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ORIGINAL ARTICLE

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Hepatobiliary circulation and dominant urinary excretion of homogentisic acid in a mouse model of alkaptonuria

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Abstract

Altered activity of specific enzymes in phenylalanine-tyrosine (phe-tyr) metabolism results in incomplete breakdown of various metabolite substrates in this pathway. Increased biofluid concentration and tissue accumulation of the phe-tyr pathway metabolite homogentisic acid (HGA) is central to pathophysiology in the inherited disorder alkaptonuria (AKU). Accumulation of metabolites upstream of HGA, including tyrosine, occurs in patients on nitisinone, a licenced drug for AKU and hereditary tyrosinaemia type 1, which inhibits the enzyme responsible for HGA production. The aim of this study was to investigate the phe-tyr metabolite content of key biofluids and tissues in AKU mice on and off nitisinone to gain new insights into the biodistribution of metabolites in these altered metabolic states. The data show for the first time that HGA is present in bile in AKU (mean [±SD] = 1003[±410] μmol/L; nitisinone-treated AKU mean [±SD] = 45[±23] μmol/L). Biliary tyrosine, 3 (4-hydroxyphenyl)pyruvic acid (HPPA) and 3(4-hydroxyphenyl)lactic acid (HPLA) are also increased on nitisinone. Urine was confirmed as the dominant elimination route of HGA in untreated AKU, but with indication of biliary excretion. These data provide new insights into pathways of phe-tyr metabolite biodistribution and metabolism, showing for the first time that hepatobiliary excretion contributes to the total pool of metabolites in this pathway. Our data suggest that biliary elimination of organic acids and other metabolites may play an underappreciated role in disorders of metabolism. We propose that our finding of approximately 3.8 times greater urinary HGA excretion in AKU mice compared with patients is one reason for the lack of extensive tissue ochronosis in the AKU mouse model.

KEYWORDS

hepatobiliary circulation, homogentisic acid, organic acids, tyrosine disorders, tyrosine metabolites

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1 | INTRODUCTION

The phenylalanine-tyrosine (phe-tyr) pathway is the major metabolic route through which excess phenylalanine and tyrosine is catabolised. Phe-tyr metabolism predominantly occurs in the liver and involves the conversion of these amino acids through several intermediate steps, resulting in the metabolite products fumarate and acetoacetate. Mutations in genes encoding the various enzymes of the phe-tyr pathway have deleterious consequences, resulting in the accumulation of metabolites that directly cause a range of specific and potentially fatal diseases (Figure S1).[1]

Alkaptonuria (AKU; OMIM #203500) is an inherited disorder of the phe-tyr pathway, in which mutations in the gene encoding the homogentisate 1,2-dioxygenase enzyme (HGD; E.C.1.12.11.5) result in its inability to metabolise the intermediate homogentisic acid (HGA).[2] Despite maximal urinary excretion of HGA in untreated AKU, HGA concentration remains elevated in plasma and accumulates in tissues throughout the body.[3] HGA accumulation in tissues gives rise to a pigment formed from oxidation of HGA, a slowly progressing process called ochronosis.[4,5] Ochronosis is central to the chronic multisystem features of AKU, which include cardiac valve disease, premature arthritis, bone fractures and rupture of ligament, tendon and muscle.[6–9]

In recent years, nitisinone (2-(2-nitro-4-(trifluoromethyl)benzoyl)cyclohexane-1,3-dione) has been established as an effective agent for treatment of AKU. Nitisinone reduces plasma and urinary HGA, slows progression of disease in patients[10,11] and completely arrests ochronosis in AKU mice.[12,13] Nitisinone is the only known disease modifying agent for AKU and hereditary tyrosinaemia type 1 (HT-1) but its inhibition of HGA production causes the accumulation of metabolites upstream of HGA in phe-tyr metabolism, including tyrosine, 4-hydroxyphenylpyruvic acid (HPPA) and 4-hydroxyphenyllactic acid (HPLA). Accumulation of tyrosine may lead to keratopathy, skin lesions and adverse neuropsychological effects.[14–19]

