

Research Paper

Plasma and urinary NMR metabolic profiling of the phenylalanine-tyrosine pathway in nitisinone-treated alkaptonuria: comparison of two dosing regimens and identification of a candidate pharmacodynamic biomarker

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ABSTRACT

Alkaptonuria is an ultra-rare autosomal recessive inborn error of metabolism caused by a deficiency of homogentisate 1,2-dioxygenase, leading to the systemic accumulation of homogentisate and progressive ochronotic deposition in connective tissues. Nitisinone, an inhibitor of 4-hydroxyphenylpyruvate dioxygenase, effectively reduces homogentisate production but causes secondary hyper-tyrosinaemia, posing a risk of ocular complications. This study employed ¹H NMR spectroscopy to characterise the metabolic profiles of plasma and urine samples collected from 12 patients with alkaptonuria before and after three months of nitisinone treatment. Quantification of key intermediates in the tyrosine catabolism pathway revealed a marked reduction in urinary homogentisate, confirming drug efficacy, alongside a substantial rise in plasma tyrosine levels. Urinary metabolites upstream of 4-hydroxyphenylpyruvate dioxygenase showed significant accumulation, consistent with an enzymatic block. Comparison of two dosage regimens (10 mg once daily vs 10 mg on every other day) identified 4-hydroxyphenylpyruvate as the only metabolite exhibiting a statistically significant inter-regimen difference, while plasma tyrosine remained elevated in both groups. These findings highlight the complementary diagnostic value of plasma and urinary NMR profiling and underscore the limitations of current dosage strategies in controlling nitisinone-induced hyper-tyrosinaemia. NMR metabolomics provides a non-invasive, simultaneous snapshot of pathway-wide metabolic changes and represents a powerful tool for personalised therapeutic monitoring in AKU.

1. Introduction

1.1. Alkaptonuria: discovery, clinical history and epidemiology

1.1.1. Genetics and molecular epidemiology

Alkaptonuria [1] (AKU; OMIM 203500) is an autosomal recessive inborn error of tyrosine catabolism caused by deficiency of homogentisate 1,2-dioxygenase (HGD; EC 1.13.11.5), resulting in the systemic accumulation of homogentisate (HG) and its progressive deposition in connective tissues. AKU is an ultra-rare disease with a global prevalence of approximately 1:100,000–250,000 live births, but with notable geographical “hot spots” [2]. The HGD gene shows remarkable allelic heterogeneity, with more than 157 distinct pathogenic variants identified to date. The prevalence in Slovakia is estimated at 1:19,000, largely

due to a founder effect traceable to the Kysuce region of the Carpathian mountains. Elevated prevalence rates have also been reported in the Dominican Republic, Jordan, and specific communities in India. The disease affects males and females equally, as expected for an autosomal recessive condition, but males tend to develop arthritic symptoms earlier and with greater severity, for reasons that remain unclear.

1.1.2. Historical background

The first written clinical account in the modern era dates to 1584, when the German physician Scribonius described a patient whose urine turned black upon standing in the air. In 1859, Dr. Carl Boedeker observed that adding alkali to the urine of affected patients produced characteristic dark discolouration, attributing this to the reducing properties of a urinary substance he named “alkapton”, a term derived

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from Arabic alkali and Greek haptēin (“to fasten”), in reference to its avid oxygen uptake in alkaline solution, and so coined the disease name “alkaptonuria”. In 1866, Rudolf Virchow introduced the term “ochronosis” after observing under microscopy that the HG-derived pigment deposited in connective tissues appeared ochre-yellow in colour, despite its macroscopic blue-black appearance. AKU occupies a unique and foundational position in the history of human genetics: it was the first disease interpreted as a Mendelian recessive trait by Archibald Garrod in 1902. In his landmark paper “The incidence of alkaptonuria: a study in chemical individuality” [3], Garrod identified a familial pattern of inheritance and proposed that an inherited biochemical abnormality, rather than an infectious or environmental cause, must be responsible for the appearance of an abnormal intermediate in the urine. Remarkably, he conceived of a discrete metabolic intermediate at a time when virtually nothing was known of serial biochemical reactions in nutrient disposal. In his subsequent *Croonian Lectures on Inborn Errors of Metabolism* and his monograph of the same title [4], Garrod systematised the concept of heritable enzymatic defects, describing how a stable biochemical equilibrium, properly balanced enzymatic reactions, was fundamental to health, and that AKU represented a localised “error in the penultimate step” of tyrosine catabolism. The enzymatic basis of AKU was definitively established only in 1958, when La Du demonstrated the absence of HGD activity in liver tissue from a patient with AKU [5], and the gene was localised to chromosome 3q21-q23 by Pollak et al. in 1993 [6].

1.1.3. Pathophysiology

The clinical triad of AKU consists of homogentisic aciduria, ochronosis, and ochronotic osteoarthropathy [7]. Dark urine upon standing, due to oxidative polymerisation of HG, is the only manifestation present from birth and throughout childhood. Ochronosis, the deposition of HG-derived polymers in connective tissues, generally becomes apparent after age 30, manifesting as blue-black pigmentation visible in the sclerae, ear cartilage, and skin of the hands, as well as in earwax and sweat. Ochronotic arthropathies, tendon ruptures, pathological fractures, cardiac valvular dysfunction, and interstitial renal disease represent the principal sources of morbidity. Prior to the approval of nitisinone, management was entirely symptomatic, relying on analgesics and joint replacement surgery [8]. Although life expectancy is not reduced, the disease causes substantial morbidity through a predictable, age-dependent cascade of tissue damage that profoundly impairs quality of life [7,8].

1.2. Nitisinone: pharmacology and clinical use

Nitisinone (2-[2-nitro-4-(trifluoromethyl)benzoyl]cyclohexane-1,3-dione; Orfadin®; NTBC) was originally derived as a triketone herbicide from leptospermone, a natural compound produced by the Australian bottlebrush plant (*Callistemon* spp.), and was first discovered by Zeneca Agrochemicals in 1982 [9]. Its pharmacological potential was identified serendipitously during animal toxicity studies, when subherbicidal dietary doses caused keratopathy in rats through a mechanism involving plasma tyrosine elevation, prompting investigation of the underlying enzyme target [10]. Five patients with hereditary tyrosinaemia type 1 were treated with nitisinone provided by Zeneca Agrochemicals, and the first critically ill patient made a remarkable recovery [11].

