, 568-74.

Clarke, J. T. & Bier, D. M. 1982. The conversion of phenylalanine to tyrosine in man. Direct measurement by continuous intravenous tracer infusions of L-[ring-2H5]phenylalanine and L-[1-13C] tyrosine in the postabsorptive state. Metabolism: Clinical and Experimental, 31, 999-1005.

Cobelli, C. & Carson, E. 2019. Introduction to modeling in physiology and medicine, Academic Press.

Cobelli, C., Foster, D. & Toffolo, G. 2007. Tracer kinetics in biomedical research: from data to model, Springer Science & Business Media.

compendium, T. e. m. 2016. Orfadin 2mg hard capsule: Summaries of Product Characteristics.

Cortiella, J., Marchini, J. S., Branch, S., Chapman, T. E. & Young, V. R. 1992. Phenylalanine and tyrosine kinetics in relation to altered protein and phenylalanine and tyrosine intakes in healthy young men. The American Journal of Clinical Nutrition, 56, 517-525.

Cox, T., Psarelli, E. E., Taylor, S., Shepherd, H. R., Robinson, M., Barton, G., Mistry, A., Genovese, F., Braconi, D., Giustarini, D., Rossi, R., Santucci, A., Khedr, M., Hughes, A., Milan, A., Taylor, L. F., West, E., Sireau, N., Dillon, J. P., Rhodes, N., Gallagher, J. A. & Ranganath, L. 2019. Subclinical ochronosis features in alkaptonuria: a cross-sectional study. BMJ Innovations, 5, 82-91.

Cox, T. F. & Ranganath, L. 2011. A quantitative assessment of alkaptonuria: testing the reliability of two disease severity scoring systems. Journal of Inherited Metabolic Disease, 34, 1153-1162.

Curtis, S. L., Roberts, N. B. & Ranganath, L. R. 2014. Interferences of homogentisic acid (HGA) on routine clinical chemistry assays in serum and urine

and the implications for biochemical monitoring of patients with alkaptonuria. Clin Biochem, 47, 640-7.

Davison, A. & Norman, B. 2023. Alkaptonuria – Past, present and future. Advances in Clinical Chemistry,114:47-81. doi: 10.1016/bs.acc.2023.02.005. Epub 2023 Mar 28.

Davison, A. S., Harrold, J. A., Hughes, G., Norman, B. P., Devine, J., Usher, J., Hughes, A. T., Khedr, M., Gallagher, J. A., Milan, A. M., Halford, J C G & Ranganath, L. R. 2018a. Clinical and biochemical assessment of depressive symptoms in patients with Alkaptonuria before and after two years of treatment with nitisinone. Molecular Genetics and Metabolism, 125, 135-143.

Davison, A. S., Hughes, A., Milan, A. M., Sireau, N., Gallagher, J. & Ranganath, L. 2019a. ANNALS EXPRESS: Alkaptonuria - many questions answered, further challenges beckon. Ann Clin Biochem, 4563219879957.

Davison, A. S., Hughes, G., Harrold, J. A., Clarke, P., Griffin, R. & Ranganath, L. R. 2022a. Long-term low dose nitisinone therapy in adults with alkaptonuria shows no cognitive decline or increased severity of depression. JIMD Rep, 63, 221-230.

Davison, A. S., Luangrath, E., Selvi, E. & Ranganath, L. R. 2020. Fatal acute haemolysis and methaemoglobinaemia in a man with renal failure and Alkaptonuria – Is nitisinone the solution? Molecular Genetics and Metabolism Reports, 23, 100588.

Davison, A. S., Milan, A. M. & Dutton, J. J. 2013. Potential problems with using deuterated internal standards for liquid chromatography-tandem mass spectrometry. Annals of Clinical Biochemistry, 50, 274.

Davison, A. S., Milan, A. M., Gallagher, J. A. & Ranganath, L. R. 2016. Acute fatal metabolic complications in alkaptonuria. Journal of Inherited Metabolic Disease, 39, 203-210.

Davison, A. S., Norman, B. P., Smith, E. A., Devine, J., Usher, J., Hughes, A. T., Khedr, M., Milan, A. M., Gallagher, J. A. & Ranganath, L. R. 2018b. Serum Amino Acid Profiling in Patients with Alkaptonuria Before and After Treatment with Nitisinone. JIMD reports, 41, 109-117.

Davison, A. S., Norman, B. P., Sutherland, H., Milan, A. M., Gallagher, J. A., Jarvis, J. C. & Ranganath, L. R. 2022b. Impact of Nitisinone on the Cerebrospinal Fluid Metabolome of a Murine Model of Alkaptonuria. Metabolites, 12.

Davison, A. S., Strittmatter, N., Sutherland, H., Hughes, A. T., Hughes, J., Bou-Gharios, G., Milan, A. M., Goodwin, R. J. A., Ranganath, L. R. & Gallagher, J. A. 2019b. Assessing the effect of nitisinone induced hypertyrosinaemia on monoamine neurotransmitters in brain tissue from a murine model of alkaptonuria using mass spectrometry imaging. Metabolomics, 15, 68.

Davison, A. S., Strittmatter, N., Sutherland, H., Hughes, A. T., Hughes, J., Bou-Gharios, G., Milan, A. M., Goodwin, R. J. A., Ranganath, L. R. & Gallagher, J. A. 2019c. Assessing the effect of nitisinone induced hypertyrosinaemia on monoamine neurotransmitters in brain tissue from a murine model of alkaptonuria using mass spectrometry imaging. Metabolomics: Official Journal of the Metabolomic Society, 15, 68.

de Betue, C. T., van Waardenburg, D. A., Deutz, N. E., van Eijk, H. M., van Goudoever, J. B., Luiking, Y. C., Zimmermann, L. J. & Joosten, K. F. 2011. Increased protein-energy intake promotes anabolism in critically ill infants with viral bronchiolitis: a double-blind randomised controlled trial. Arch Dis Child, 96, 817-22.

Durbin, P. W., Jeung, N., Kullgren, B. & Clemons, G. K. 1992. Gross Composition and Plasma and Extracellular Water Volumes of Tissues of a Reference Mouse. Health Physics, 63, 427-442.

EMA. 2019. Palynziq [Online]. EMA. Available: https://www.ema.europa.eu/en/medicines/human/EPAR/palynziq [Accessed 1/1/2023 2023].

EMA. 2020. First treatment for rare metabolic disorder alkaptonuria [Online]. Available: https://www.ema.europa.eu/en/news/first-treatment-rare-metabolic-disorder-alkaptonuria [Accessed 1/07/2022 2022].

Endo, F., Katoh, H., Yamamoto, S. & Matsuda, I. 1991. A murine model for type III tyrosinaemia: lack of immunologically detectable 4-hydroxyphenylpyruvic acid dioxygenase enzyme protein in a novel mouse strain with hypertyrosinaemia. American journal of human genetics, 48, 704-709.

Engelen, M., Ten Have, G. A. M., Thaden, J. J. & Deutz, N. E. P. 2019. New advances in stable tracer methods to assess whole-body protein and amino acid metabolism. Curr Opin Clin Nutr Metab Care, 22, 337-346.

Engelen, M. P., Deutz, N. E., Mostert, R., Wouters, E. F. & Schols, A. M. 2003. Response of whole-body protein and urea turnover to exercise differs between

patients with chronic obstructive pulmonary disease with and without emphysema. Am J Clin Nutr, 77, 868-74.

Engelen, M. P., Deutz, N. E., Wouters, E. F. & Schols, A. M. 2000. Enhanced levels of whole-body protein turnover in patients with chronic obstructive pulmonary disease. American Journal of Respiratory and Critical Care Medicine, 162, 1488-1492.

Fairney, A., Francis, D., Ersser, R. S., Seakins, J. W. & Cottom, D. 1968. Diagnosis and treatment of tyrosinosis. Archives of disease in childhood, 43, 540-547.

FDA, U. 2018a. Bioanalytical method validation guidance for industry, US Department of Health and Human Services. Center for Drug Evaluation and Research (CDER), Center for Veterinary Medicine (CVM), Biopharmaceutics. Available from: https://www. fda. gov/downloads/drugs/guidances/ucm070107. Pdf [last accessed 20 Sep 2018).

FDA, U. 2018b. FDA approves a new treatment for PKU, a rare and serious genetic disease [Online]. Available: https://www.fda.gov/news-events/press-announcements/fda-approves-new-treatment-pku-rare-and-serious-genetic-disease [Accessed 1/1/2023 2023].

FDA, U. 2022. FDA Approves First Gene Therapy to Treat Adults with Hemophilia B [Online]. [Accessed 02/12/2022 2022].

Fernández-Cañón, J. M., Granadino, B., De Bernabé, D. B. V., Renedo, M., Fernández-Ruiz, E., Peñalva, M. A. & De Córdoba, S. R. 1996. The molecular basis of alkaptonuria. 14, 19-24.