Ochronosis is well established as the central cause of disease pathogenesis in AKU, but there remains much to learn about this metabolic pathway. It is clear that ochronotic pigment is an oxidation product of HGA, but the exact chemical structure of the pigment remains unknown.[20,21] The molecular mechanism through which HGA-derived pigment becomes bound to cartilaginous tissue, the preferential site of pigment accumulation, is also not fully understood.[22–24] Metabolite data from the 'Suitability of Nitisinone in AKU' (SONIA 1[25] and SONIA 2[10]) clinical trials have provided insights into the metabolism of HGA, including identification of the major HGA biotransformation pathways,[26] and have enabled inferences on the daily biodistribution rates of HGA in plasma and total body water compared with urinary excretion.[27,28] It is not currently possible to directly measure all the HGA-derived products in the ochronotic pathway, but the blockade of HGA production by nitisinone in these studies has been studied as a way to unmask the amount of flux that would otherwise be directed down this pathway in untreated AKU. SONIA 1 and SONIA 2 both showed a marked increase in the summed concentrations of phe-tyr pathway metabolites in total body water, but not in urine, in patients on nitisinone treatment. The interpretation of this finding was that approximately half of the total daily HGA produced is excreted in the urine. It is possible that the remaining half of the daily HGA accumulates in tissues as HGA and/or pigment, but estimates of lifetime accumulation based on this rate of deposition seem implausible. An alternative, but as yet unconfirmed, explanation for the total phe-tyr flux unaccounted for in untreated AKU is that biliary secretion is a significant elimination route of HGA from the liver.[27]

The enterohepatic circulation is the circulation of bile and its constituents between the liver and gastrointestinal tract. It is well known that endogenous substrates and xenobiotics, including drugs and metabolites are excreted into bile from hepatocytes.[29] The liver is the major site of phe-tyr metabolism[30] and the transporter machinery for small molecule anions such as HGA exists at the canalicular surface of hepatocytes for secretion into the bile, namely transmembrane transporter multidrug resistance protein 2 (MRP2).[31,32] Once secreted into bile, it is proposed that HGA is secreted into the small intestine, from which both secondary absorption and faecal excretion are possible. We tested the hypothesis that bile HGA is a contributor to the total phe-tyr metabolite pool in AKU. Phe-tyr metabolites have not been directly measured in bile in patients with AKU due to the invasiveness of accessing this biofluid. The $Hgd^{-/-}$ mouse is a useful model in which to study AKU biochemistry, as it recapitulates the marked increases in circulating (a) HGA in untreated AKU, (b) tyrosine and associated metabolites in nitisinone-treated AKU, and as it (c) reproduces the earliest stages of tissue ochronosis.[30] This mouse model of AKU provides an opportunity to systematically investigate bile metabolite composition both in untreated AKU and in response to nitisinone treatment under controlled conditions. Thyroid was investigated as an additional potential site of HGA accumulation; there are reports of thyroid tissue ochronosis in patients with AKU[33] but to our knowledge, HGA concentration has not yet been measured in thyroid.

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2 | METHODS

2.1 | Chemicals and reagents

Deionised water was purified in-house by DIRECT-Q 3UV water purification system (Millipore, Watford, UK). Methanol, isopropanol (Sigma–Aldrich, Poole, UK), formic acid (Biosolve, Valkenswaard, Netherlands) and ammonium formate (Fisher Scientific, Schwerte, Germany) were LC/MS grade. Nitisinone, perchloric acid and H_2SO_4 were from Sigma-Aldrich.

2.2 | Animal details

All mice studied were on a C57BL/6 genetic background. AKU mice were generated from targeted knockout of Hgd ($Hgd^{-/-}$).[30] For AKU mice, the nitisinone-treated and no treatment groups each comprised four mice (2 male, 2 female). Mean age (±SD) was 13.5(±0.9) weeks in the nitisinone group and 12.5 (±1.6) weeks in the no treatment group. Wild type mice were three females all aged 29.1 weeks. For bile measurements, samples from an additional two AKU untreated mice (1 male and 1 female, both aged 9 weeks) were available and included in the analysis to enable investigation of potential sex differences in metabolites.

All mice were housed, maintained and experimental procedures carried out within the University of Liverpool Biological Services Unit in specific pathogen-free conditions, under project licence PP8132802, in accordance with UK Home Office guidelines, under the Animals (Scientific Procedures) Act 1986. Mice were culled by Schedule 1 approved methods and tissues harvested without delay. All mice were housed in cages of up to five mice, with 12-h light/dark cycle, and food and water available ad libitum. Mice were drug/test-naïve at baseline. In the nitisinone-treated group, nitisinone was added to all drinking water to a final concentration of 4 mg/L.

2.3 | Sample collection and analysis

Blood samples were collected from venous tail bleeds into lithium heparin microvettes (CB 300 LH, Sarstedt, UK) and kept on ice until processed. Whole blood was centrifuged at 1500× *g* for 10 min at 4°C and plasma supernatant acidified with 10% v/v 5.8 M perchloric acid then vortexed. Acidified plasma samples were centrifuged at 1500× *g* for 10 min at 4°C and the supernatant removed.