Nitisinone acts as a potent, time-dependent, reversible inhibitor of 4-hydroxyphenylpyruvate dioxygenase (HPD; EC 1.13.11.27), the second enzyme in the tyrosine catabolic pathway, which converts 4-HPP to homogentisate. Studies on rat liver cytosol demonstrated a time-dependent reversible inhibition of HPD with median inhibitory concentrations (IC₅₀) of 40–173 nM and a very slow dissociation of the NTBC–enzyme complex: a single oral dose of 0.1 mg/kg produced complete inhibition of hepatic HPD within one hour, with only 40–50% of enzyme activity restored four days after dosing [10]. Nitisinone is further characterised by a long elimination half-life, on average

approximately 54 h, which provides the pharmacokinetic rationale for considering administration on alternate days, the every-other-day regimen investigated in the present study alongside once-daily dosing. By blocking HPD upstream of the HGD enzymatic defect, nitisinone prevents HG formation and thereby reduces urinary HG excretion by up to 95–99%, substantially slowing ochronosis progression [12]. Importantly, nitisinone does not inhibit tyrosine hydroxylase (TH, EC 1.14.16.2) or tyrosine aminotransferase (TAT; EC 2.6.1.5), as established in early pharmacological characterisation at Zeneca Central Toxicology Laboratories [10].

The FDA first approved nitisinone for hereditary tyrosinaemia type 1 (HT-1) [13] in 2002 [14]. Following the results of the SONIA 2 clinical trial, the EMA approved Orfadin® for the treatment of adult patients with alkaptonuria in September 2020 [12]. In 2025, the FDA also approved nitisinone for the treatment of adult patients with alkaptonuria, extending to the United States an indication already available in Europe. SONIA 2 (Suitability Of Nitisinone In Alkaptonuria 2) was a 4-year, open-label, evaluator-blind, randomised, no-treatment-controlled, parallel-group study conducted at three sites in the UK, France and Slovakia, randomly assigning patients aged 25 years or older (1:1) to receive either oral nitisinone 10 mg daily or no treatment, with daily urinary HG excretion (u-HG₂₄) as the primary endpoint at 12 months and the clinical Alkaptonuria Severity Score Index (cAKUSI) as a key secondary outcome [12]. The trial demonstrated a 98.85% reduction in urinary HG and a statistically significant slowdown in clinical disease progression as measured by cAKUSI. By inhibiting HPD, nitisinone prevents the catabolism of tyrosine beyond 4-HPP, resulting in accumulation of tyrosine in plasma across all treated patients [15]. This elevation of plasma tyrosine is analogous to tyrosinaemia type 2 [16], a related condition that causes painful palmoplantar hyperkeratosis and ocular pathology manifesting as conjunctivitis and herpetic-like corneal ulceration [17]. Following a single dose, plasma tyrosine peaked at approximately 2000 nmol/mL at 24 h, while tyrosine in the aqueous humour of the eye reached 3500 nmol/mL, substantially higher than plasma levels, returning to baseline only after three days [18]. This preferential accumulation in ocular tissues provides a direct mechanistic explanation for the predominance of corneal toxicity as the primary dose-limiting adverse effect. Clinically, keratopathy typically manifests as dendritiform sub-epithelial corneal opacities visible on slit-lamp examination, which are generally reversible upon discontinuation of nitisinone [17]. According to the EMA Summary of Product Characteristics, in AKU patients who develop keratopathy, plasma tyrosine levels should be monitored, and a diet restricted in tyrosine and phenylalanine should be implemented to keep plasma tyrosine below 500 µM; nitisinone should be temporarily discontinued and may be reintroduced upon resolution of symptoms.

1.3. The tyrosine catabolic pathway blockades

The branched catabolic network downstream of phenylalanine and tyrosine, as reconstructed from KEGG pathway databases, is illustrated in Fig. 1, and reports the dual enzymatic blocks imposed by AKU (HGD deficiency) and Orfadin (HPD inhibition). The eleven metabolites of this study are marked along the pathways to contextualise their biochemical inter-relationships. The primary degradation route is the TAT route: phenylalanine (Phe) is converted to tyrosine (Tyr) by phenylalanine hydroxylase (PAH), and tyrosine is subsequently transaminated to 4-hydroxyphenylpyruvate (4-HPP) by tyrosine aminotransferase (TAT). TAT initiates the primary hepatic catabolic route of tyrosine and has been characterised as the rate-limiting enzyme of this pathway in isolated hepatocytes [19]. 4-HPP is then the direct substrate of HPD (4-hydroxyphenylpyruvate dioxygenase), which converts it to homogentisate (HG). This is the step blocked by nitisinone: by reversibly inhibiting HPD, the drug prevents HG accumulation, thereby interrupting the cascade that leads to ochronotic pigment deposition. A secondary branch from 4-HPP leads, via MDH1, to 4-