Ferreira, G. K., Scaini, G., Carvalho-Silva, M., Gomes, L. M., Borges, L. S., Vieira, J. S., Constantino, L. S., Ferreira, G. C., Schuck, P. F. & Streck, E. L. 2013. Effect of L-tyrosine in vitro and in vivo on energy metabolism parameters in brain and liver of young rats. Neurotoxicity Research, 23, 327-335.

Fox, J. G., Barthold, S., Davisson, M., Newcomer, C. E., Quimby, F. W. & Smith, A. 2006. The Mouse in Biomedical Research: Normative Biology, Husbandry, and Models, Elsevier Science.

García, M. I., de la Parra, A., Arias, C., Arredondo, M. & Cabello, J. F. 2017. Long-term cognitive functioning in individuals with tyrosinaemia type 1 treated with nitisinone and protein-restricted diet. Molecular Genetics and Metabolism Reports, 11, 12-16.

Garlick, P. J., McNurlan, M. A. & Ballmer, P. E. 1991. Influence of dietary protein intake on whole-body protein turnover in humans. Diabetes Care, 14, 1189-98.

Garlick, P. J., McNurlan, M. A. & Caso, G. 1997. Critical Assessment of Methods Used to Measure Protein Synthesis In Human Subjects. The Yale Journal of Biology and Medicine, 70, 65-76.

Garrod, A. 1908. The Croonian Lectures ON INBORN ERRORS OF METABOLISM. The Lancet, 172, 1-7.

Gentz, J., Lindblad, B., Lindstedt, S., Levy, L., Shasteen, W. & Zetterstrom, R. 1967. Dietary treatment in tyrosinaemia (tyrosinosis): with a note on the possible recognition of the carrier state. American Journal of Diseases of Children, 113, 31-37.

Granados, J. Z., Ten Have, G. A. M., Letsinger, A. C., Thaden, J. J., Engelen, M., Lightfoot, J. T. & Deutz, N. E. P. 2020. Activated whole-body arginine pathway in high-active mice. PLoS One, 15, e0235095.

Grompe, M., Lindstedt, S., Al-Dhalimy, M., Kennaway, N. G., Papaconstantinou, J., Torres-Ramos, C. A., Ou, C.-N. & Finegold, M. 1995. Pharmacological correction of neonatal lethal hepatic dysfunction in a murine model of hereditary tyrosinaemia type I. 10, 453-460.

Halvorsen, S. 1967. Dietary Treatment of Tyrosinosis. JAMA Pediatrics, 113, 38-40.

Halvorsen, S., Pande, H., Loken, A. C. & Gjessing, L. R. 1966. Tyrosinosis. A study of 6 cases. Arch Dis Child, 41, 238-49.

Harper, P. S. & Bradley, D. M. 1978. ... AND IN WALES. The Lancet, 312, 576-577. doi.org/10.1016/S0140-6736(78)92911-2.

Harries, J. T., Seakins, J. W., Ersser, R. S. & Lloyd, J. K. 1969. Recovery after dietary treatment of an infant with features of tyrosinosis. Arch Dis Child, 44, 258-67.

Helliwell, T. R., Gallagher, J. A. & Ranganath, L. 2008. Alkaptonuria--a review of surgical and autopsy pathology. Histopathology, 53, 503-512.

Hendrikse, N. M., Holmberg Larsson, A., Svensson Gelius, S., Kuprin, S., Nordling, E. & Syrén, P.-O. 2020. Exploring the therapeutic potential of modern and ancestral phenylalanine/tyrosine ammonia-lyases as supplementary treatment of hereditary tyrosinaemia. Scientific Reports, 10, 1315.

Honour, J. W. 2011. Development and validation of a quantitative assay based on tandem mass spectrometry. Ann Clin Biochem, 48, 97-111.

Hughes, A. T. 2021. Development and validation of liquid chromatography mass spectrometry methods to study the effects of nitisinone in Alkaptonuria. PhD, University of Liverpool.

Hughes, A. T., Milan, A. M., Christensen, P., Ross, G., Davison, A. S., Gallagher, J. A., Dutton, J. J. & Ranganath, L. R. 2014. Urine homogentisic acid and tyrosine: simultaneous analysis by liquid chromatography tandem mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci, 963, 106-12.

Hughes, A. T., Milan, A. M., Davison, A. S., Christensen, P., Ross, G., Gallagher, J. A., Dutton, J. J. & Ranganath, L. R. 2015. Serum markers in alkaptonuria: simultaneous analysis of homogentisic acid, tyrosine and nitisinone by liquid chromatography tandem mass spectrometry. Annals of Clinical Biochemistry, 52, 597-605.

Hughes, A. T., Milan, A. M., Shweihdi, E., Gallagher, J. & Ranganath, L. 2022. Method development and validation for analysis of phenylalanine, 4-hydroxyphenyllactic acid and 4-hydroxyphenylpyruvic acid in serum and urine. JIMD Reports, 63, 341-350.

Hughes, J. H., Wilson, P. J. M., Sutherland, H., Judd, S., Hughes, A. T., Milan, A. M., Jarvis, J. C., Bou-Gharios, G., Ranganath, L. R. & Gallagher, J. A. 2020. Dietary restriction of tyrosine and phenylalanine lowers tyrosinaemia associated with nitisinone therapy of alkaptonuria. J Inherit Metab Dis, 43, 259-268.

Hyde, R., Taylor, P. M. & Hundal, H. S. 2003. Amino acid transporters: roles in amino acid sensing and signalling in animal cells. Biochem J, 373, 1-18.

Introne, W. J., Perry, M. B., Troendle, J., Tsilou, E., Kayser, M. A., Suwannarat, P., O'Brien, K. E., Bryant, J., Sachdev, V., Reynolds, J. C., Moylan, E., Bernardini, I. & Gahl, W. A. 2011. A 3-year randomized therapeutic trial of nitisinone in alkaptonuria. Molecular Genetics and Metabolism, 103, 307-314.

Introne WJ, P. M., Chen M. 2003 Alkaptonuria. In: ADAM MP, M. G., PAGON RA (ed.) GeneReviews® [Internet]. Seattle (WA): University of Washington.

James, W. P., Garlick, P. J., Sender, P. M. & Waterlow, J. C. 1976. Studies of amino acid and protein metabolism in normal man with L-[U-14C]tyrosine. Clinical Science and Molecular Medicine, 50, 525-532.

Jeevanandam, M., Lowry, S., Horowitz, G. & Brennan, M. 1984. Cancer cachexia and protein metabolism. The Lancet, 323, 1423-1426.

Jiang, L., Cao, L., Fang, J., Yu, X., Dai, X. & Miao, X. 2019. Ochronotic arthritis and ochronotic Achilles tendon rupture in alkaptonuria: A 6 years follow-up case report in China. Medicine (Baltimore), 98, e16837.

Jones, M. R., Kopple, J. D. & Swendseid, M. E. 1978. Phenylalanine metabolism in uremic and normal man. Kidney International, 14, 169-179.

Jones, P. J. & Leatherdale, S. T. 1991. Stable isotopes in clinical research: safety reaffirmed. Clin Sci (Lond), 80, 277-80.

Judd, S., Khedr, M., Milan, A. M., Davison, A. S., Hughes, A. T., Needham, A., Psarelli, E. E., Shenkin, A. & Ranganath, L. R. 2020. The nutritional status of people with alkaptonuria: An exploratory analysis suggests a protein/energy dilemma. JIMD Reports, 53, 45-60.

Jurecki, E. R., Cederbaum, S., Kopesky, J., Perry, K., Rohr, F., Sanchez-Valle, A., Viau, K. S., Sheinin, M. Y. & Cohen-Pfeffer, J. L. 2017. Adherence to clinic recommendations among patients with phenylketonuria in the United States. Mol Genet Metab, 120, 190-197.

Keenan, C. M., Preston, A. J., Sutherland, H., Wilson, P. J., Psarelli, E. E., Cox, T. F., Ranganath, L. R., Jarvis, J. C. & Gallagher, J. A. 2015. Nitisinone Arrests but Does Not Reverse Ochronosis in Alkaptonuric Mice. JIMD Reports, 24, 45-50.

Khedr, M., Cooper, M. S., Hughes, A. T., Milan, A. M., Davison, A. S., Norman, B. P., Sutherland, H., Jarvis, J. C., Fitzgerald, R., Markinson, L., Psarelli, E.-E., Ghane, P., Deutz, N. E. P., Gallagher, J. A. & Ranganath, L. R. 2020. Nitisinone causes acquired tyrosinosis in alkaptonuria. Journal of Inherited Metabolic Disease, 43, 1014-1023.

Khedr, M., Judd, S., Briggs, M. C., Hughes, A. T., Milan, A. M., Stewart, R. M. K., Lock, E. A., Gallagher, J. A. & Ranganath, L. R. 2018. Asymptomatic Corneal Keratopathy Secondary to Hypertyrosinaemia Following Low Dose Nitisinone and a Literature Review of Tyrosine Keratopathy in Alkaptonuria. JIMD reports, 40, 31-37.