Urine was sampled on a single-collection basis into 0.5 mL microcentrifuge tubes. Urine samples were acidified by addition of 5% v/v 1 M H_2SO_4.

Post schedule 1 cull, the thyroid and gall bladder were excised. Bile was removed from the gall bladder. Faecal pellets were freshly collected. All samples were snap frozen in liquid nitrogen. All tissues and biofluid samples were stored at −80°C prior to analysis.

Whole thyroid tissues, which were pooled among groups to provide sufficient material, and individual faeces samples were thawed on ice in 1.5 mL microcentrifuge tubes prior to metabolite extraction. Extraction was performed by addition of 50% of the final volume of 0.58 M perchloric acid to samples and homogenised in tubes by manual handheld homogeniser. The homogeniser tip was then washed in the second 50% volume of perchloric acid, and the wash transferred to the tissue homogenate. Tissue extract concentrations (tissue weight/extraction buffer volume) were 250 mg/mL for pooled thyroid and 125 mg/mL for faeces. All tissue homogenates, urine and bile samples were centrifuged at 1500× *g* for 10 min at 4°C, supernatant was removed and diluted 1:1000 sample: internal standard-containing diluent as with plasma and urine samples. Plasma and urine samples were prepared and quantified for phe-tyr pathway metabolites by liquid chromatography triple-quadrupole tandem mass spectrometry (LC-QQQ-MS) following published plasma (serum) and urine protocols.[34–36] Bile, faeces and thyroid samples were analysed using the aforementioned serum phe-tyr protocol.

2.4 | Data processing and statistical analysis

Raw LC/MS data files were processed in MassHunter QQQ Quantitative Analysis software (v. B.09.00; Agilent) following published protocols.[34–36] Statistical analyses were performed in Prism (v. 6.01; Graphpad). Statistical significance tests employed were paired t-tests for paired data (plasma, urine and faeces) and Kruskal-Wallis tests for unpaired data (bile). *p*-Values <0.05 were considered significant.

Summed values for the total phe-tyr metabolite pool measured here were calculated for each plasma, urine and bile sample. The combined raw μmol/L concentrations of all phe-tyr metabolites were calculated and summed values derived for each biofluid or tissue sample as follows. Summed plasma μmol/L concentrations were multiplied by 60% of body weight (g)[37] to reflect total body water concentrations. Total 24-h urine metabolite output was estimated by multiplying the summed μmol/L

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urine concentrations by published 24-h urine output volume (in L/100 g body weight) in C57BL/6 mice, which equates to 7.1 mL/100 g body weight in males and 9.9 mL/100 g body weight in females.[38] The gall bladder bile pool was also estimated using published data on total gall bladder bile volume in C57BL/6 mice; 4 μL bile per g liver weight.[39] Liver weight estimates were calculated as 4.3% of total body weight in males and 4.1% of total body weight in females (C57BL/6 mean organ weight data available from the Jackson Laboratory: https://www.jax.org/jax-mice-and-services). Summed bile μmol/L concentrations were then multiplied by the estimated total gall bladder bile volume (L) to derive total gall bladder metabolites.

3 | RESULTS

3.1 | Individual phe-tyr pathway metabolites

For plasma, urine and faeces, metabolite concentrations were compared between paired samples taken from the same AKU mice at baseline then at 1 week on nitisinone or no treatment. Terminal bile metabolites were compared between three groups of mice: untreated AKU mice, AKU mice at 1 week on nitisinone and untreated wild type mice. Faeces and Thyroid concentrations are expressed as μmol/g tissue. A summary of measured metabolite concentrations is shown in Table S1.

Terminal bile HGA concentration was markedly higher in untreated AKU compared with both nitisinone-treated AKU and wild type (Figure 1). There was a more than 20-fold difference between untreated AKU (mean [±SD] = 1003[±410] μmol/L) and nitisinone-treated AKU (mean = 45[±23] μmol/L). All bile HGA concentrations in wild type mice fell below the lower limit of quantification (LLOQ; 3.1 μmol/L). Further analysis within the untreated AKU group revealed a clear sex difference in bile HGA, with higher concentration in male mice ($p = 0.018$). Analysis of the other phe-tyr pathway bile metabolites showed clear increases in tyrosine, HPPA and HPLA in nitisinone-treated AKU mice (Figure 2), and nitisinone-treated males also had increased concentrations of these metabolites (Figure S7). Bile phenylalanine was also statistically significantly higher in untreated AKU compared with treated AKU.

