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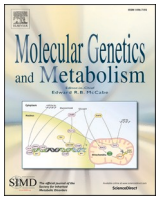

Two new cases of KYNU deficiency: Further delineation of the phenotypic and biochemical spectrum and exploration of treatment options

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ABSTRACT

Congenital NAD^+ deficiency disorders (CNDD) represent a novel category of hereditary diseases that affect NAD^+ biosynthesis from tryptophan. CNDD include kynureninase (KYNU) deficiency with only ten documented patients to date. Here, we report two new cases. In addition to the previously described clinical phenotype of congenital heart defects, skeletal abnormalities, hearing loss and airway malacia, our patients also had hypothyroidism, recurrent infections, and necrotizing enterocolitis. The first patient's exome analysis identified compound heterozygosity for two novel *KYNU* variants, pathogenicity being supported by extensive metabolic profiling. The second patient was homozygous for a known *KYNU* missense variant. For the first time we describe a potential treatment of KYNU deficiency as both patients were treated with oral nicotinamide (a B3 vitamer and NAD^+ precursor) and pyridoxine (a vitamin B6, cofactor of KYNU) from young age, which improved biochemistry without evident short-term adverse effects. Furthermore, we show that maternal supplementation with nicotinamide riboside (another B3 vitamer and NAD^+ precursor) during pregnancy appears to be safe. In conclusion, KYNU deficiency is an extremely rare disorder associated with severe congenital defects, demonstrating the indispensable role of *de novo* NAD^+ biosynthesis in foetal development. Metabolomic profiling is a valuable tool in the analysis of new genetic variants. NAD^+ precursor supplementation in the form of nicotinamide, combined with pyridoxine, may be a novel therapeutic approach.

1. Introduction

Nicotinamide adenine dinucleotide (NAD^+) and its derivates play a central role in energy metabolism and are involved in over 450 intracellular reactions. The redox couple of NAD^+ (oxidized form) and NADH (reduced form) functions in various catabolic pathways, including the tricarboxylic cycle, glycolysis, and fatty acid beta-oxidation. In these reactions, NAD^+ acts as a cofactor for numerous dehydrogenases, while the generated NADH shuttles electrons to the electron transport chain, yielding ATP. NAD^+ is also phosphorylated to NADP(H) which drives anabolic reactions and regulates oxidative stress. Finally, NAD^+ is also a substrate for NAD^+-consuming enzymes, including sirtuins, poly(ADP-ribose)-polymerases (PARPs), and cyclic ADP-ribose (cADPr) synthases. Together, these NAD^+-consuming enzymes regulate energy

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metabolism and gene expression and preserve DNA integrity (reviewed by [1]).

NAD^+ can be produced from (a) tryptophan *via* the *de novo* biosynthesis pathway, (b) from dietary precursors (niacin), and (c) salvaged NAD^+ precursors (Fig. 1; (reviewed by [1])). The term niacin is used to define the family of B3 vitamers, consisting of nicotinic acid (NA), nicotinamide (NAM), nicotinamide riboside (NR) and nicotinamide mononucleotide (NMN). Niacin is found in a wide variety of foods, including meat, fish, vegetables, whole-grain products and potatoes. The *de novo* biosynthesis pathway, also referred to as the kynurenine pathway, occurs mainly in the liver, where all its enzymes are expressed. *Via* this pathway, approximately 90 % of available tryptophan is catabolized to yield either NAD^+ or provide energy by degradation to acetyl-CoA (reviewed by [2]).

In recent years, a new group of genetic disorders have been elucidated affecting NAD^+ synthesis from tryptophan, collectively named congenital NAD deficiency disorders (CNDD) [3]. CNDD include genetic deficiencies of kynureninase (*KYNU*), 3-hydroxyanthranilate 3,4-dioxygenase (*HAAO*), and NAD synthetase 1 (*NADSYN1)* [3,4]. Patients affected by these disorders share congenital defects of the heart, vertebrae, limbs, kidneys and trachea, illustrating an indispensable role of *de novo* NAD^+ biosynthesis during embryonic development [5]. Given the multitude of reactions dependent on NAD^+ and its derivatives, it is a formidable challenge to disentangle the pathophysiology underlying CNDD-related malformations. Moreover, most studies of NAD^+ metabolism have been performed in postnatal models and not in the context of pregnancy and embryonic development. Nevertheless, it is speculated that NAD^+ deficiency interferes with crucial processes such as patterning, cell fate determination, coordinated growth, morphogen gradients, boundary formation and branching morphogenesis (reviewed by [5]).