Kilani, R. A., Cole, F. S. & Bier, D. M. 1995. Phenylalanine hydroxylase activity in preterm infants: is tyrosine a conditionally essential amino acid? The American journal of clinical nutrition, 61, 1218-1223.

Kogut, M. D., Shaw, K. N. & Donnell, G. N. 1967. Tyrosinosis. American Journal of Diseases of Children, 113, 47-53.

Koletzko, B., Demmelmair, H., Hartl, W., Kindermann, A., Koletzko, S., Sauerwald, T. & Szitanyi, P. 1998. The use of stable isotope techniques for nutritional and metabolic research in paediatrics. Early Hum Dev, 53 Suppl, S77-97.

Koletzko, B., Sauerwald, T. & Demmelmair, H. 1997. Safety of stable isotope use. Eur J Pediatr, 156 Suppl 1, S12-7.

Korou, L. M., Doulamis, I. P., Tzanetakou, I. P., Mikhailidis, D. P. & Perrea, D. N. 2013. The effect of biological age on the metabolic responsiveness of mice fed a high-fat diet. Lab Anim, 47, 241-4.

Krempf, M., Hoerr, R. A., Marks, L. & Young, V. R. 1990. Phenylalanine flux in adult men: estimates with different tracers and route of administration. Metabolism, 39, 560-2.

Lei, Y., Han, H., Yuan, F., Javeed, A. & Zhao, Y. 2017. The brain interstitial system: Anatomy, modeling, in vivo measurement, and applications. Progress in Neurobiology, 157, 230-246.

Lewis, R. 2018. Consequences and Prevention of Elevated Circulating Tyrosine during Nitisinone Therapy in Alkaptonuria. PhD Doctoral, Liverpool John Moores University.

Lindner, M. & Bertelmann, T. 2014. On the ocular findings in ochronosis: a systematic review of literature. BMC Ophthalmology, 14, 12.

Lindstedt, S., Holme, E., Lock, E. A., Hjalmarson, O. & Strandvik, B. 1992. Treatment of hereditary tyrosinaemia type I by inhibition of 4-hydroxyphenylpyruvate dioxygenase. Lancet (London, England), 340, 813-817.

Liu, W. & Prayson, R. A. 2001. Dura mater involvement in ochronosis (alkaptonuria). Archives of pathology & laboratory medicine, 125, 961-963.

Lock, E. A., Ellis, M. K., Gaskin, P., Robinson, M., Auton, T. R., Provan, W. M., Smith, L. L., Prisbylla, M. P., Mutter, L. C. & Lee, D. L. 1998. From toxicological problem to therapeutic use: the discovery of the mode of action of 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione (NTBC), its toxicology and development as a drug. J Inherit Metab Dis, 21, 498-506.

Lock, E. A., Gaskin, P., Ellis, M. K., McLean Provan, W., Robinson, M. & Smith, L. L. 2000. Tissue distribution of 2-(2-nitro-4-trifluoromethylbenzoyl)-cyclohexane-1,3-dione (NTBC) and its effect on enzymes involved in tyrosine catabolism in the mouse. Toxicology, 144, 179-187.

Macallan, D. C., McNurlan, M. A., Milne, E., Calder, A. G., Garlick, P. J. & Griffin, G. E. 1995. Whole-body protein turnover from leucine kinetics and the response to nutrition in human immunodeficiency virus infection. The American Journal of Clinical Nutrition, 61, 818-826.

Macsai, M. S., Schwartz, T. L., Hinkle, D., Hummel, M. B., Mulhern, M. G. & Rootman, D. 2001. Tyrosinaemia type II: nine cases of ocular signs and symptoms. Am J Ophthalmol, 132, 522-7.

Madsen, B. W., Everett, A. W., Sparrow, M. P. & Fowkes, N. D. 1977. Linear kinetic model to estimate protein synthesis rate after [14C]tyrosine infusion in dogs. FEBS Letters, 79, 313-316.

Manoj Kumar, R. & Rajasekaran, S. 2003. Spontaneous tendon ruptures in alkaptonuria. The Journal of bone and joint surgery. British volume, 85, 883-886.

Marchini, J. S., Castillo, L., Chapman, T. E., Vogt, J. A., Ajami, A. & Young, V. R. 1993. Phenylalanine conversion to tyrosine: Comparative determination with l-[ring-2H5]phenylalanine and l-[1-13C]phenylalanine as tracers in man. Metabolism, 42, 1316-1322.

Mason, A., Engelen, M. P. K. J., Ivanov, I., Toffolo, G. M. & Deutz, N. E. P. 2017. A four-compartment compartmental model to assess net whole body protein breakdown using a pulse of phenylalanine and tyrosine stable isotopes in humans. American Journal of Physiology. Endocrinology and Metabolism, 313, E63-E74.

Masoud, H. M. F., Alhawari, H. H., Alryalat, N. T., Murshidi, M. M. & Murshidi, M. M. 2017. A rare presentation of alkaptonuria: Extensive prostatic calculi with highlight of stones found in a unique paraprostatic urethral diverticulum. International Journal of Surgery Case Reports, 38, 192-195.

Matalon, R., Matthews, D. E., Michals, K. & Bier, D. 1982. The use of deuterated phenylalanine for the in vivo assay of phenylalanine hydroxylase activity in children. J Inherit Metab Dis, 5, 17-9.

Matthews, D. E. 2016. Heated-Hand Boxes for Obtaining "Arterialized"-Venous Blood [Online]. Available: https://www.uvm.edu/~dmatthew/heated_hand.html [Accessed 29/4/2023 2023].

McKiernan, P. J. 2006. Nitisinone in the treatment of hereditary tyrosinaemia type 1. Drugs, 66, 743-750.

McKiernan, P. J. 2013. Nitisinone for the treatment of hereditary tyrosinaemia type I. Expert Opinion on Orphan Drugs, 1, 491-497.

Medes, G. 1932. A new error of tyrosine metabolism: tyrosinosis. The intermediary metabolism of tyrosine and phenylalanine. The Biochemical journal, 26, 917-940.

Meesters, R. J. W., Wolfe, R. R. & Deutz, N. E. P. 2009. Application of liquid chromatography-tandem mass spectrometry (LC-MS/MS) for the analysis of stable isotope enrichments of phenylalanine and tyrosine. Journal of chromatography. B, Analytical technologies in the biomedical and life sciences, 877, 43-49.

Meissner, T., Betz, R. C., Pasternack, S. M., Eigelshoven, S., Ruzicka, T., Kruse, R., Laitenberger, G. & Mayatepek, E. 2008. Richner-Hanhart syndrome detected by expanded newborn screening. Pediatric Dermatology, 25, 378-380.

Melville, S., McNurlan, M. A., Calder, A. G. & Garlick, P. J. 1990. Increased protein turnover despite normal energy metabolism and responses to feeding in patients with lung cancer. Cancer Research, 50, 1125-1131.

Milan, A. M., Hughes, A. T., Davison, A. S., Devine, J., Usher, J., Curtis, S., Khedr, M., Gallagher, J. A. & Ranganath, L. R. 2017. The effect of nitisinone on homogentisic acid and tyrosine: a two-year survey of patients attending the National Alkaptonuria Centre, Liverpool. Annals of Clinical Biochemistry, 54, 323-330.

Milan, A. M., Hughes, A. T., Davison, A. S., Khedr, M., Rovensky, J., Psarelli, E. E., Cox, T. F., Rhodes, N. P., Gallagher, J. A. & Ranganath, L. R. 2019. Quantification of the flux of tyrosine pathway metabolites during nitisinone treatment of Alkaptonuria. Scientific Reports, 9, 10024.

Milch, R. A. 1960. Studies of Alcaptonuria: Inheritance of 47 Cases in Eight Highly Inter-related Dominican Kindreds. American journal of human genetics, 12, 76-85.

Millucci, L., Braconi, D., Bernardini, G., Lupetti, P., Rovensky, J., Ranganath, L. & Santucci, A. 2015. Amyloidosis in alkaptonuria. Journal of Inherited Metabolic Disease, 38, 797-805.

Millucci, L., Spreafico, A., Tinti, L., Braconi, D., Ghezzi, L., Paccagnini, E., Bernardini, G., Amato, L., Laschi, M., Selvi, E., Galeazzi, M., Mannoni, A., Benucci, M., Lupetti, P., Chellini, F., Orlandini, M. & Santucci, A. 2012. Alkaptonuria is a novel human secondary amyloidogenic disease. Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease, 1822, 1682-1691.

Mohiuddin., A. T. B. D. B. S. S. 2022. Physiology, Water Balance. Treasure Island (FL):.

Møller, N., Meek, S., Bigelow, M., Andrews, J. & Nair, K. S. 2000. The kidney is an important site for in vivo phenylalanine-to-tyrosine conversion in adult humans: A metabolic role of the kidney. Proc Natl Acad Sci U S A, 97, 1242-6.

Morais, J. A., Chevalier, S. & Gougeon, R. 2006. Protein turnover and requirements in the healthy and frail elderly. The journal of nutrition, health & aging, 10 4, 272-83.