Plasma and urine showed the expected clear decrease in HGA and increases in tyrosine, HPPA and HPLA on nitisinone treatment. Signals for these metabolites in faecal extracts were more variable. Faeces HGA showed no clear trend across serial samples in treated and untreated groups. There were two outlier samples with HGA at 1971 μmol (at baseline) and 268 μmol (at 1 week on nitisinone) per g tissue, but the other faecal samples remained close to the LLOQ. Faecal phenylalanine and tyrosine were above the LLOQ but no statistically significant differences were observed from baseline in treated or untreated groups, despite a decrease in these metabolites in 3/4 mice at 1 week on nitisinone. HPPA and HPLA were not consistently detected across samples, except for one mouse on nitisinone (Figures S2–S6).

Data from thyroid homogenates pooled within the same three groups of mice showed clear group differences generally consistent with those observed for bile (Table S1). Thyroid HGA was detected only in the untreated AKU group, and tyrosine and HPLA were greater in nitisinone-treated AKU compared with untreated AKU and wild type.

3.2 | Summed phe-tyr pathway metabolites

Summed data were analysed for the paired samples at baseline then at 1 week on nitisinone/no treatment based on the approach previously described in patients with AKU[27,28] (Figure 3). In line with previous reports in

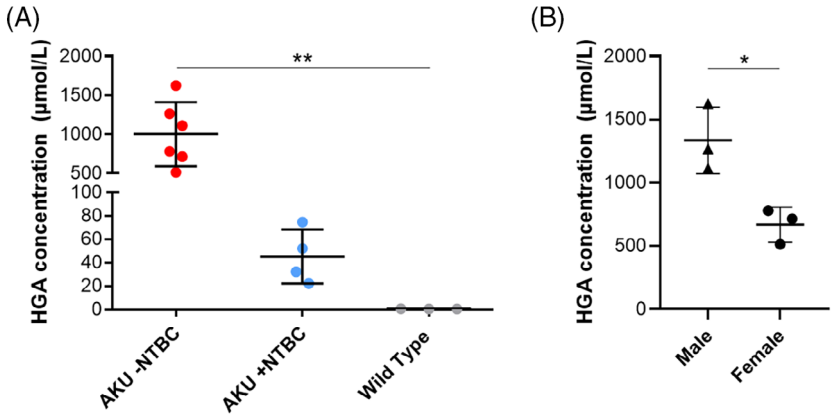

FIGURE 1 Bile HGA is increased in AKU. Bile HGA showed a marked increase in Hgd$^{-/-}$ untreated AKU mice compared with AKU mice at 7 days on nitisinone (NTBC) and wild type mice (A). Bile HGA is increased in male versus female untreated Hgd$^{-/-}$ mice (B). *$p < 0.05$; **$p < 0.01$ (B).

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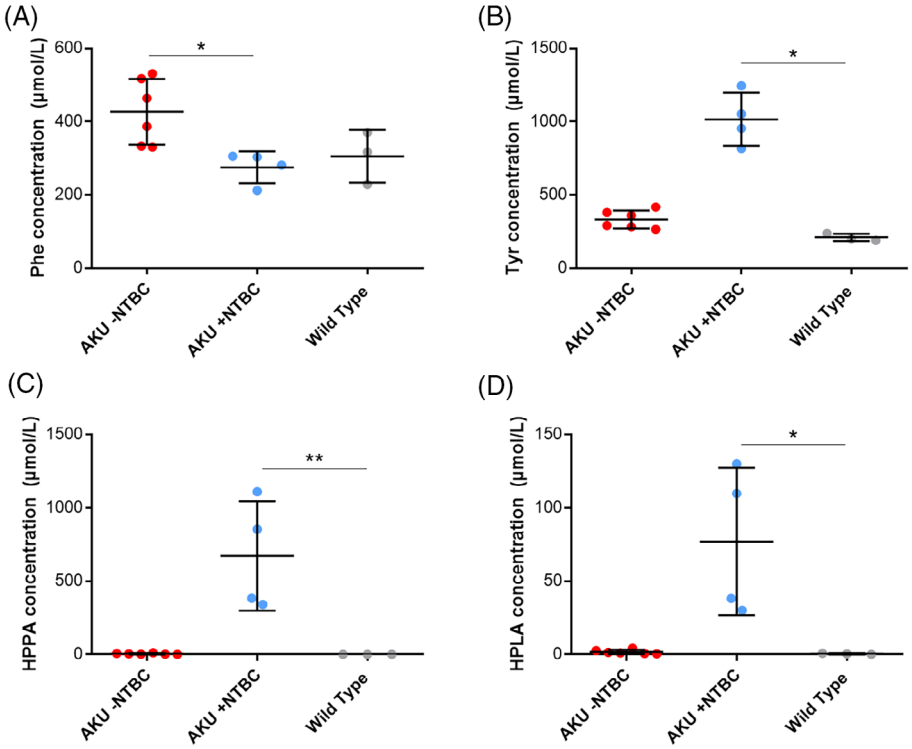