To date, ten cases of patients with KYNU deficiency have been published [3,6–8]. Here we report two new cases of KYNU deficiency, with severe congenital malformations, including heart defects and skeletal abnormalities. We describe the results of metabolic profiling that supported the pathogenicity of the novel genetic variants, and discuss the potential effects of treatment with NAM and vitamin B6, as the latter is a cofactor of KYNU.

2. Results

2.1. Case descriptions

The features and clinical course of the two patients are outlined in Table 1 and Fig. 2.

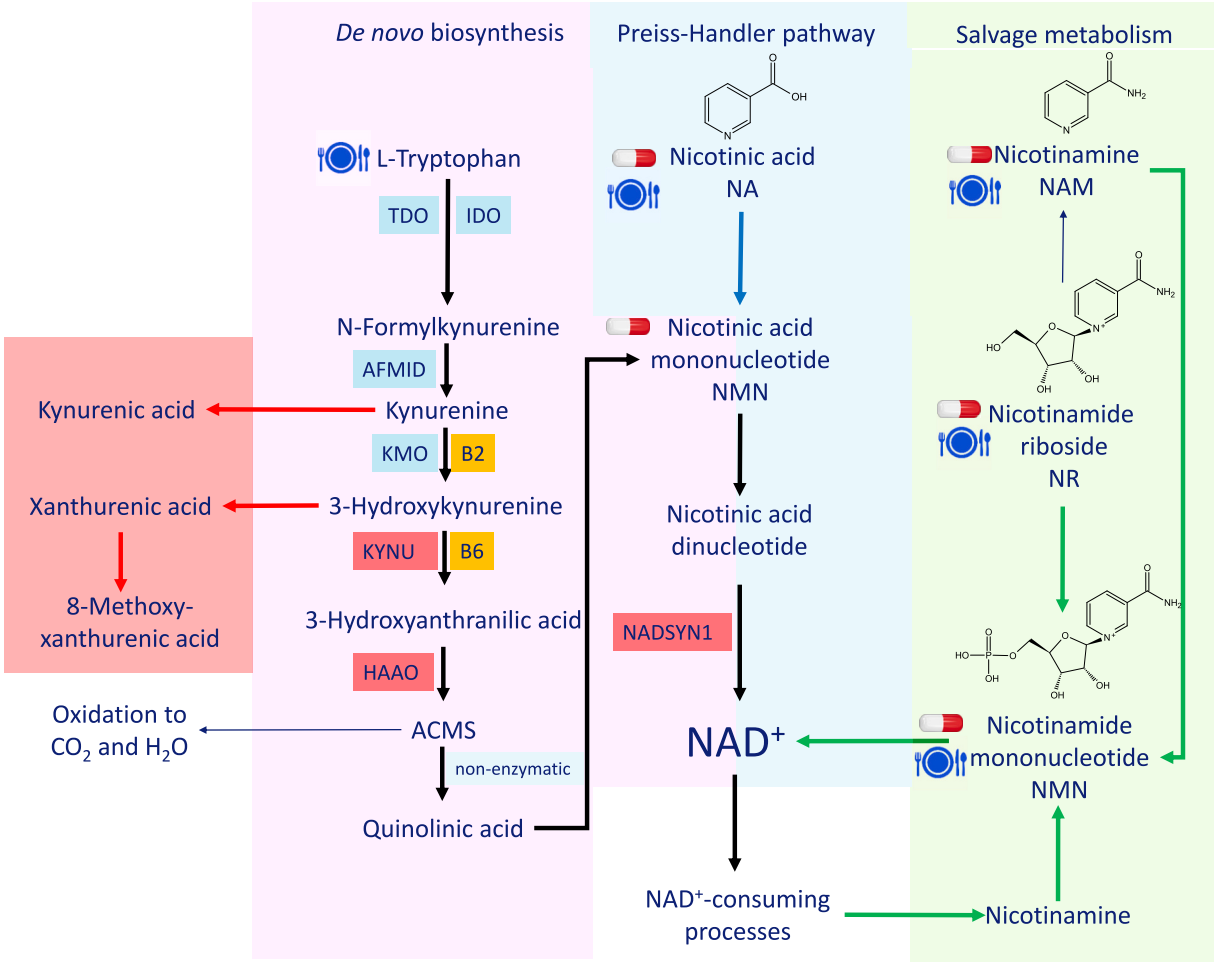

Fig. 1. KYNU pathway and NAD^+ precursors.
Three biochemical pathways contribute to NAD^+ biosynthesis; the *de novo* biosynthetic pathway from tryptophan is also referred to as the kynurenine pathway. Nicotinic acid from dietary or supplementary origin is channelled toward NAD^+ in the Preiss-Handler pathway. Finally, salvage metabolism ensures the flux of nicotinamine released by NAD^+-consuming enzymes back to NAD^+. Icons: plate-and-cutlery: dietary sources; capsule: food supplements and medication.
ACMS: aminocarboxymuconate-semialdehyde; AFMID: arylformamidase; HAAO: 3-hydroxyanthranilate 3,4-dioxygenase; IDO: indoleamine 2,3-dioxygenase; KMO: kynurenine 3-monooxygenase; KYNU: kynureninase; NAD^+: nicotinamide adenine dinucleotide; NADSYN: NAD^+ synthase; TDO: tryptophan 2,3-dioxygenase.

Table 1
Features of patients with KYNU deficiency.

Patient characteristics	Patient 1	Patient 2
Sex	Male	Male
Ethnicity	Dutch	Afghan
Consanguinity	No	Yes
Gestational age	40 + 4 weeks	37 + 2 weeks
Birth weight	3480 g (percentile 30)	2560 g (percentile 10)
Birth length	42 cm (<−3 SDS)	47 cm (−2 SDS)
Head circumference	34.5 cm (−0.9 SDS)	33 cm (−2 SDS)
Apgar score	7, 9, 10 after 1, 5, 10 min	8, 10, 10 after 1, 5, 10 min
Maternal history	Three early miscarriages	No miscarriages
Genetics		
KYNU mutations	Compound heterozygous c.256dup, p.(Tyr86Leufs*3) c.1301G > A, p.(Arg434Gln)	Homozygous c.989G > A, p.(Arg330Gln)
Symptoms		