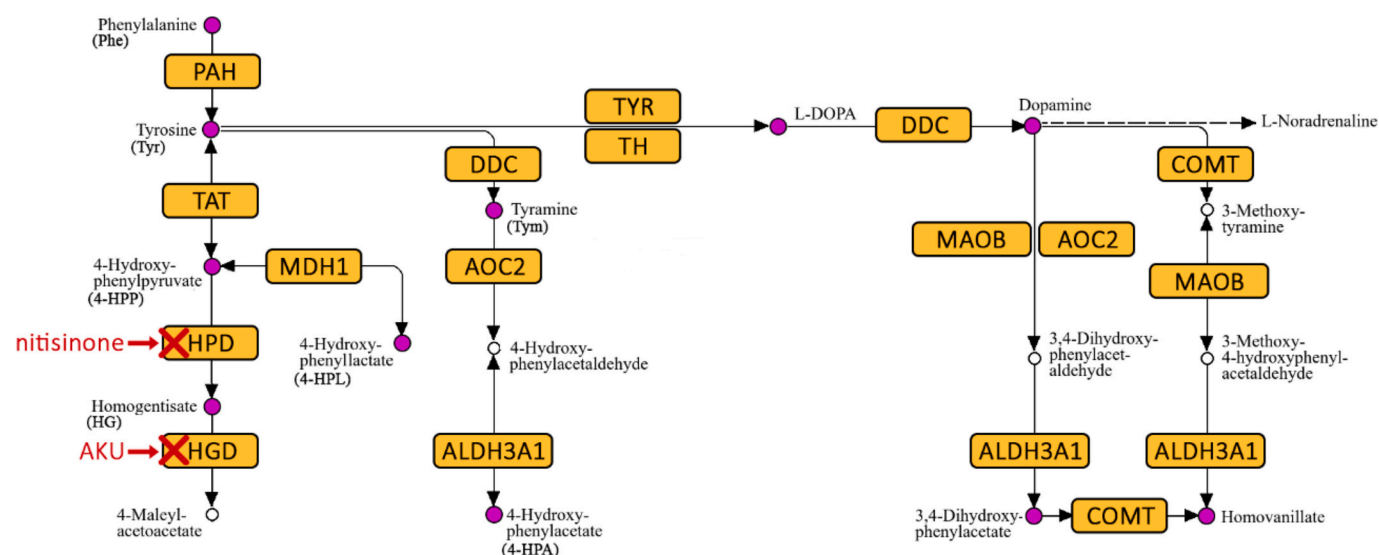


Fig. 1. Tyrosine catabolic pathway and pharmacological target of nitisinone. Enzymatic reactions are represented by orange boxes, metabolites by nodes, and the direction of metabolic flux by arrows. Metabolites quantified by ^1H NMR spectroscopy in this study are highlighted in magenta; open circles denote intermediates not directly measured. Two red crosses mark the critical points of enzymatic disruption central to this work. TAT: tyrosine aminotransferase, HPD: 4-hydroxyphenylpyruvate dioxygenase, HGD: homogentisate 1,2-dioxygenase, MDH1: malate dehydrogenase, DDC: dopa decarboxylase, AOC2: amine oxidase copper-containing 2, ALDH3A1: aldehyde dehydrogenase 3 family member A1, TYR: tyrosinase, TH: tyrosine hydroxylase, MAOB: monoamine oxidase B, COMT: catechol-O-methyltransferase. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

hydroxyphenyllactate (4-HPL), a metabolite whose increase is observable by NMR in nitisinone treatment [20]. HG undergoes ring cleavage by HGD to form 4-maleylacetoacetate, which is subsequently isomerised to 4-fumarylacetoacetate by maleylacetoacetate isomerase (GSTZ1) and finally hydrolysed by fumarylacetoacetate hydrolase to yield fumarate and acetoacetate. This last step is the one disrupted in HT-1. In alkaptonuria, loss-of-function mutations in HGD abolish this step (see Fig. 1), causing HG to accumulate and polymerise [21,22] into the ochronotic pigment responsible for the disease's progressive tissue damage. A secondary catabolic branch, the DDC route, diverges from tyrosine itself via decarboxylation by DDC (DOPA decarboxylase) to form tyramine (Tym), a biogenic amine rapidly catabolised by amine oxidase copper containing 2 (AOC2) and further transformed into 4-hydroxy-phenylacetate (4-HPA), a homogentisate similar but non-reactive [21]. Tyrosine can also be hydroxylated by tyrosine hydroxylase (TH) to L-DOPA, which is further decarboxylated by DDC to dopamine, entering the catecholamine biosynthetic cascade and degraded to 3,4-dihydroxyphenylacetate and homovanillate (via COMT and MAOB-mediated pathways). These branches are secondary under physiological conditions but become metabolically relevant during HPD inhibition or HGD deficiency, when the primary TAT route is blocked, and tyrosine substrate pressure increases [20].

Taken together, the figure captures the biochemical rationale of this study: nitisinone shifts the metabolic burden upstream of HPD, while AKU independently blocks HG clearance at HGD. The dual pharmacological and genetic disruption of this pathway, and the NMR-measurable metabolic consequences in plasma and urine, form the mechanistic backbone of the present work, aiming to (i) characterise the NMR metabolic profiles of plasma and urine in AKU patients at baseline and after three months of nitisinone therapy; (ii) quantify changes in key tyrosine catabolism metabolites to assess drug efficacy and side-effect profile; and (iii) compare metabolic responses between two clinically adopted dosage regimens to inform future personalised dosing strategies.

2. Methods

2.1. Biological samples

12 patients with Alkaptonuria (8 males, 4 females; mean age 48) were enrolled to receive nitisinone treatment (clinical characteristics are summarised in Table 1). All patients received nutritional counselling and followed a tyrosine- and phenylalanine-restricted diet as part of standard care under nitisinone, restriction of tyrosine intake necessarily entailing restriction of phenylalanine as its metabolic precursor. The entire study was conducted with approval from the Siena University Hospital Ethics Committee (n° CELAOUS100721). The informed consent obtained from all participants conformed to the standards set by the latest revision of the Declaration of Helsinki. Plasma and urine samples were collected before (T0) and three months after the treatment (T1), following overnight fasting, and promptly stored at -80°C . Patients were stratified by dosage regimen: six received 10 mg every other day (e.o.d), six received 10 mg once daily (o.d.).

2.2. Sample preparation

All reagents and solvents were purchased at analytical grade from Merck Life Science (Darmstadt, Germany): deuterium oxide (D_2O), Tris-d_{11} (Tris(hydroxymethyl- d_3)amino- d_2 -methane), calcium formate, potassium hydrogen phosphate (K_2HPO_4), potassium dihydrogen phosphate (KH_2PO_4), sodium azide (NaN_3), and trimethylsilylpropionic acid- d_4 (TSP). Deionised water was obtained using a Milli-Q® Millipore system (Burlington, MA, USA). A stock solution of phosphate buffer 333 mM (PB) was prepared from K_2HPO_4 and KH_2PO_4 , and pH was adjusted to 7.0 with NaOH 1 M. NMR urine samples were prepared with 300 μL of

Table 1
Clinicopathological characteristics of the AKU patient cohort.

Clinicopathological characteristics	Cohort (n = 12)
Sex	8 males (67%), 4 females (33%)
Mean age (years)	48
Dosage Regimen e.o.d (n = 6)	10 mg every other day
Dosage Regimen o.d. (n = 6)	10 mg once daily

urine, 30 μ L TSP 10 mM and 270 μ L PB to final concentrations of 0.5 mM TSP and 150 mM PB. NaN_3 0.1% (p/p) was added to samples to prevent microbial proliferation. NMR plasma samples were prepared with 250 μ L of plasma, 310 μ L TRIS- d_{11} buffer 150 mM (final concentration of 65 mM), and 40 μ L formate 45 mM (final concentration of 3 mM). The total deuterium concentration in all samples was maintained at 10% for NMR purposes.

2.3. NMR data acquisition and processing

^1H 1D NMR spectra were acquired at 298 K on a Bruker Avance III 600 MHz spectrometer (Bruker BioSpin, Karlsruhe, Germany) operating at 14.1 T. Urine samples were acquired with a spectral width of 16 MHz and 32 scans digitised over 32 k points and RG of 128 using an optimised-PURGE pulse sequence. Solvent signal removal was achieved with a pre-saturation power of 55 dB during repetition delay (4 s). For plasma, the PROJECT sequence (Periodic Refocusing of J Evolution by Coherence Transfer) [23,24] was used with an echo time (τ) of 0.3 ms, and 128 loops to get a T2 filter delay of 153.6 ms. To allow full magnetisation recovery for the compound, an additional delay (20s) was added to T2 pulse sequences before repetition/presaturation delay [25]. The spectra were acquired with a spectral width of 10 kHz. All spectra were obtained with 16 scans, digitalised over 32 k points and zero-filled to 128 k points. Solvent signal removal was achieved with a presaturation power of 55 dB during delay d1 (4s). The NMR data were processed with TopSpin 4.0.8 software and analysed with Chenomx 10 (Chenomx Inc., Edmonton, CA). Metabolite concentrations were determined by spectral library fitting, using TSP and formate concentrations as internal standards. Urinary metabolite concentrations were normalised to urinary creatinine, quantified from the same ^1H NMR spectra, and are reported throughout as analyte-to-creatinine ratios ($\mu\text{mol}/\text{mmol}$ creatinine).