FIGURE 2 Bile phe-tyr pathway metabolites in AKU and their response to nitisinone. Statistically significant differences were observed for each of the phe-tyr pathway metabolites analysed beside HGA. Phenylalanine was increased in untreated $Hgd^{-/-}$ versus $Hgd^{-/-}$ at 7 days on nitisinone (NTBC) (A). Tyrosine, HPPA and HPLA were markedly increased in $Hgd^{-/-}$ on nitisinone (B–D). $^{*}p < 0.05$; $^{**}p < 0.01$.

patients, a clear increase in summed plasma metabolites from baseline was observed at 1 week on nitisinone ($p < 0.01$) but not at 1 week of no treatment ($p = 0.14$). For the one-week samples, summed plasma plus bile was also increased on nitisinone treatment compared with no treatment ($p < 0.001$). This increase in summed metabolites on nitisinone was no longer observed when urine data were considered; no difference was observed from baseline for summed urine, summed urine plus plasma, or summed urine, plasma plus bile.

3.3 | Estimated total daily HGA output in urine, bile and faeces

From the HGA concentrations established here, it is possible to estimate rates of daily HGA output via bile, faeces and urine in untreated mice using the published total 24-h outputs of these biofluids/tissues in mice[40,41] (Table 1). These estimates show urine as the dominant route of HGA excretion, accounting for 96.4%–99.8% of total daily HGA output. Biliary HGA excretion was estimated to account for up to 3.5% of total daily HGA output. Estimated daily faecal HGA output values were minimal.

4 | DISCUSSION

Untreated AKU and nitisinone-treated AKU result in two contrasting altered metabolic states characterised by differential accumulation of phe-tyr pathway metabolites. In untreated AKU, ochronotic pathway activity occurs due to accumulation and oxidation of HGA, the direct cause of multisystem disease. Nitisinone inhibits the 4-hydroxyphenylpyruvate dioxygenase (HPPD) enzyme, which blocks HGA formation and ochronosis, but with invariable accumulation of metabolites upstream of HGA: tyrosine, HPPA and HPLA. Biliary HGA and tyrosine concentrations of approximately 1 mmol/L in untreated and treated AKU, respectively, indicate that bile is an important excretory route for the accumulating phe-tyr pathway metabolites in these conditions. To our knowledge, this is the first direct observation of biliary excretion of HPPA and HPLA, the metabolites of tyrosine, which accumulate in patients on nitisinone. Our observations in mice provide new fundamental insights into the tissue and biofluid distribution of HGA. We confirm that urine is the major excretion route of HGA but show for the first time that HGA is also excreted via the biliary system and alimentary tract. Hepatobiliary excretion is referred to as 'phase III' detoxification of molecules,[42] which adds to our previous observations that HGA undergoes detoxification by phase I and II metabolism.[26,43] Our data may underestimate HGA biliary excretion, as previously reported HGA biotransformation products may also be excreted with HGA from the liver and therefore contribute to the total HGA pool.

Bile is a complex aqueous secretion formed in the liver, the major site of phe-tyr metabolism. Bile is approximately 95% water with constituents including bile salts,