Moran, A., Milla, C., Ducret, R. & Nair, K. S. 2001. Protein metabolism in clinically stable adult cystic fibrosis patients with abnormal glucose tolerance. Diabetes, 50, 1336-43.

Motulsky, H. J. n.d.-a. "Advice: Don't automate the decision to use a nonparametric test", GraphPad Statistics Guide [Online]. Available: https://www.graphpad.com/guides/prism/8/statistics/using_a_normality_test_to_choo.htm [Accessed 16 May 2020].

Motulsky, H. J. n.d.-b. "Advice: When to choose a nonparametric test", GraphPad Statistics Guide [Online]. Available: https://www.graphpad.com/guides/prism/8/statistics/when_to_choose_a_nonparametric.htm?q=parametric [Accessed 7 March 2020].

O'Brien, W. M., La Du, B. N. & Bunim, J. J. 1963. Biochemical, pathologic and clinical aspects of alcaptonuria, ochronosis and ochronotic arthropathy: Review of world literature (1584-1962). The American Journal of Medicine, 34, 813-838.

Pannemans, D. L., Halliday, D., Westerterp, K. R. & Kester, A. D. 1995. Effect of variable protein intake on whole-body protein turnover in young men and women. The American Journal of Clinical Nutrition, 61, 69-74.

Paoli, A. 2014. Ketogenic Diet for Obesity: Friend or Foe? International Journal of Environmental Research and Public Health, 11, 2092-2107.

Pettit, S. J., Fisher, M., Gallagher, J. A. & Ranganath, L. R. 2011. Cardiovascular manifestations of Alkaptonuria. Journal of Inherited Metabolic Disease, 34, 1177-1181.

Phornphutkul, C., Introne, W. J., Perry, M. B., Bernardini, I., Murphey, M. D., Fitzpatrick, D. L., Anderson, P. D., Huizing, M., Anikster, Y., Gerber, L. H. & Gahl, W. A. 2002. Natural history of alkaptonuria. The New England Journal of Medicine, 347, 2111-2121.

Pickering, D. & Bower, B. 1972. Temporary tyrosinosis. Arch Dis Child, 47, 151.

Pollak, M. R., Wu Chou, Y.-H., Cerda, J. J., Steinmann, B., La Du, B. N., Seidman, J. G. & Seidman, C. E. 1993. Homozygosity mapping of the gene for alkaptonuria to chromosome 3q2. Nature Genetics, 5, 201-204.

Pons, G. & Rey, E. 1999. Stable isotopes labeling of drugs in pediatric clinical pharmacology. Pediatrics, 104, 633-9.

Preston, A. J., Keenan, C. M., Sutherland, H., Wilson, P. J., Wlodarski, B., Taylor, A. M., Williams, D. P., Ranganath, L. R., Gallagher, J. A. & Jarvis, J. C. 2014. Ochronotic osteoarthropathy in a mouse model of alkaptonuria, and its inhibition by nitisinone. Annals of the Rheumatic Diseases, 73, 284-289.

Rall, L. C., Rosen, C. J., Dolnikowski, G., Hartman, W. J., Lundgren, N., Abad, L. W., Dinarello, C. A. & Roubenoff, R. 1996. Protein metabolism in rheumatoid arthritis and aging. Effects of muscle strength training and tumor necrosis factor alpha. Arthritis and Rheumatism, 39, 1115-1124.

Ranganath, L., Khedr, M., Evans, L. A., Bygott, H., Luangrath, E. & West, E. 2021a. Vitiligo, alkaptonuria, and nitisinone-A report of three families and review of the literature. JIMD Rep, 61, 25-33.

Ranganath, L., Khedr, M., Milan, A. M., Davison, A. S., Norman, B. P., Janssen, M. C. H., Lock, E., Bou-Gharios, G. & Gallagher, J. A. 2023. Increased prevalence of Parkinson's disease in alkaptonuria. JIMD Reports, 11;64(4):282-292. doi: 10.1002/jmd2.12367.

Ranganath, L. R. & Cox, T. F. 2011. Natural history of alkaptonuria revisited: analyses based on scoring systems. J Inherit Metab Dis, 34, 1141-51.

Ranganath, L. R., Gallagher, J. A., Davidson, J. & Vinjamuri, S. 2021b. Characterising the arthroplasty in spondyloarthropathy in a large cohort of eighty-seven patients with alkaptonuria. Journal of Inherited Metabolic Disease, 44, 656-665.

Ranganath, L. R., Heseltine, T., Khedr, M. & Fisher, M. F. 2021c. Evaluating the aortic stenosis phenotype before and after the effect of homogentisic acid lowering therapy: Analysis of a large cohort of eighty-one alkaptonuria patients. Molecular Genetics and Metabolism, 133, 324-331.

Ranganath, L. R., Hughes, A. T., Davison, A. S., Khedr, M., Imrich, R., Rudebeck, M., Olsson, B., Norman, B. P., Bou-Gharios, G., Gallagher, J. A. & Milan, A. M. 2022a. Revisiting Quantification of Phenylalanine/Tyrosine Flux in the Ochronotic Pathway during Long-Term Nitisinone Treatment of Alkaptonuria. Metabolites, 29;12(10):920. doi: 10.3390/metabo12100920.

Ranganath, L. R., Hughes, A. T., Davison, A. S., Khedr, M., Olsson, B., Rudebeck, M., Imrich, R., Norman, B. P., Bou-Gharios, G., Gallagher, J. A. & Milan, A. M. 2022b. Temporal adaptations in the phenylalanine/tyrosine pathway and related factors during nitisinone-induced tyrosinaemia in alkaptonuria. Mol Genet Metab, 1:S1096-7192(22)00325-0. doi: 10.1016/j.ymgme.2022.05.006. Epub ahead of print.

Ranganath, L. R., Jarvis, J. C. & Gallagher, J. A. 2013. Recent advances in management of alkaptonuria (invited review; best practice article). Journal of Clinical Pathology, 66, 367-373.

Ranganath, L. R., Khedr, M., Milan, A. M., Davison, A. S., Hughes, A. T., Usher, J. L., Taylor, S., Loftus, N., Daroszewska, A., West, E., Jones, A., Briggs, M., Fisher, M., McCormick, M., Judd, S., Vinjamuri, S., Griffin, R., Psarelli, E. E., Cox, T. F., Sireau, N., Dillon, J. P., Devine, J. M., Hughes, G., Harrold, J., Barton, G. J., Jarvis, J. C. & Gallagher, J. A. 2018. Nitisinone arrests ochronosis and decreases rate of progression of Alkaptonuria: Evaluation of the effect of nitisinone in the United Kingdom National Alkaptonuria Centre. Molecular Genetics and Metabolism, 125, 127-134.

Ranganath, L. R., Khedr, M., Vinjamuri, S. & Gallagher, J. A. 2020a. Frequency, diagnosis, pathogenesis, and management of osteoporosis in alkaptonuria: data analysis from the UK National Alkaptonuria Centre. Osteoporosis International, ;32(5):927-938. doi: 10.1007/s00198-020-05671-y. Epub 2020 Oct 29.

Ranganath, L. R., Khedr, M., Vinjamuri, S. & Gallagher, J. A. 2021d. Characterizing the alkaptonuria joint and spine phenotype and assessing the effect of homogentisic acid lowering therapy in a large cohort of 87 patients. J Inherit Metab Dis, 44, 666-676.

Ranganath, L. R., Milan, A. M., Hughes, A. T., Davison, A. S., Khedr, M., Imrich, R., Rudebeck, M., Olsson, B., Norman, B. P., Bou-Gharios, G. & Gallagher, J. A. 2022c. Comparing the Phenylalanine/Tyrosine Pathway and Related Factors between Keratopathy and No-Keratopathy Groups as Well as between Genders in Alkaptonuria during Nitisinone Treatment. Metabolites, 12, 772.

Ranganath, L. R., Milan, A. M., Hughes, A. T., Davison, A. S., M, K., Norman, B. P., Bou-Gharios, G., Gallagher, J. A., Imrich, R., Arnoux, J. B., Rudebeck, M. & Olsson, B. 2022d. Determinants of tyrosinaemia during nitisinone therapy in alkaptonuria. Scientific Reports, 12, 16083.

Ranganath, L. R., Milan, A. M., Hughes, A. T., Dutton, J. J., Fitzgerald, R., Briggs, M. C., Bygott, H., Psarelli, E. E., Cox, T. F., Gallagher, J. A., Jarvis, J. C., van Kan, C., Hall, A. K., Laan, D., Olsson, B., Szamosi, J., Rudebeck, M., Kullenberg, T., Cronlund, A., Svensson, L., Junestrand, C., Ayoob, H., Timmis, O. G., Sireau, N., Le Quan Sang, K.-H., Genovese, F., Braconi, D., Santucci, A., Nemethova, M., Zatkova, A., McCaffrey, J., Christensen, P., Ross, G., Imrich, R. & Rovensky, J. 2016. Suitability Of Nitisinone In Alkaptonuria 1 (SONIA 1): an international, multicentre, randomised, open-label, no-treatment controlled, parallel-group, dose-response study to investigate the effect of once daily nitisinone on 24-h urinary homogentisic acid excretion in patients with alkaptonuria after 4 weeks of treatment. Annals of the Rheumatic Diseases, 75, 362-367.