Skeletal abnormalities	Metaphyseal widening Shortened long bones Lumbar hemivertebrae Thoracic butterfly vertebrae Short ribs Bell-shaped thorax	Thoracic butterfly vertebrae Thoracic hemivertebrae Costal deformities
Heart defect	Double outlet right ventricle, Fallot type	Small left ventricle Small bicuspid aortic valve Hypoplastic aortic arch with aortic coarctation Left superior caval vein draining into coronary sinus Atrial septal defect type II
Dysmorphic features	High philtrum Thin lips Micrognathia	High philtrum Low ears
Hearing loss	Sensorineural	No
Feeding difficulties	Periodic tube feeding	Periodic tube feeding
Hypothyroidism	No	Yes
Kidney abnormalities	No	No
Ocular abnormalities	No	No
Respiratory tract abnormalities	Tracheolaryngobronchomalacia Vocal cord paresis after intubation	Malacia left main bronchus
Other	Germinolytic cyst	None
Clinical course		
Age initial presentation	Antenatal, first trimester	Antenatal, second trimester
Surgery	2 months: central shunt placement to alleviate cyanotic spells Complications: chylothorax for which medium chain triglycerides diet, and mediastinitis for which antibiotics and surgical exploration 3 months: surgical exploration mediastinitis Complication: resuscitation due to difficult intubation	4 days: bilateral pulmonary artery banding to prevent pulmonary overflow 1 month: reduction of the atrial septal defect size 2 months: aortic arch reconstruction, pulmonary artery debanding, and ligation of arterial duct
Inflammation/infection	2.5 months: Staphylococcal Aureus mediastinitis 3 months: Rhinovirus airway infection 4 months: Necrotizing enterocolitis 4 months: Candida sepsis	1.5 month: *Enterobacter Cloacae* airway infection 2 months: *Escherichia Coli* sepsis 2.5 months: Klebsiella sepsis
Latest follow-up	Death at age 4 months	Age 8 months
Treatment		
Vitamin B3	Nicotinamide 2 weeks: 25 mg twice daily	Nicotinamide 3 weeks: 25 mg twice daily
Pyridoxine	2 months: 25 mg once daily 3.5 months: 50 mg once daily	5 weeks: 25 mg once daily 8 weeks: 25 mg twice daily
Surgery	Nicotinamide 9 mg twice daily and pyridoxine intravenous	Nicotinamide 9 mg twice daily and pyridoxine intravenous
Possible side effects	None	None