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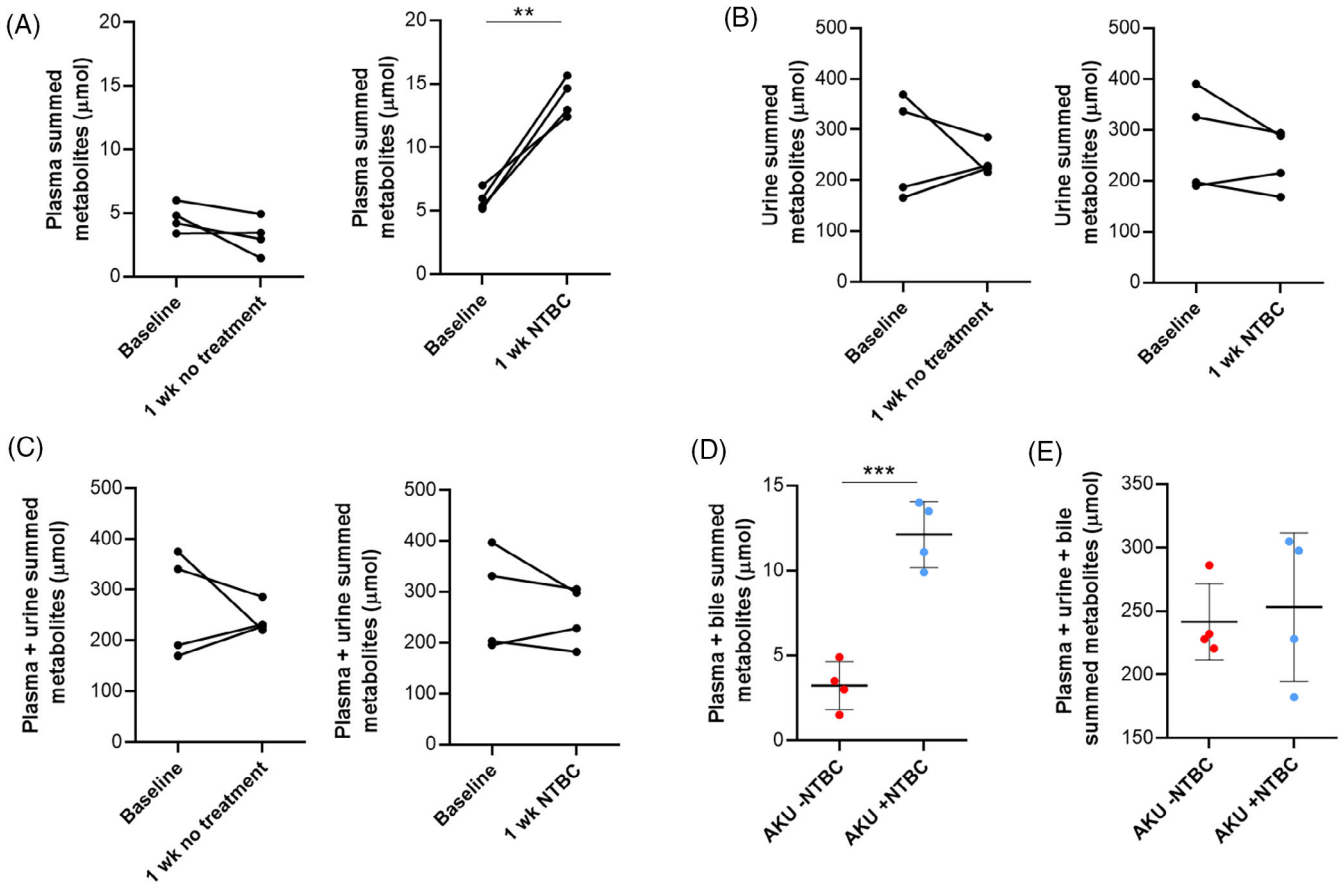

FIGURE 3 Summed phe-tyr metabolite data in treated versus untreated AKU. Summed phe-tyr metabolite data (phe, tyr, HPPA, HPLA and HGA) in AKU Hgd$^{-/-}$ mice. (A)–(C) show paired data; the same mice at baseline then at 7 days on nitisinone (NTBC) or no treatment. (D, E) show unpaired data incorporating bile measurements. (A): plasma summed, (B): urine summed, (C): plasma summed + urine summed, (D): plasma summed + bile summed, (E): plasma summed + urine summed + bile summed. $^{**}p < 0.01$; $^{***}p < 0.001$.

bilirubin, phospholipids, cholesterol, amino acids, steroids and hormones. Hepatobiliary secretion is an excretory route for diverse substrates including xenobiotics and endogenous small molecules.[29] The literature on biliary phe-tyr metabolite content is scarce. A case report of a Danish patient with AKU suggested secretion of HGA in bile. This patient had recurrent gallstones over a 30-year period, and upon starting a low phenylalanine and tyrosine diet, bile duct stone formation and associated abdominal pain immediately subsided. Remission on the low phenylalanine and tyrosine diet strongly implicates HGA in formation of bile stones.[44] Compared with HGA, hepatobiliary circulation of amino acids is better understood.[45] Amino acids including phenylalanine and tyrosine from biliary secretions undergo subsequent reabsorption throughout the intestine via the solute carrier transporter SLC6A19.[46] Our finding of generally low or undetectable HGA in faeces indicates that HGA secreted in bile is reabsorbed in the intestine and/or metabolised by the gut microbiome. There is evidence that HGA is absorbed from the intestine when given orally to humans.[47] It is also possible that HGA is metabolised in the intestine by gut microbiota, since phenolic acids are widely metabolised by bacterial strains native to the gut microbiome.[48]