Ranganath, L. R., Milan, A. M., Hughes, A. T., Khedr, M., Davison, A. S., Shweihdi, E., Norman, B. P., Hughes, J. H., Bygott, H., Luangrath, E., Fitzgerald, R., Psarelli, E. E., van Kan, C., Laan, D., Olsson, B., Rudebeck, M., Mankowitz, L., Sireau, N., Arnoux, J.-B., Le Quan Sang, K.-H., Jarvis, J. C., Genovese, F., Braconi, D., Santucci, A., Zatkova, A., Glasova, H., Stančík, R., Imrich, R., Rhodes, N. P. & Gallagher, J. A. 2020b. Homogentisic acid is not only eliminated by glomerular filtration and tubular secretion but also produced in the kidney in alkaptonuria. Journal of Inherited Metabolic Disease, 43, 737-747.

Ranganath, L. R., Milan, A. M., Hughes, A. T., Khedr, M., Davison, A. S., Wilson, P. J., Dillon, J. P., West, E. & Gallagher, J. A. 2020c. Reversal of ochronotic pigmentation in alkaptonuria following nitisinone therapy: Analysis of data from the United Kingdom National Alkaptonuria Centre. JIMD Rep, 55, 75-87.

Ranganath, L. R., Norman, B. P. & Gallagher, J. A. 2019. Ochronotic pigmentation is caused by homogentisic acid and is the key event in Alkaptonuria leading to the destructive consequences of the disease – a review. Journal of Inherited Metabolic Disease, 42(5):776-792. doi: 10.1002/jimd.12152. Epub 2019 Aug 5.

Ranganath, L. R., Psarelli, E. E., Arnoux, J. B., Braconi, D., Briggs, M., Broijersen, A., Loftus, N., Bygott, H., Cox, T. F., Davison, A. S., Dillon, J. P., Fisher, M., FitzGerald, R., Genovese, F., Glasova, H., Hall, A. K., Hughes, A. T., Hughes, J. H., Imrich, R., Jarvis, J. C., Khedr, M., Laan, D., Le Quan Sang, K. H., Luangrath, E., Lukacova, O., Milan, A. M., Mistry, A., Mlynarikova, V., Norman, B. P., Olsson, B., Rhodes, N. P., Rovensky, J., Rudebeck, M., Santucci, A., Shweihdi, E., Scott, C., Sedlakova, J., Sireau, N., Stancik, R., Szamosi, J., Taylor, S., van Kan, C., Vinjamuri, S., Vrtikova, E., Webb, C., West, E., Zanova, E., Zatkova, A. & Gallagher, J. A. 2020d. Efficacy and safety of once-daily nitisinone for patients with alkaptonuria (SONIA 2): an international, multicentre, open-label, randomised controlled trial. Lancet Diabetes Endocrinol, 8, 762-772.

Reinoso, R. F., Telfer, B. A. & Rowland, M. 1997. Tissue water content in rats measured by desiccation. Journal of Pharmacological and Toxicological Methods, 38, 87-92.

Robert, J. J., Bier, D., Schoeller, D., Wolfe, R., Matthews, D. E., Munro, H. N. & Young, V. R. 1984. Effects of intravenous glucose on whole body leucine dynamics, studied with 1-13C-leucine, in healthy young and elderly adults. J Gerontol, 39, 673-81.

Roberts, S. A., Ball, R. O., Filler, R. M., Moore, A. M. & Pencharz, P. B. 1998. Phenylalanine and Tyrosine Metabolism in Neonates Receiving Parenteral Nutrition Differing in Pattern of Amino Acids. Pediatric Research, 44, 907-914.

Roberts, S. A., Ball, R. O., Moore, A. M., Filler, R. M. & Pencharz, P. B. 2001. The effect of graded intake of glycyl-L-tyrosine on phenylalanine and tyrosine metabolism in parenterally fed neonates with an estimation of tyrosine requirement. Pediatr Res, 49, 111-9.

Roche 2010. Creatinine Jaffé Gen.2 [kit insert].Mannheim, Germany: Roche Diagnostics; 2010. 2010-01, V 10 English ed.: Roche Diagnostics.

Sakthivel, S., Zatkova, A., Nemethova, M., Surovy, M., Kadasi, L. & Saravanan, M. P. 2014. Mutation Screening of theHGDGene Identifies a Novel Alkaptonuria

Mutation with Significant Founder Effect and High Prevalence. Annals of Human Genetics, 78, 155-164.

Schellekens, R. C., Stellaard, F., Woerdenbag, H. J., Frijlink, H. W. & Kosterink, J. G. 2011. Applications of stable isotopes in clinical pharmacology. Br J Clin Pharmacol, 72, 879-97.

Scott, C. R. 2006. The genetic tyrosinaemias. Am J Med Genet C Semin Med Genet, 142C, 121-6.

Sršeň, Š., Cisárik, F., Pásztor, L., Harmečko, L. & Opitz, J. M. 1978. Alkaptonuria in the Trenčín District of Czechoslovakia. American Journal of Medical Genetics, 2, 159-166.

Sršeň, Š. & Varga, F. 1978. Screening for alkaptonuria in the newborn in Slovakia. The Lancet, 312, 576.

Srsen, S., Vondrácek, J., Srsnová, K. & Svác, J. 1985. [Analysis of the life span of alkaptonuric patients]. Cas Lek Cesk, 124, 1288-91.

Stenn, F. F., Milgram, J. W., Lee, S. L., Weigand, R. J. & Veis, A. 1977. Biochemical identification of homogentisic acid pigment in an ochronotic egyptian mummy. Science, 197, 566-8.

Steven, R. A., Kinshuck, A. J., McCormick, M. S. & Ranganath, L. R. 2015. ENT manifestations of alkaptonuria: report on a case series. J Laryngol Otol, 129, 1004-8.

Stewart, R. M. K., Briggs, M. C., Jarvis, J. C., Gallagher, J. A. & Ranganath, L. 2014. Reversible keratopathy due to hypertyrosinaemia following intermittent low-dose nitisinone in alkaptonuria: a case report. JIMD reports, 17, 1-6.

Suwannarat, P., O'Brien, K., Perry, M. B., Sebring, N., Bernardini, I., Kaiser-Kupfer, M. I., Rubin, B. I., Tsilou, E., Gerber, L. H. & Gahl, W. A. 2005. Use of nitisinone in patients with alkaptonuria. Metabolism: Clinical and Experimental, 54, 719-728.

Tang, J. E., Manolakos, J. J., Kujbida, G. W., Lysecki, P. J., Moore, D. R. & Phillips, S. M. 2007. Minimal whey protein with carbohydrate stimulates muscle protein synthesis following resistance exercise in trained young men. Applied Physiology, Nutrition, and Metabolism = Physiologie Appliquee, Nutrition Et Metabolisme, 32, 1132-1138.

Taylor, A. M., Preston, A. J., Paulk, N. K., Sutherland, H., Keenan, C. M., Wilson, P. J. M., Wlodarski, B., Grompe, M., Ranganath, L. R., Gallagher, J. A. & Jarvis, J. C. 2012. Ochronosis in a murine model of alkaptonuria is synonymous to that in the human condition. Osteoarthritis and Cartilage, 20, 880-886.

Tessari, P., Deferrari, G., Robaudo, C., Vettore, M., Pastorino, N., Biasi, L. D. & Garibotto, G. 1999. Phenylalanine hydroxylation across the kidney in humans Rapid Communication. Kidney International, 56, 2168-2172.

Therapeutics, S. 2019. Dose-Ranging Study of ST-920, an AAV2/6 Human Alpha Galactosidase A Gene Therapy in Subjects With Fabry Disease [Online]. [Accessed 02/12/2022 2022].

Thimm, E., Richter-Werkle, R., Kamp, G., Molke, B., Herebian, D., Klee, D., Mayatepek, E. & Spiekerkoetter, U. 2012. Neurocognitive outcome in patients with hypertyrosinaemia type I after long-term treatment with NTBC. Journal of Inherited Metabolic Disease, 35, 263-268.

Thompson, G. N., Pacy, P. J., Merritt, H., Ford, G. C., Read, M. A., Cheng, K. N. & Halliday, D. 1989. Rapid measurement of whole body and forearm protein turnover using a [2H5]phenylalanine model. American Journal of Physiology-Endocrinology and Metabolism, 256, E631-E639.