2.4. Statistical analysis

Within the regimen, metabolite concentrations (the same patient's T0 vs T1) were compared using the paired Wilcoxon signed-rank test. Between-regimen (o.d. vs e.o.d.) concentration deltas (T1-T0) were compared using the Mann-Whitney test. Given the small cohort size, non-parametric tests that do not assume normality of the distribution were preferred to parametric tests. In both cases, adjustment for multiple comparisons was applied using the false discovery rate according to Benjamini, Krieger, and Yekutieli, with a Q of 5%. All statistical analyses were performed in GraphPad and visualised as box plots.

3. Results

3.1. NMR metabolomic profiling

^1H NMR spectroscopy exploits the magnetic properties of hydrogen nuclei to yield quantitative, structurally informative profiles of complex biological matrices without the need for exogenous tracers [26]. Key advantages include robustness, high reproducibility, minimal sample preparation, and the capacity to detect and quantify dozens of metabolites simultaneously in a single acquisition [27]. These properties make NMR particularly suited to longitudinal clinical studies and metabolic phenotyping. Urine is especially amenable to metabolomic profiling, given its abundance of small-molecule catabolites and ease of access; plasma reflects the systemic physiological state and complements the urinary snapshot. In rare diseases such as AKU, where sample volumes are limited and patient numbers are small, the untargeted nature of NMR offers unparalleled efficiency by capturing the entire accessible metabolome in a single measurement [28]. The pheno-metabonomic concept, predicting drug response from pre-treatment metabolic profiles, positions NMR metabolomics as a forerunner of personalised medicine and patient stratification in rare metabolic diseases [28,29].

Superimposed ^1H NMR spectra of urine from a representative AKU patient (o.d.) at T0 and T1 are shown in Fig. 2. Before treatment (T0, black), the spectral region 6.7–6.8 ppm is dominated by intense resonances characteristic of HG, consistent with the high urinary HG load in untreated AKU [20]. After treatment (T1, red), these signals collapse dramatically, while new multiplets in the 6.9–7.0 ppm region emerge from tyrosine, phenylalanine, and aromatic metabolites (4-hydroxyphenylacetate, 4-hydroxyphenyllactate, 4-hydroxyphenylpyruvate, tyramine) that accumulate as a consequence of HPD inhibition (Fig. 1). The qualitative shift in spectral pattern is thus a direct visual readout of nitisinone's mechanism. However, despite the high clearance of such metabolites through urine, tyrosine increases its concentration more than 10-fold in plasma, as shown by comparison of samples from the same patient before and after the treatment (Fig. 3). Out of the eleven metabolites confirmed by spiking in samples, seven gave NMR signals above the limit of detection (LoD) in urine and two in plasma: Phe, Tyr, Tym, 4-HPA, 4-HPL, 4-HPP, HG and Phe and Tyr, respectively. They were quantified via spectral integration across all samples, and statistical results are reported by biofluid (plasma, urine) and dose regimen (o.d., e.o.d.) in Fig. 4. They can be divided into precursors (Phe, Tyr), the TAT route (4-HPP, 4HPL, HG), and the DDC route (Tym, 4-HPA). Metabolites from the TH route showed no relevance in biofluids.

3.1.1. Phenylalanine and tyrosine — Precursors

Plasma tyrosine and phenylalanine levels increased markedly in the continuous dosing group, consistent with effective and sustained HPD blockade that prevents tyrosine catabolism via the TAT route (Fig. 1) and exerts stronger upstream pressure on the phenylalanine-to-tyrosine conversion pathway. The rise in plasma tyrosine (Fig. 3) was mirrored by a significant increase in urinary tyrosine excretion, indicating that the degree of drug-induced tyrosinaemia exceeded the renal reabsorption threshold, resulting in overflow tyrosinuria. The magnitude of the urinary tyrosine increase was disproportionately larger than the plasma increase, acting as a relief valve for the plasma tyrosine burden imposed by nitisinone. The every other day regimen reproduced the same pattern for tyrosine, with significant increases in both plasma and urine, and a non-significant trend for plasma phenylalanine, indicating that the pharmacodynamic effect on the tyrosine pool is largely preserved even with the less intensive dosing schedule, whereas the upstream effect on phenylalanine metabolism appears more sensitive to differences in drug exposure.

3.1.2. Homogentisate, 4-hydroxyphenylpyruvate and 4-hydroxyphenyllactate — TAT route

Along the TAT route, nitisinone at 10 mg once daily clearly exerts its effect, reducing urinary homogentisate levels below the LoD. Concurrently, the HPD blockade by nitisinone caused significant accumulation of its substrate 4-hydroxyphenylpyruvate (4-HPP) in urine, consistent with upstream metabolite trapping at the site of enzyme inhibition. The increase in urinary 4-hydroxyphenyllactate (4-HPL) reflects the compensatory reduction of accumulated 4-HPP by MDH1, representing an alternative catabolic outlet that partially relieves the 4-HPP burden. The every other day regimen reproduced the same pattern along the TAT route, with the same reductions in urinary HG and significant increases in 4-HPP and 4-HPL, demonstrating that HPD inhibition is sufficiently sustained even with alternate-day dosing to effectively suppress HG production.