Estimated total daily HGA output values (Table 1) show urine to be the dominant excretion route; 13.4–115.7 mg HGA/24 h. Estimated bile HGA output was an order of magnitude lower than urinary output; 0.28–0.49 mg HGA/24 h. Most 24-h faecal HGA output estimates were minimal due to measured HGA concentrations below or close to the assay LLOQ. The reason for variability in faecal HGA is not clear, but potential factors include individual differences in both metabolism by the gut microbiome and reabsorption efficiency of the HGA secreted into the intestine from bile.

Summed phe-tyr metabolite data indicate important differences between mice and humans (Figure 3). While summed plasma metabolites showed the clear increase in nitisinone-treated subjects that has been characterised in multiple AKU patient cohorts,[27,28] summed plasma plus urine metabolites showed no difference in mice on

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TABLE 1 Estimated range of 24-h HGA output in urine, faeces and bile in untreated AKU Hgd$^{-/-}$ mice. Values represent 24-h output of HGA calculated from measured tissue/biofluid concentrations and literature outputs of bile,[40] faeces and urine.[41] For urine, the range estimations are based on the lowest measured HGA concentration in untreated Hgd$^{-/-}$ mice combined with the lower limit 24-h urine output (0.85 mL); upper range calculated from the greatest measured urine HGA concentration combined with the upper limit 24-h urine output (3.375 mL). 24-h faecal output is based on estimated 14.5 mg total output of faeces per g of body weight.[41] 24-h bile output is based on the mean bile flow rate reported in mice, taking into account established sex differences in this measure: 2.5 mL/24-h for females and 1.8 mL/24-h for males.[40]

	Estimated 24-h HGA output range		
Biofluid/tissue	**mg**	**μmol**	**% of total HGA output**
Urine	13.4–115.7	80–689	96.4–99.8
Faeces	0–0.01	0–0.06	0
Bile	0.28–0.49	2–3	0.2–3.5

versus off nitisinone. Increases on nitisinone were only observed for plasma summed metabolites and plasma plus bile summed metabolites, suggesting that in AKU mice, consideration of urinary metabolite clearance negates the increase in summed plasma phe-tyr metabolites on nitisinone. This finding contrasts reports in humans that summed phe-tyr metabolites in total body water plus urine doubled in AKU patients on nitisinone, which was maintained across 48 months of treatment.[10] We propose decreased activity in mice of the HGA ochronotic pigment pathway as the reason for this species difference, meaning that there is not the same degree of 'unmasking' of this pathway when on nitisinone as observed in humans. Mice have nitisinone-induced tyrosinaemia to a similar degree as humans, and our data suggest that the major metabolic difference between the two species in this context is greater urinary excretion of HGA in AKU mice. Mean urine HGA (random urine) was 133.7 mmol/L in mice, which is approximately 3.8 times greater than the concentrations of 35 mmol/L (24-h collection urine) reported in SONIA 2. Increased urinary HGA excretion is a possible reason for the observation that mice do not develop such extensive macroscopic tissue ochronosis and joint destruction as in human AKU, although other factors have been proposed including the shorter lifespan of mice and protection against HGA autoxidation by endogenous vitamin C production.[12,49] Renal elimination of HGA has not yet been studied systematically in mice but the importance of the kidney to HGA detoxification in patients is well known, with renal HGA elimination rate exceeding theoretical renal plasma flow.[3]

We report the first direct observation of HGA accumulation in thyroid in AKU and thyroid tyrosine accumulation on nitisinone. Thyroid HGA and tyrosine were several orders of magnitude greater than their plasma concentrations when comparing the equivalent concentrations per weight of tissue/biofluid; tissue concentrations are in μmol per g of tissue, biofluid concentrations expressed in μmol per L of fluid (Table S1). Thyroid HGA is consistent with reports of ochronotic thyrocytes in AKU patient tissue.[33] Primary hypothyroidism has greater prevalence in AKU, in which thyroid gland dysfunction appears mainly attributable to exposure to HGA and/or HGA-derived pigment, over tyrosine. In a recent analysis, it was observed that for all cases of hypothyroidism reported in nitisinone-treated AKU patients, the hypothyroidism preceded the administration of nitisinone.[33]