Tuvdendorj, D., Chinkes, D. L., Bahadorani, J., Zhang, X.-j., Sheffield-Moore, M., Killewich, L. A. & Wolfe, R. R. 2014. Comparison of bolus injection and constant infusion methods for measuring muscle protein fractional synthesis rate in humans. Metabolism: Clinical and Experimental, 63, 1562-1567.

van Eijk, H. M., Rooyakkers, D. R., Soeters, P. B. & Deutz, N. E. 1999. Determination of amino acid isotope enrichment using liquid chromatography-mass spectrometry. Anal Biochem, 271, 8-17.

van Eijk, H. M., Suylen, D. P., Dejong, C. H., Luiking, Y. C. & Deutz, N. E. 2007. Measurement of amino acid isotope enrichment by liquid chromatography mass spectroscopy after derivatization with 9-fluorenylmethylchloroformate. J Chromatogr B Analyt Technol Biomed Life Sci, 856, 48-56.

van Ginkel, W. G., Jahja, R., Huijbregts, S. C. J., Daly, A., MacDonald, A., De Laet, C., Cassiman, D., Eyskens, F., Körver-Keularts, I. M. L. W., Goyens, P. J., McKiernan, P. J. & van Spronsen, F. J. 2016a. Neurocognitive outcome in tyrosinaemia type 1 patients compared to healthy controls. Orphanet Journal of Rare Diseases, 11, 87.

van Ginkel, W. G., Jahja, R., Huijbregts, S. C. J., Daly, A., MacDonald, A., De Laet, C., Cassiman, D., Eyskens, F., Körver-Keularts, I. M. L. W., Goyens, P. J., McKiernan, P. J. & van Spronsen, F. J. 2016b. Neurocognitive outcome in tyrosinaemia type 1 patients compared to healthy controls. Orphanet Journal of Rare Diseases, 11.

van Ginkel, W. G., van Vliet, D., Burgerhof, J. G. M., de Blaauw, P., Rubio Gozalbo, M. E., Heiner-Fokkema, M. R. & van Spronsen, F. J. 2017. Presumptive brain influx of large neutral amino acids and the effect of phenylalanine supplementation in patients with Tyrosinaemia type 1. PLoS One, 12, e0185342.

van Ginkel, W. G., van Vliet, D., van der Goot, E., Faassen, M., Vogel, A., Heiner-Fokkema, M. R., van der Zee, E. A. & van Spronsen, F. J. 2019. Blood and Brain Biochemistry and Behaviour in NTBC and Dietary Treated Tyrosinaemia Type 1 Mice. Nutrients, 11.

van Vliet, K., van Ginkel, W. G., Jahja, R., Daly, A., MacDonald, A., De Laet, C., Vara, R., Rahman, Y., Cassiman, D., Eyskens, F., Timmer, C., Mumford, N., Bierau, J., van Hasselt, P. M., Gissen, P., Goyens, P. J., McKiernan, P. J., Wilcox, G., Morris, A. A. M., Jameson, E. A., Huijbregts, S. C. J. & van Spronsen, F. J. 2019. Emotional and behavioral problems, quality of life and metabolic control in NTBC-treated Tyrosinaemia type 1 patients. Orphanet J Rare Dis, 14, 285.

Wadman, S. K., van Sprang, F. J., Maas, J. W. & Ketting, D. 1968. An exceptional case of tyrosinosis. 12, 269.

Walter, J. H., White, F. J., Hall, S. K., MacDonald, A., Rylance, G., Boneh, A., Francis, D. E., Shortland, G. J., Schmidt, M. & Vail, A. 2002. How practical are recommendations for dietary control in phenylketonuria? Lancet, 360, 55-7.

White, A. & C Tchan, M. 2018. Nitisinone-Induced Keratopathy in Alkaptonuria: A Challenging Diagnosis Despite Clinical Suspicion. JIMD reports, 40, 7-9.

Wilkinson, D. J. 2018. Historical and contemporary stable isotope tracer approaches to studying mammalian protein metabolism. Mass Spectrometry Reviews, 37, 57-80.

Wolfe, R. R. & Chinkes, D. L. 2004. Isotope Tracers in Metabolic Research: Principles and Practice of Kinetic Analysis, Wiley.

Wolkow, M. & Baumann, E. 1891. Ueber das Wesen der Alkaptonurie. Z Physiol Chem 15, 228-85.

Womack, M. & Rose, W. C. 1934. Feeding experiments with mixtures of highly purified amino acids. 6. The relation of phenylalanine and tyrosine to growth. Journal of Biological Chemistry, 107, 449-458.

Yanai, S. & Endo, S. 2021. Functional Aging in Male C57BL/6J Mice Across the Life-Span: A Systematic Behavioral Analysis of Motor, Emotional, and Memory Function to Define an Aging Phenotype. Frontiers in Aging Neuroscience, 13.

Zaleski, W. A. & Hill, A. 1973. Tyrosinosis: a new variant. Can Med Assoc J, 108, 477 passim.

Zannoni, V. G., Lomtevas, N. & Goldfinger, S. 1969. Oxidation of homogentisic acid to ochronotic pigment in connective tissue. Biochimica Et Biophysica Acta, 177, 94-105.

Zatkova, A., Olsson, B., Ranganath, L. R. & Imrich, R. 2022. Analysis of the Phenotype Differences in Siblings with Alkaptonuria. Metabolites, 12, 990.

Zatkova, A., Ranganath, L. & Kadasi, L. 2020. Alkaptonuria: Current Perspectives. Appl Clin Genet, 13, 37-47.

Zatkova, A., Sedlackova, T., Radvansky, J., Polakova, H., Nemethova, M., Aquaron, R., Dursun, I., Usher, J. L. & Kadasi, L. 2012. Identification of 11 Novel Homogentisate 1,2 Dioxygenase Variants in Alkaptonuria Patients and Establishment of a Novel LOVD-Based HGD Mutation Database. Springer Berlin Heidelberg.

Zhang, X.-J., Chinkes, D. L. & Wolfe, R. R. 2002. Measurement of muscle protein fractional synthesis and breakdown rates from a pulse tracer injection. American Journal of Physiology. Endocrinology and Metabolism, 283, E753-764.

7.0 Appendices

7.1 Appendix 1: Study protocol

7.1.1 Screening procedures

Subjects underwent study-specific screening within 28 days prior to the intravenous administration of tracers. At the screening visit, subjects were assessed against the exclusion criteria.

The following information and procedures were recorded and performed as part of the screening assessments:

- medical history
- gender, race/ethnic origin, age, height, weight, and BMI
- vital signs, including supine blood pressure, supine pulse rate and oral body temperature.
- resting 12-lead ECG
- physical examination
- clinical laboratory and serology investigations for the evaluations to be performed)

7.1.2 Study procedures

7.1.2.1 *Vital signs*

Supine blood pressure, supine pulse rate and oral body temperature were measured at the times indicated in the Study Plan (Appendix 2).

Vital signs were performed at other times if judged to be clinically appropriate.

Measurements were repeated once if outside the relevant clinical reference ranges. If repeated, the repeat value was used in the data analysis.

Blood pressure and pulse rate were measured using automated monitors. Subjects must be supine for at least 5 minutes before blood pressure and pulse rate measurements. Oral body temperature was measured singly using a digital thermometer.

7.1.2.2 *12-Lead electrocardiogram*

A single 12-lead resting ECG was recorded after the subject has been supine for at least 5 minutes at the times indicated in the Study Plan (Appendix 2).

Additional 12-lead ECGs were performed at other times if judged to be clinically appropriate or if the ongoing review of the data suggested a more detailed assessment of ECGs was required.

The study physician performed a clinical assessment of each 12-lead ECG. CRU reference ranges were applied to all ECG parameters determined throughout the study.

7.1.2.3 *Clinical laboratory evaluations*

Blood and urine samples were collected for clinical laboratory evaluations at the times indicated in the Study Plan (Appendix 2). Clinical laboratory parameters were analysed at local laboratories.

Additional clinical laboratory evaluations were performed at other times if judged to be clinically appropriate or if the ongoing review of the data suggested a more detailed assessment of clinical laboratory safety evaluations was required.

The study Investigator performed a clinical assessment of all clinical laboratory data.

Table 62: Clinical laboratory evaluations

Serum biochemistry	Units	Haematology	Units
Aspartate aminotransferase (AST)	IU/L	White blood cell count (WBC)	10^9/L
Alanine aminotransferase (ALT)	IU/L	Red blood cell count (RBC)	10^{12}/L
Alkaline phosphatase (ALP)	IU/L	Haemoglobin	g/L
Gamma glutamyl transferase (GGT)	IU/L	Haematocrit (PCV)	%
Sodium	mmol/L	Mean cell volume (MCV)	fL
Potassium	mmol/L	haemoglobin (MCH)	pg
Chloride	mmol/L	Mean cell MCH concentration	g/L
Calcium	mmol/L	(MCHC) Platelet count	10^9/L
Inorganic phosphate	mmol/L	Differential WBC	10^9/L
Glucose	mmol/L		10^9/L & %
Urea	mmol/L		
Uric acid	µmol/L		
Total Bilirubin	µmol/L		
Direct Bilirubin	µmol/L		
Creatinine	µmol/L		
Total protein	g/L		
Albumin	g/L		
Creatinine phosphokinase (CPK)	IU/L		
Hormone Panel: [a]			
Follicle stimulating hormone (FSH)	IU/L		
Human chorionic gonadotropin (hCG) (serum pregnancy test)	IU/L		
Urinalysis	**Units**		
Specific gravity	NA		
pH	NA		
Protein	+		
Glucose	+		
Ketones	+		
Blood	+		
Urobilinogen	+		
Urine Pregnancy Test [b]	neg/pos		

[a] Hormone Panel was performed if urine pregnancy test is positive; [b] Females only aged 18-64 years. Positive pregnancy result would result in hormone panel analysed in serum; neg = Negative, pos = Positive

7.1.2.4 *Physical examination*

A full physical examination was performed at the times indicated in the Study Plan (see Appendix 2).