3.1.3. Tyramine and 4-hydroxyphenylacetate — DDC route

Along the DDC route, nitisinone at 10 mg once daily induced a significant increase in urinary tyramine, consistent with enhanced flux through DDC-mediated decarboxylation of tyrosine as an alternative catabolic outlet when the TAT axis is pharmacologically blocked at HPD step [21]. The increased availability of tyrosine secondary to HPD inhibition appears to drive greater substrate delivery to DDC, thereby augmenting tyramine production. Urinary 4-hydroxyphenylacetate (4-

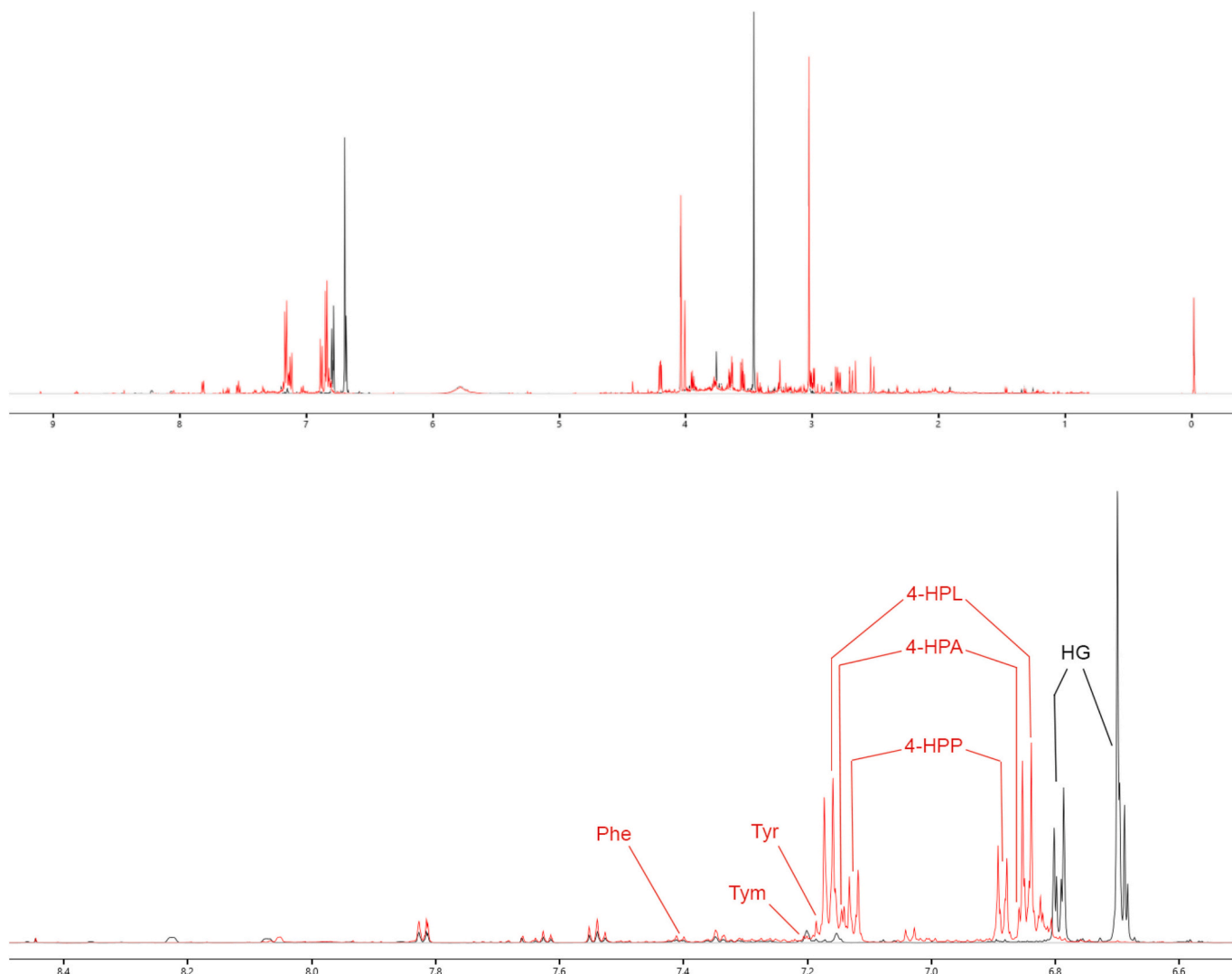


Fig. 2. Representative ^1H NMR spectra of urine from an AKU patient collected at baseline (T0, black) and following nitisinone treatment (T1, red). The upper panel displays the full spectral range, while the lower one shows an expanded view of the aromatic region (δ 6.5–8.5 ppm). Prior to treatment (T0), homogentisate (HG) resonance dominated the spectrum. Following nitisinone administration, HG signals decreased below the limit of detection (LoD), consistent with the mechanism of action of nitisinone. Alongside, upstream metabolites (4-HPL, 4-HPA, 4-HPP, Tyr, Tym, and Phe), accumulated at T1, as evidenced by the intensification of their signals in the aromatic region. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

HPA) also increased significantly in the continuous dosing group (o.d.), reflecting enhanced activity of the downstream AOC2-ALDH3A1 axis converting tyramine-derived intermediates to 4-HPA, further supporting increased overall flux through the DDC branch. The e.o.d. regimen reproduced a significant increase in 4-HPA, confirming that the rerouting of tyrosine catabolism toward the DDC pathway occurs under both dosing schedules.

3.2. Dose regimen effects

Taken together, activation of both the TAT alternative branch and the DDC route under nitisinone treatment at both doses indicates broad metabolic adaptation to sustained HPD blockade, with both schedules achieving equivalent suppression of the primary therapeutic target, urinary HG. To assess whether the magnitude of nitisinone-induced metabolic change differed between the two dosing regimens, within-patient differences (ΔC , T1 – T0) for each metabolite were compared between the o.d. and e.o.d. groups using the Mann-Whitney test with false discovery rate correction, see Fig. 5 Fig. 4 (two-stage step-up method, Benjamini, Krieger and Yekutieli; desired FDR 5%). After FDR

correction, urinary 4-hydroxyphenylpyruvate (4-HPP) was the only metabolite showing a statistically significant difference between regimens. This finding is mechanistically informative: 4-HPP is the direct substrate of HPD, the enzyme inhibited by nitisinone, and its urinary accumulation therefore reflects the degree and continuity of HPD blockade. The significant decrease in urinary 4-HPP in the e.o.d. group compared to the o.d. group is consistent with intermittent HPD inhibition allowing partial enzymatic recovery during the drug-free day, resulting in a transient but measurable increase in 4-HPP flux through the TAT route before conversion to homogentisate is again suppressed upon the next dose. This pharmacodynamic signature at the immediate site of drug action, with 4-HPP uniquely sensitive to dosing schedule while all other pathway metabolites, including HG, tyrosine and the DDC route products, remained statistically indistinguishable between regimens, suggests that the e.o.d. schedule preserves sufficient overall HPD inhibition to achieve comparable therapeutic efficacy in terms of HG suppression, while producing a distinct upstream metabolic fingerprint at the level of the direct enzymatic substrate. All remaining metabolites, including urinary HG, plasma and urinary tyrosine, phenylalanine, 4-HPL, 4-HPA, and tyramine, showed no significant