Clear sex differences were observed in bile metabolites. HGA was higher in untreated male AKU mice, and tyrosine, HPLA and HPPA were higher in males at 1 week on nitisinone (Figure S7). This finding may reflect greater phe-tyr pathway flux in males due to higher dietary intake and is consistent with greater AKU disease severity in male patients.[7] The reason for higher bile phenylalanine in untreated AKU mice is not clear and requires further investigation; this finding is inconsistent with previous reports of increased plasma phenylalanine on nitisinone (vs. untreated baseline) in AKU mice.[26]

Constituents of bile, such as bile acids, show diurnal variation in mammals. In mice, this pattern is in phase with the fasting/feeding cycle, which results in release of gall bladder bile to the intestine and reabsorption to the liver during night-time feeding hours. Gall bladder bile acid concentration peaks during daytime hours in mice,[50] therefore the daytime gall bladder bile metabolite concentrations reported here may represent values towards the maximal diurnal range. It was not possible to measure diurnal bile metabolite fluctuations without much more invasive procedures. We acknowledge that studying the mice in metabolic cages would have enabled a more detailed readout of pathway flux and rates of specific metabolite elimination. Metabolic cages were avoided for several reasons, including the difficulty in acidifying urine for HGA stabilisation in this approach. Metabolic

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cages are also known to induce stress in rodents, which can affect the evaluation of important factors in phe-tyr metabolism including feeding habits and renal function.[41] We acknowledge the greater age in the wild-type control mice (29 weeks) compared to Hgd knockout mice (10–14 weeks) studied for comparison of bile and thyroid metabolites. It is unlikely that age is a major confounder in these data given the striking metabolite increases associated with Hgd gene modification and HPPD enzyme inhibition with nitisinone.

It is important to note that our targeted analysis does not capture the entirety of phe-tyr pathway flux. We have shown in previous studies, using a semi-quantitative untargeted metabolomic approach, that each metabolite of the phe-tyr transamination pathway undergoes metabolism via various alternative pathways, including phase I and II biotransformations, decarboxylation of tyrosine to products including tyramine and 4-hydroxyphenylacetic acid, and conversion of phenylalanine to various phenyl-acid metabolites.[26,43,51] We focused on the phe-tyr transamination metabolites due to the availability of established quantitative LC/MS assays for accurate measurement of these compounds. The establishment of quantitative assays for the array of phe-tyr metabolite products is currently challenging due to unavailability of the range of reference standards required but will aid future endeavours to comprehensively quantify total flux and metabolite biodistribution in this pathway.

In conclusion, our observations provide new insights into the biodistribution of phe-tyr pathway metabolites in the altered metabolic states of alkaptonuria and tyrosinaemia induced by nitisinone. For the first time, we have shown that there is biliary secretion of each of the metabolites that accumulate in these conditions, including amino acids and organic acids. Urinary excretion is confirmed as the dominant route of HGA elimination in untreated AKU, but the findings highlight that bile and tissue metabolite content should be considered in attempts to model or quantify total pathway flux in AKU and disorders of metabolism more broadly.

AUTHOR CONTRIBUTIONS

Conceptualisation: BPN, JAG, GB, LRR. *Data curation*: BPN. *Formal analysis*: BPN, AMM, ATH, ASD. *Funding acquisition*: BPN. *Investigation*: BPN, HS, PJMW, DAR. *Resources*: JCJ, GB, LRR. *Writing—original draft*: BPN. *Writing—review and editing*: all authors. All authors have read and agreed to the published version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

Lakshminarayan Ranganath received fees for lectures and consultations from Swedish Orphan Biovitrum. Brendan Norman, Hazel Sutherland, Peter Wilson, Dominic Rutland, Anna Milan, Andrew Hughes, Andrew Davison, Milad Khedr, Jonathan Jarvis, James Gallagher and George Bou-Gharios declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Raw data reported in the manuscript will be shared by the corresponding author upon reasonable request.

ETHICS STATEMENT

Research on laboratory animals reported in the manuscript was approved under project licence PP8132802 and performed in accordance with UK Home Office guidelines, under the Animals (Scientific Procedures) Act 1986.

ANIMAL RIGHTS

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

INFORMED CONSENT STATEMENT

N/A: no studies on humans were undertaken.

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