2.1.1. Patient 1

The first patient was a Dutch boy and the first child of non-consanguineous parents. An ultrasound was performed antenatally at gestational age 11 weeks and 5 days, which showed an enlarged nuchal fold (4.9 mm, reference <3.5 mm) and raised suspicion of a congenital heart defect. A structural ultrasound performed at gestational age 16 weeks and 5 days confirmed the congenital heart defect, specifically a Fallot-type double outlet right ventricle, and additionally revealed several skeletal abnormalities, including shortened long bones, hemivertebrae, and butterfly vertebrae. Using exome sequencing on DNA obtained from amniotic fluid, two novel compound heterozygous variants were identified in the *KYNU* gene. A maternally inherited frameshift variant NM_003937.2: c.256dup (p.Tyr86Leufs*3) was identified leading to an early premature stopcodon (frequency for heterozygous carriers in gnomAD v4.1 (GRCh38): 250/1179312 European (non-Finnish) controls; no homozygous carriers). This variant was classified as pathogenic using the ACMG criteria (PVS1, PM2, PP4). In addition, a paternally inherited missense variant NM_003937.2: c.1301G > A (p.Arg434Gln) was identified concerning a highly conserved amino acid which seems to have an important role in substrate binding [9]

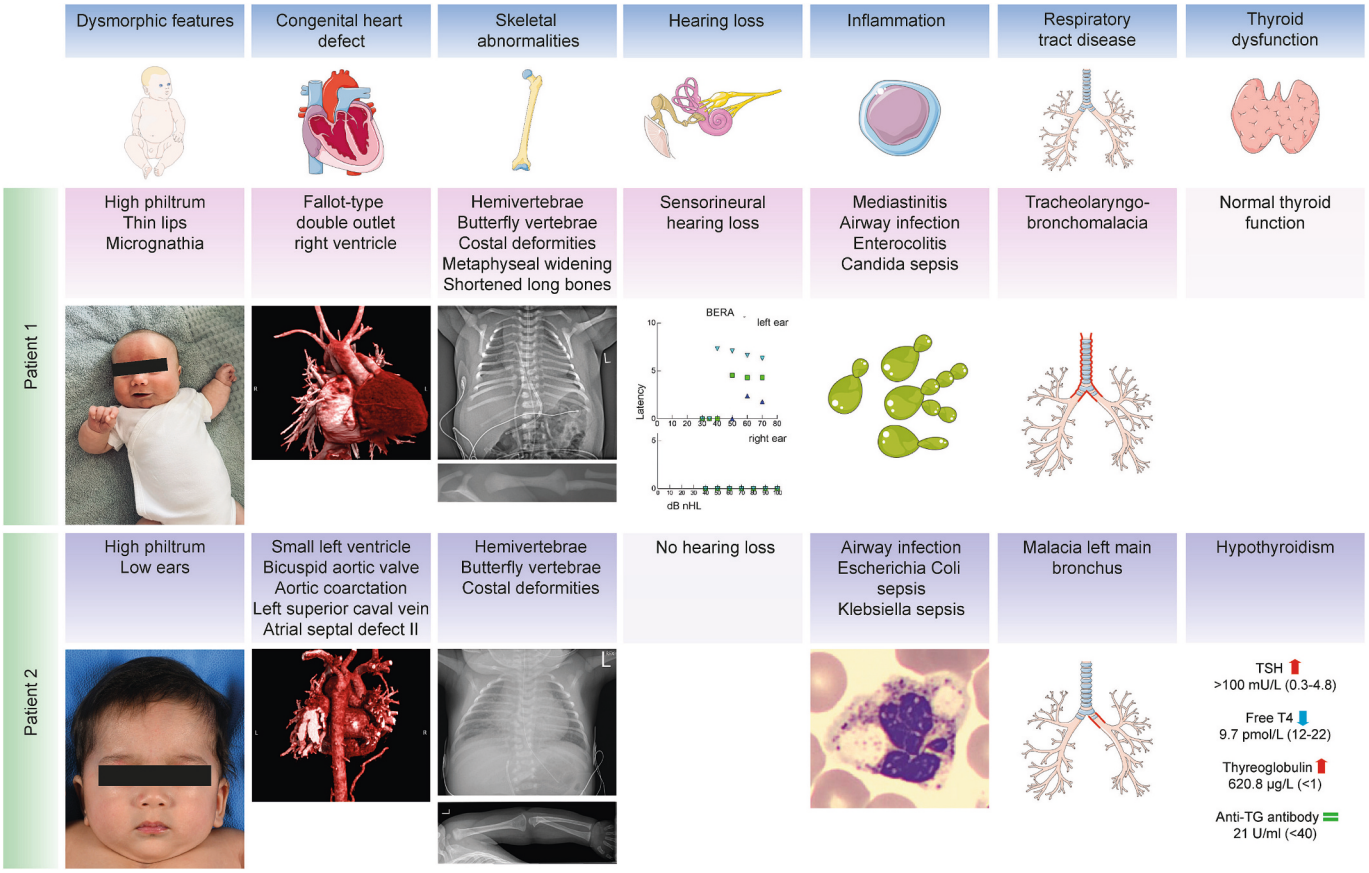

Fig. 2. Clinical features of both patients with KYNU deficiency.
Blue boxes indicate the affected organs and tissues. Pink boxes and purple boxes describe the detailed symptoms of the first patient and second patient respectively. If available, pictures (dysmorphic features) and results from diagnostic tests from the patients (congenital heart defects, skeletal abnormalities, hearing loss patient 1, inflammation patient 2 and thyroid dysfunction patient 2) were shown. The leucocyte that illustrates inflammation in patient 2 concerns a neutrophilic granulocyte with vacuoles. To designate inflammation in patient 1, an illustration of *Candida Albicans* was used. Airway malacia is shown by the red line in both patients.

(frequency for heterozygous carriers in gnomAD v4.1 (GRCh38): 40/1178390 European (non-Finnish) controls; no homozygous carriers). This variant was assessed as likely pathogenic according to the ACMG criteria (PM2, PM3, PP3, PP4). Since the clinical phenotype was similar to other reported CNDD cases, a diagnosis of KYNU deficiency was considered very likely.

When the genetic result became known at 20 weeks of gestational age, we considered high-dose vitamin B3 supplementation of the pregnant mother as an antenatal treatment of KYNU deficiency, because maternal supplementation of NA prevented malformations in *Kynu*-null mouse embryos [3]. However, due to potential toxicity risks for the mother, we refrained from high-dose vitamin B3 supplementation. Furthermore, it was unclear whether any improvement in the birth defects would still be possible at this late stage of pregnancy. Of note, the mother had been taking a daily commercial multivitamin supplement containing 33 mg NAM from early pregnancy on (starting at gestational age 3 weeks and 4 days).