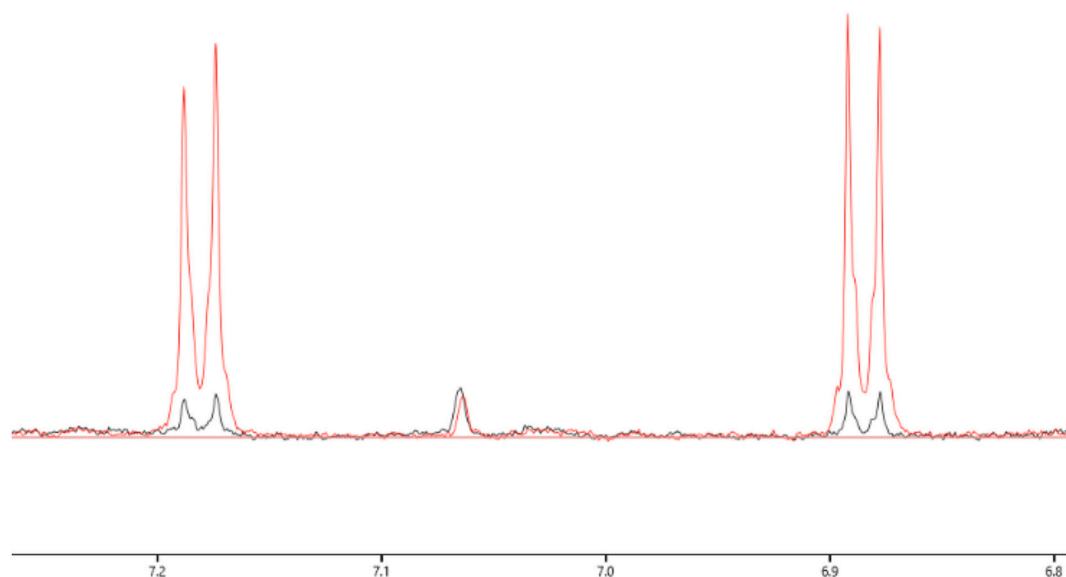


Fig. 3. ^1H NMR spectra of an AKU patient plasma before (T0, black) and after nitisinone treatment (T1, red). The signals displayed correspond to the characteristic Tyrosine system. Following nitisinone treatment, a drastic increase in plasma tyrosine signal intensity is observed, consistent with the upstream accumulation of this metabolite resulting from HPD inhibition. This elevation, together with the increase in urinary tyrosine excretion, indicates that the degree of drug-induced tyrosinaemia exceeded the renal reabsorption threshold. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

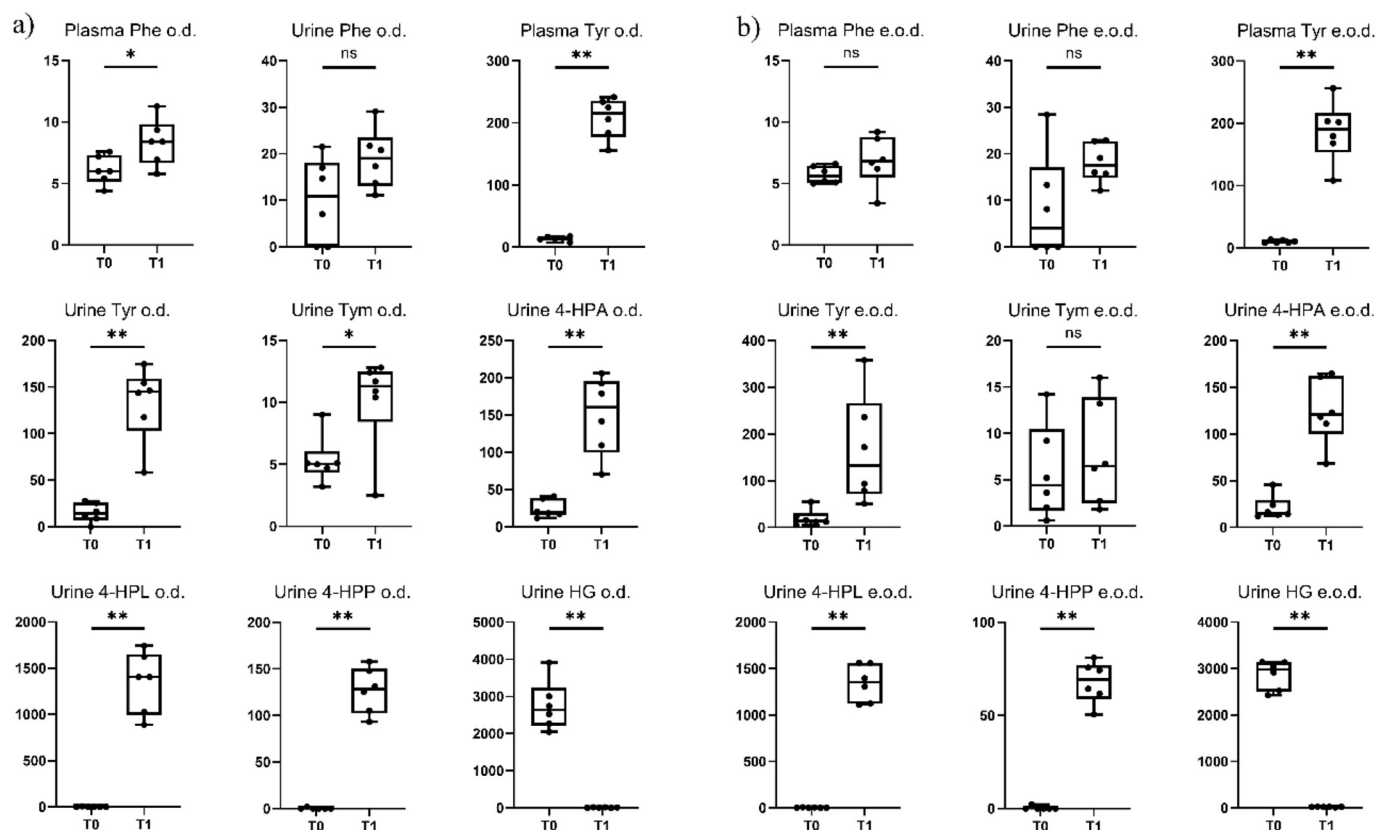


Fig. 4. Plasma and urinary metabolite concentrations before (T0) and after (T1) nitisinone treatment in AKU patients. Boxplots show the distribution of plasma (μM) and urinary ($\mu\text{mol}/\text{mmol}$ creatinine) concentrations of phenylalanine (Phe), tyrosine (Tyr), homogentisate (HG), 4-hydroxyphenylpyruvate (4-HPP), 4-hydroxyphenyllactate (4-HPL), 4-hydroxyphenylacetate (4-HPA), and tyramine (Tym) in patients treated with nitisinone 10 mg once daily (o.d., $n = 6$) in panel a) and 10 mg on every other day (e.o.d., $n = 6$) in panel b). Statistical comparisons between T0 and T1 were performed using the Wilcoxon matched-pairs signed-rank test with exact p -value calculation. P -values were corrected for multiple comparisons using the two-stage step-up false discovery rate method (Benjamini, Krieger, and Yekutieli) at a desired FDR of 5%. Asterisks indicate the level of statistical significance after FDR correction: * $q < 0.05$, ** $q < 0.01$; ns, not significant.