7.1.2.5 *Body weight*

Body weight (without shoes) were recorded at the times indicated in the Study Plan (see Appendix 2).

7.1.2.6 *Bioelectrical Impedance Analysis (BIA):*

This was assessed using a stand on scale to estimate the fat free body mass (see Appendix 2).

7.1.2.7 *Tyrosine and phenylalanine tracers' kinetic studies*

7.1.2.8 *Serial blood sampling*

The timings of all serial blood sampling assessments performed during the study were subject to change based on the ongoing review of the data. Furthermore, it was possible to perform up to two additional assessments for each tracers' kinetic study on each subject per treatment period. The maximum volume of blood withdrawn per subject did not exceed the limit detailed in Table 63. Changes to the scheduled times of the tracers' kinetic studies were agreed with the Sponsor and documented in the Site Master File.

For each time point one serum sample and one plasma sample were taken. For serum samples, aliquots were acidified and frozen and the remainder was frozen without acidification.

Accurate measurements of amino acids in the whole body requires direct arterial sampling which is quite uncomfortable to participants and has its own risks and complications. Fortunately, an alternative method has been validated and adopted in the metabolic research (Abumrad et al., 1981). It employs venous sampling after warming the hand using a heated hand device to obtain arterialised blood samples (Matthews, 2016). Confirmation that the blood sample was arterialised was obtained by measuring O_2 saturation in a venous sample taken from the warmed arm/ hand. An ABG analyser was used to measure O2 saturation. The tracers' injection proceeded after confirmation of O_2 saturation.

Blood samples were collected in non-gel serum tubes and lithium heparin tubes. Sample processing, labelling, transportation, and storage were performed as stipulated in biochemistry laboratory standard operating procedures. NTBC levels were measured in the pre-tracer injection sample in AKU patients.

7.1.2.9 *Urine collections for the analysis of tyrosine, phenylalanine, and their tracers*

Urine was collected into standard-weight polyethylene containers over the time intervals indicated in the Study Plan (Appendix 2) for an exploratory analysis of the tyrosine and phenylalanine tracers and their compounds.

Two –hour Urine collection started by emptying of the bladder (urine was discarded or used for biomarkers and metabolomics). The last portion of the 2-hour sample was collected when emptying the bladder exactly 2 hours following the start of the collection period.

Urine was collected into 2.5-L bottles. During collection, bottles were stored away from bright light or warm conditions. At the end of the collection period, the bottles were weighed, and the total weight was recorded. Urine was acidified using 0.5N H_2SO_4. The weight of the empty bottles, before adding the H_2SO4, was also recorded. Sample processing, labelling, transportation, and storage were performed as stipulated in biochemistry laboratory standard operating procedures.

7.1.2.10 *List of Analytes that were measured:*

In addition to the clinical laboratory evaluations summarised in Table 62, the following were measured:

a. Serum L-[$^{13}C_9$]tyrosine (to enable the determination of volume of distribution)

b. Serum L-[d_8]phenylalanine

c. Serum L-[d_7]tyrosine

d. Serum phenylalanine

e. Serum tyrosine

f. Serum homogentisic acid (HGA)

g. Serum hydroxyphenyl pyruvic acid (HPPA)

h. Serum hydroxyphenyl lactic acid (HPLA)

i. Urine phenylalanine

j. Urine tyrosine

k. Urine HGA

l. Urine HPPA and HPLA

7.1.2.11 *Measurement of tracers in urine and serum*

State of the art LC-MS/MS analyser was used in analysing urine and serum samples in the Department of Clinical Biochemistry at the Royal Liverpool University Hospital.

7.1.2.12 *Total blood volume*

The following blood volumes withdrawn for each subject are summarised below.

Table 63: Total blood volume to be withdrawn for each participant

a Includes up to an additional 2 samples that may be taken per sampling period for tracers' kinetic studies purposes, if required.

b Clinical laboratories evaluation was not repeated if done previously within the previous 28 days. HV: Healthy volunteers

	Volume per blood sample (mL)	Planned total number of samples (Maximum number of samples [a])	Planned total amount of blood (Maximum amount of blood) (mL)
Clinical laboratory evaluations	10	HV: 1 AKU patients: 3[b]	HV 10 AKU patients: 30
Blood oxygen saturation check	1	HV: 1(4)[a] AKU patients: 2(8)	HV: 1(4) AKU patients: 2(8)
Tracers' Kinetic Studies	12.5	HV: 12 (14)[a] AKU patients: 24(28)	HV: 150 (175) AKU patients: 300 (350)
Total:			HV: 161 (189) AKU patients: 332 (388)

[a] Includes up to an additional 2 samples that may have be taken per sampling period for tracers' kinetic studies purposes, if required.

[b] Clinical laboratories evaluation were not repeated if done previously within the previous 28 days.

HV: Healthy volunteers

7.1.2.13 *Study residency/ visits*

7.1.2.13.1 Visit 1 (screening and enrolment):

Study participants reported to the CRU in the morning and left once all the assessments are done.

7.1.2.13.2 Visit 2:

Study participants reported to the CRU in the morning, and they remained there until the sampling procedure is completed.

Participant were discharged as soon as the sampling procedure was completed if they felt well. The Investigator checked on all subjects' wellbeing prior to their discharge from the CRU. Where necessary, subjects remained at the CRU until any adverse events of concern have resolved.

7.1.2.14 *Participant withdrawal:*

Participant safety was paramount and was a priority over adhesion to the protocol.

NTBC was stopped if the patient:

- Developed ocular signs or symptoms, or
- Developed a skin rash which are judged by the investigator to be related to elevated tyrosine

Subjects were withdrawn from the study if:

- Became pregnant.

- ALT increased to > 3 x upper limit of normal.
- Haemoglobin decreased to < 100 g/L.
- Platelets decreased to < 100 x 109/L.

- Any clinically relevant signs or symptoms that in the opinion of the Investigator warranted subject withdrawal
- Non-compliance with the study restrictions, as considered applicable by the Investigator
- Subject self-withdrawal

7.1.3 Specific restrictions/requirements on participants

7.1.3.1 *Diet:*

On the days of tracers administrations, participants had nothing to eat (water was be permitted) from 00:00 the night prior to dosing until approximately 2 hours after the intravenous administration of the tracers.

7.1.3.1.1 Healthy volunteers:

All healthy participants were asked to maintain their usual diet.

7.1.3.1.2 AKU patients:

7.1.3.1.2.1 NTBC Naïve AKU patients:

Prior to first infusion of tracers, participants were asked to maintain their usual diet. All patients were under the care of the NAC and have had specialist dietary

counselling regarding low protein diet. They were advised to maintain this diet when they start NTBC.

7.1.3.1.2.2 NTBC treated AKU patients:

This group was already be on a low tyrosine diet, and were advised to maintain it throughout the study

7.1.3.2 *Smoking (applies to AKU patients only)*

Smoking was not permitted from waking until after completion of study procedures. Subjects were permitted to smoke their normal daily number of cigarettes between study visits. Smoking was not permitted on site.

7.1.3.3 *Exercise*

Subjects were requested not to undertake vigorous exercise from 48 hours before each study visit.

7.1.3.4 *Blood donation*

Subjects were advised not to donate blood for 3 months after the post-study visit.

7.1.3.5 *Contraception*

For AKU female patients, they were advised to use a reliable method of contraception during the study and for one month after the study. This was to be done using at least one highly effective method of contraception' which would be defined as barrier methods with spermicide gel / foam / cream, oral contraceptive pill, contraceptive hormonal implant, Mirena coil. This also applies to male

patients as well, and excludes women who are surgically sterile (except for women who had tubal ligation)

7.1.3.6 *Concomitant Medication*

7.1.3.6.1 Healthy Subjects:

Any drug, herbal remedy, or vitamin/mineral supplement which, in the opinion of the principal investigator, precluded their participation in the study.

7.1.3.6.2 AKU patients not on NTBC:

No restrictions

7.1.3.6.3 AKU patients on NTBC:

They were asked stop NTBC for month prior to the first administration of the intravenous tracers. There are no other restrictions.

7.1.3.7 *Contact lenses:*

All AKU Patients were recommended to avoid using contact lenses during the study.

7.1.4 Safety and tolerability assessments

The timings of all measurements to be performed during the study were subject to change based on the ongoing review of the safety, tolerability of NTBC and tracers.