inter-regimen difference after FDR correction, supporting the conclusion that the two dosing schedules are compatible with a broadly similar

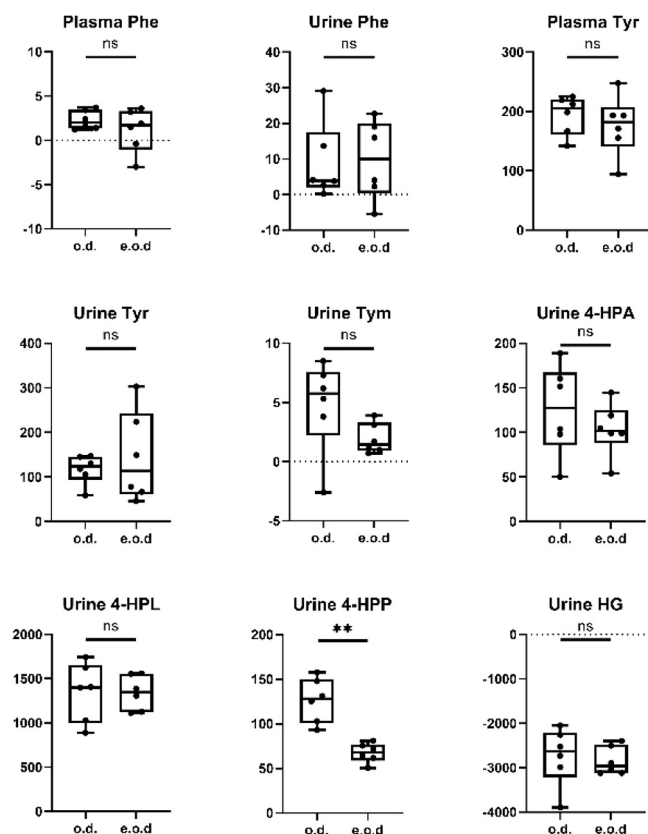


Fig. 5. Plasma (μM) and urine ($\mu\text{mol}/\text{mmol}$ creatinine) levels change after treatment with nitisonone compared side by side for the o.d. and e.o.d. regimens.

response across the Phe-Tyr pathway, with the notable exception of the immediate HPD substrate pool.

4. Discussion

This study provides a comprehensive NMR metabolomic characterisation of the Phe-Tyr catabolic perturbations induced by nitisonone in AKU patients, integrating both plasma and urinary data from a well-characterised cohort and comparing two clinically relevant dosage regimens.

The most robust and clinically meaningful finding is the near-complete suppression of urinary HG following initiation of nitisonone, corroborating the data from the SONIA 2 trial and earlier metabolic flux analyses [12] by using our nuclear magnetic resonance approach. The near-complete suppression of urinary HG is mechanistically attributable to nitisonone's near-complete blockade of HPD, which eliminates the substrate available to the defective HGD enzyme. The tight clustering of T1 HG values near zero, in contrast to the broad T0 distribution spanning up to 28,000 μM , confirms that this pharmacological effect is highly consistent across patients despite substantial inter-individual variability in upstream metabolic responses. Crucially, HG suppression was equivalent between the o.d. and e.o.d. regimens, suggesting the alternate-day schedule as an effective therapeutic alternative for the primary clinical endpoint.

The secondary rise in plasma tyrosine is the principal pharmacological liability of nitisonone therapy in AKU. Our data show that this hyper-tyrosinaemia is not attenuated by reducing the dosing frequency to every other day.

The marked accumulation of urinary 4-hydroxyphenyllactate (4-HPL) in both treatment groups reflects redirection of the elevated 4-HPP pool through the MDH1-mediated side branch of the TAT route. Under normal conditions, 4-HPP is rapidly converted to HG by HPD, and 4-HPL

remains a minor metabolite; when HPD is blocked, accumulating 4-HPP undergoes compensatory enzymatic reduction to 4-HPL. This is consistent with SONIA 1 data [30], in which serum 4-HPL showed the greatest proportional, dose-dependent increase among TAT-route metabolites, interpreted as preferential conversion of accumulated 4-HPP to 4-HPL rather than to tyrosine. The extremely wide interquartile range of urinary 4-HPL at T1 in our cohort, with individual values ranging from near-zero to over 20,000 μM , likely reflects substantial inter-individual variability in MDH1 activity and dietary substrate load, cautioning against its use as a quantitative pharmacodynamic marker in small cohorts. The absence of a significant inter-regimen difference for 4-HPL, in contrast to 4-HPP, further suggests that MDH1-mediated conversion acts as a metabolic buffer, partially equalising the upstream 4-HPP signal between dosing schedules.

The increase in urinary tyramine is consistent with enhanced flux through the decarboxylation branch (DDC) of tyrosine catabolism. As nitisonone blocks the TAT-HPD axis, the increased availability of tyrosine as a substrate drives augmented DDC-mediated decarboxylation to tyramine. This is in agreement with Norman et al. [31], who reported a statistically significant increase in urinary tyramine in SONIA 2 nitisonone-treated patients, with a fold change of 2.1 against baseline at two years. The moderate effect size of urinary tyramine compared to TAT-route metabolites reflects the secondary nature of the DDC route: although tyrosine substrate pressure increases substantially under nitisonone, the enzymatic capacity of DDC appears limited. The absence of a significant inter-regimen difference for tyramine is consistent with this interpretation: DDC-route activation is driven primarily by overall tyrosine availability, which does not differ significantly between o.d. and e.o.d. dosing. The substantial increase in urinary 4-hydroxyphenyllactate (4-HPA) represents the clearest signature of DDC route activation in our dataset, with median values rising from near-zero at T0 to approximately 500–700 μM at T1 and individual outliers approaching 2500 μM . 4-HPA is formed downstream of tyramine via AOC2-mediated oxidative deamination followed by ALDH3A1-mediated oxidation, and its urinary excretion therefore reflects cumulative flux through the entire DDC branch. The magnitude of the 4-HPA increase is disproportionately larger than that of tyramine, suggesting that downstream oxidative steps efficiently convert the modest tyramine surplus into 4-HPA, which accumulates in urine due to its hydrophilic nature and poor tubular reabsorption. This finding extends the observations of Norman et al. [31] and, to our knowledge, provides the first quantitative NMR-based characterisation of 4-HPA dynamics in an Italian AKU cohort at the clinically approved 10 mg dose. The absence of a significant inter-regimen difference for 4-HPA mirrors the tyramine result, reinforcing the conclusion that DDC-route activation is governed by the common tyrosine substrate pool rather than by the degree of HPD inhibition per se.