7.1.4.1 *Adverse events*

The condition of each subject was monitored throughout the study. In addition, any signs or symptoms were observed and elicited at least once a day by open questioning, such as

“How have you been feeling since you were last asked?”

Subjects were encouraged to spontaneously report AEs occurring at any other time during the study. Any AEs and remedial action required were recorded in the subject's source data. The nature, time of onset, duration and severity were documented, together with the Investigator’s opinion of the relationship to study drug administration. Any clinically significant abnormalities identified during the study were followed up until they return to normal or can be clinically explained. Adverse event definitions, assignment of severity and causality, and procedures for reporting.

7.1.4.1.1 Stable isotopes related adverse events

Stable isotopes are naturally present in human body. For example, the natural abundance of ^{13}C is 1.1%, while for deuterium it is 0.0115. This translates, in the latter, to 15 mg kg of body weight. Of note, biochemical studies have employed deuterium tissue enrichment up to 95 mg/kg of body weight (Jones and Leatherdale, 1991).

As far as infusing stable isotopes, the risk to participants is minimal (Koletzko et al., 1997, Koletzko et al., 1998). The use of phenylalanine and tyrosine stable

isotopes in humans is safe and well documented in healthy volunteers (Clarke and Bier, 1982, Thompson et al., 1989, Cortiella et al., 1992, Marchini et al., 1993, van Eijk et al., 2007, Mason et al., 2017), premature infants (Clark et al., 1997), ill new-borns and infants in the Neonatal Intensive Care Unit (Castillo et al., 1994, Roberts et al., 1998, Roberts et al., 2001, de Betue et al., 2011), stable cystic fibrosis patients (Moran et al., 2001) and in critically ill patients with septic shock (Meesters et al., 2009).

Administration of ^{13}C labelled endogenous substance in metabolic studies did not result in toxicity (Schellekens et al., 2011). In the case of deuterium, toxicity can only be induced by high deuteration exceeding 15% of body water (Pons and Rey, 1999). In comparison, the dose of deuterated phenylalanine given in this study is minuscule (see also section on dose justification). Furthermore,van Eijk et al. (2007) have used 45 mL bolus volume of tracers in healthy male volunteers and no side effects were reported.

Stable isotopes infusions solutions were prepared at the Radiopharmacy Department of the Royal Liverpool University Hospital to ensure its sterility and that the process is compliant with the principles of Good Manufacturing Practice (GMP).

7.1.4.1.2 NTBC related adverse events

For over two decades NTBC has been used for the treatment of hereditary tyrosinaemia 1 (HT-1) in children using a dose of 1 mg/kg/day. This condition is

caused by an enzyme deficiency which leads to accumulation of tyrosine metabolites. Before using NTBC, death in childhood was inevitable without liver transplant (McKiernan, 2006). In adults with AKU, NTBC was generally well tolerated at a dose of 2 mg daily over a three-year period (Introne et al., 2011). In this study, only 2 mg daily was used. This much lower compared to the paediatric dose. NTBC can increase tyrosine concentration. This infrequently can lead to ocular signs and symptoms, including corneal ulcers, corneal opacities, keratitis, conjunctivitis, eye pain, and photophobia. It may also lead to hyperkeratotic skin lesions. Patients were asked to report any ocular symptoms and stop the NTBC immediately. They were brought back for a slit lamp examination. Tyrosine keratopathy is completely reversible upon discontinuation of NTBC. Side effects of NTBC are summarised in Table 64.

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Table 64: NTBC side effects.
Adapted from (compendium, 2016).

system organ class	**Frequency**	**Adverse reaction**
Blood and lymphatic system disorders	Common (≥1/100 to <1/10)	Thrombocytopenia, leucopenia, granulocytopenia
	Uncommon (≥1/1,000 to <1/100)	Leukocytosis
Eye disorders	Common	Conjunctivitis, corneal opacity, keratitis, photophobia, eye pain
	Uncommon	Blepharitis
Skin and subcutaneous tissue disorders	Uncommon	Exfoliative dermatitis, erythematous rash, pruritus
Investigations	Very common (≥1/10)	Elevated tyrosine levels

7.2 Appendix 2

7.2.1 Study plan for healthy volunteers

	Screening*	**Visit 1**
Inclusion/exclusion criteria	X	
Demographic data	X	
Medical history	X	
Informed consent	X	
Pregnancy Test [a]		
Study residency:		
Out-patient Visit	X	X
Intravenous injection of tracers:		X
Adverse event recording		X
Blood pressure, pulse rate and oral body temperature	X	X
12-lead ECG	X	
Clinical laboratory evaluations	X	
Body weight, height, and BIA	X	X
Physical examination	X	
Tracers' Kinetic Studies		
Blood sampling for tyrosine, phenylalanine, and their tracers (Taken at the following intervals: Pre- tracers injection, 5,10,15,20, 25, 30,40,50,60,90,120 minutes post-injection)		X
Urine collection [b] (pre-injection spot collection and 2 hr-collection)		X
1-hr observation period post completion of kinetic sampling [c]		X

[a] Females aged 18-64 years. Performed in urine every visit. Hormone Panel was performed if the urine pregnancy test result was positive.

[b] Participants were asked to empty their bladder just before the tracers injections. The 2-hr urine collection started after that. Subjects were asked to provide last urine sample after the last blood sample is taken after 120 minutes of the tracers' injection.

[C] Participants left as soon as the sampling procedure was completed if they felt well.

* Screening in healthy volunteers required participants to attend to visit the CRU, whereas in AKU patients it was done before their first visit

7.2.2 Study plan for NTBC naïve AKU patients

	Screening	Visit 1	Visit 2*
Inclusion/exclusion criteria	X		
Demographic data	X		
Medical history	X		
Informed consent		X	
Pregnancy Test [a]		X	X
Study residency:			
Out-patient Visit		X	X
Intravenous injection of tracers:		X	X
Adverse event recording		X	X
Blood pressure, pulse rate and oral body temperature		X	X
12-lead ECG		X	X
Clinical laboratory evaluations		X	X
Body weight, height, and BIA		X	X
Physical examination		X	X
Tracers' Kinetic Studies			
Blood sampling for tyrosine, phenylalanine, and their tracers (Taken at the following intervals: Pre- tracers injection, 5,10,15,20, 25, 30,40,50,60,90,120 minutes post-injection)		X	X
Urine collection [b] (pre-injection spot collection and 2 hr-collection)		X	X
1-hr observation period post completion of kinetic sampling [c]		X	X

[a] Females aged 18-64 years. Performed in urine every visit. Hormone Panel was performed if the urine pregnancy test result was positive.
[b] Participants were asked to empty their bladder just before the tracers injections. The 2-hr urine collection started after that. Subjects were asked to provide last urine sample after the last blood sample is taken after 120 minutes of the tracers' injection.
[C] Participants left as soon as the sampling procedure was completed if they felt well.
* NTBC was started after the tracers injection in visit 1 and stopped after the tracers injection in visit 2

7.2.3 Study plan for AKU patients already on NTBC

	Visit 1*	**Visit 2***	
Inclusion/exclusion criteria	X		
Demographic data	X		
Medical history	X		
Informed consent	X		
Pregnancy Test [a]	X	X	
Study residency:			
Out-patient Visit	X	X	
Intravenous injection of tracers:	X	X	
Adverse event recording	X	X	
Blood pressure, pulse rate and oral body temperature	X	X	
12-lead ECG	X	X	
Clinical laboratory evaluations	X	X	
Body weight, height, and BIA	X	X	
Physical examination	X	X	
Tracers' Kinetic Studies	X	X	
Blood sampling for tyrosine, phenylalanine, and their tracers (Taken at the following intervals: Pre- tracers injection, 5,10,15,20, 25, 30,40,50,60,90,120 minutes post-injection)	X	X	
Urine collection [b] (pre-injection spot collection and 2 hr- collection)	X	X	
1-hr observation period post completion of kinetic sampling [c]	X	X	
Notes: [a] Females aged 18-64 years. Performed in urine every visit. Hormone Panel was performed if the urine pregnancy test result was positive.[b] Participants were asked to empty their bladder just before the tracers injections. The 2-hr urine collection started after that. Subjects were asked to provide last urine sample after the last blood sample was taken after 120 minutes of the tracers' injection.[C] Participants left as soon as the sampling procedure was completed if they felt well. * NTBC was stopped after the tracers injection in visit 1 and restarted after the tracers injection in visit 2			

8.0 Publication based on this thesis:

Results from this thesis has been published in the following article:

Khedr M, Cooper MS, Hughes AT, Milan AM, Davison AS, Norman BP, Sutherland H, Jarvis JC, Fitzgerald R, Markinson L, Psarelli EE, Ghane P, Deutz NEP, Gallagher JA, Ranganath LR. Nitisinone causes acquired tyrosinosis in alkaptonuria.*J Inherit Metab Dis.* 2020 Sep;43(5):1014-1023. doi: 10.1002/jimd.12229. Epub 2020 Mar 5. PMID: 32083330