The potential sensitivity of urinary 4-HPP to dosing schedule, in the context of overall pharmacodynamic equivalence between regimens, suggests that this metabolite may serve as a nuanced and mechanistically grounded biomarker of HPD inhibition intensity in nitisonone-treated AKU patients [32]. Unlike HG, whose suppression is near-complete at both dosages and therefore lacks discriminatory power between regimens, 4-HPP accumulates in direct proportion to the extent and continuity of HPD blockade, positioning it as a more sensitive indicator of the pathway's pharmacodynamic state at the immediate site of drug action. In this framework, urinary 4-HPP could function as a real-time readout of effective HPD inhibition, complementing HG as a therapeutic efficacy marker: while HG confirms that the primary therapeutic objective is met, 4-HPP would reflect the degree of residual enzymatic activity and the adequacy of drug exposure between doses. This distinction is of potential clinical relevance in scenarios where dosage optimisation is being considered, such as in patients experiencing nitisonone-related adverse effects, most notably corneal keratopathy secondary to hypertyrosinaemia, in whom dose reduction or interval extension is contemplated. In such cases, monitoring urinary 4-HPP

alongside HG could provide a more complete pharmacodynamic picture, allowing clinicians to identify the minimum effective dosing schedule that maintains adequate HG suppression while minimising tyrosinaemia-related toxicity. The incorporation of 4-HPP into routine biochemical monitoring panels for nitisinone-treated AKU patients, alongside the currently recommended HG and tyrosine measurements, therefore warrants prospective evaluation in larger cohorts.

A methodological limitation of the present study concerns the quantification of HG in plasma. With respect to aromatic aminoacids, a disadvantage is intrinsic to the spin systems involved: tyrosine and phenylalanine each give aromatic doublets arising from two equivalent protons, whereas the 1,2,4-trisubstituted ring of HG distributes its intensity across three non-equivalent one-proton resonances, so that at equal molar concentration the signal per peak is two- to threefold lower. The CH₂ peak at 3.5 ppm would indeed be suitable for quantification but is severely overlapped with glucose resonances. Under nitisinone, the constraint becomes absolute rather than marginal: plasma HG decreases by approximately 95% [33]. The absence of a quantifiable plasma HG signal at T1 is therefore an intrinsic consequence of the analyte's post-treatment concentration. It is also relevant that the primary endpoint of both SONIA 1 [30] and SONIA 2 [12] is 24-h urinary HG excretion rather than circulating concentration, and urinary HG is precisely the compartment in which the present approach performs best, showing near-complete, regimen-independent suppression at T1.

5. Conclusions

This study provides a comprehensive metabolomic characterisation of the biochemical response to nitisinone therapy in alkaptonuria, demonstrating that NMR enables simultaneous assessment of therapeutic efficacy, metabolic adaptation, and treatment-related metabolic perturbations across the phenylalanine and tyrosine catabolic pathway. In addition to confirming the near-complete suppression of urinary homogentisate, the primary therapeutic target of nitisinone, our results show that HPD inhibition induces a coordinated metabolic rewiring, with activation of both the alternative TAT branch and the DDC pathway, reflected by the accumulation of 4-hydroxyphenylpyruvate, 4-hydroxyphenyllactate, tyramine, and 4-hydroxyphenylacetate. The complementary analysis of plasma and urine provides an integrated view of systemic metabolic changes together with renal excretion, highlighting the marked increase in plasma tyrosine and the associated overflow tyrosinuria.

Comparison of the once daily and every other day dosing regimens showed comparable suppression of urinary homogentisate and broadly similar metabolic responses across the pathway, indicating that alternate-day administration preserves the overall pharmacodynamic effect of nitisinone. Among all quantified metabolites, urinary 4-hydroxyphenylpyruvate was the only metabolite that distinguished between the two dosing schedules, reflecting differences in the continuity of HPD inhibition, while the primary therapeutic outcome remained unchanged. This finding supports urinary 4-hydroxyphenylpyruvate as a pharmacodynamic biomarker that complements homogentisate by providing information on the degree of HPD inhibition rather than therapeutic efficacy alone.

Overall, these findings extend the current understanding of the metabolic response to nitisinone in AKU and support the integration of pathway-focused metabolomic profiling into therapeutic monitoring. Validation in larger, prospective cohorts, including evaluation of lower nitisinone doses and longitudinal clinical outcomes, will be essential to determine the clinical utility of urinary 4-hydroxyphenylpyruvate for treatment optimisation and to develop metabolomics-guided strategies that maximise therapeutic benefit while minimising tyrosinaemia-related adverse effects.

From a translational standpoint, AKU requires not maximal sensitivity for a single analyte but reliable, repeatable follow-up of chronic therapy across several metabolites at once. ¹H NMR meets this

requirement, since biofluids are analysed essentially untreated, quantification is referred to a single internal standard rather than to analyte-specific calibration curves, and the measurement is non-destructive and untargeted, so that reproducibility across serial time points is preserved and metabolites not anticipated at the outset remain accessible. Where the analyte is scarce, as for plasma homogentisate under nitisinone, mass spectrometry remains the more sensitive option, so the two approaches are complementary rather than alternative, the choice depending on the analyte and on the purpose of the measurement. In its benchtop form, moreover, NMR is operationally simpler, making it attractive for routine follow-up of patients on long-term nitisinone.

CRedit authorship contribution statement

Daniela Grasso: Methodology, Investigation. **Valentina Balloni:** Data curation. **Elena Bianchi:** Validation, Resources. **Andrea Mazzon:** Validation, Resources. **Marco Bardelli:** Validation, Resources. **Gabriella Iacomelli:** Resources. **Luana Peruzzi:** Resources. **Bruno Frediani:** Writing – review & editing. **Annalisa Santucci:** Writing – review & editing. **Andrea Bernini:** Writing – original draft, Supervision, Conceptualization.

Ethics statement

The study was conducted in accordance with the Declaration of Helsinki and approved by the local institutional review board. Informed written consent was obtained from all participants.

Ethics declaration

Written informed consent to take part in the study and to publish the article has been obtained from all participants or their legal representatives. The privacy rights of participants have been observed.

This study included organ or tissue donors.

This study includes human biological material and consent was obtained by donors, or their next of kin or legal representatives, for use in this study and for publication of the article. The samples used in this research were not sourced from executed prisoners or prisoners of conscience.

This study was performed in compliance with relevant laws, regulatory frameworks and guidelines where the research took place.

This study was approved by the Siena University Hospital Ethics Committee.

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Declaration of competing interest

The authors declare no conflicts of interest.

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Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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