The boy was born at gestational age 40 weeks and 4 days. His birth weight, height and head circumference were 3480 g, 42 cm, and 34.5 cm respectively. In addition to the congenital heart defect and skeletal abnormalities, he had sensorineural deafness and a tracheolaryngobronchomalacia. Feeding difficulties necessitated tube feeding. At the age of two months, a central shunt was placed due to cyanotic spells related to the tetralogy of Fallot. Chylothorax and a mediastinitis complicated this surgery. In the following weeks he also developed a viral airway infection and necrotizing enterocolitis.

From the age of two weeks, the boy was treated with 25 mg NAM orally twice daily. Vitamin B6 treatment (pyridoxine) was added at two months, initially at a dosage of 25 mg daily, which was increased to 50 mg daily 1.5 month later. NAM and pyridoxine were administered intravenously, when oral intake was not possible. No evident side effects were observed. Unfortunately, the patient died at the age of four months due to sepsis.

During pregnancy of her second child, a girl, the first patient's mother started nicotinamide riboside (NR) supplementation (commercial product) at 300 mg 3 times daily at 3 weeks gestational age on her own accord. At gestational age 10 weeks, the dosage was decreased to 200 mg twice daily. In addition, she had been taking the same multivitamin supplement as in her first pregnancy, containing 33 mg NAM daily. No adverse effects of this maternal NR and NAM supplementation were noticed. Maternal liver enzymes were consistently normal. The parents refrained from antenatal molecular diagnostics. Metabolic and genetic testing performed after birth confirmed that the girl was not affected by KYNU deficiency.

2.1.2. *Patient 2*

The second patient is an Afghan boy and the first child of consanguineous parents. A structural ultrasound antenatally performed at gestational age 23 weeks and 4 days revealed a congenital heart defect, including a small left ventricle, aortic arch hypoplasia with suspected coarctation of the aorta, and a persistent left superior caval vein (LSVC). Antenatal genetic testing returned negative, however postnatal reanalysis identified a known homozygous missense mutation in the *KYNU* gene (NM_003937.3: c.989G > A; (p.Arg330Gln)), consistent with KYNU deficiency [6] (frequency for heterozygous carriers in gnomAD v4.1 (GRCh38): 0/6062 Middle Eastern controls; also no

homozygous carriers).

The boy was born at gestational age 37 weeks and 2 days. His birth weight, height and head circumference were 2560 g, 47 cm, and 33 cm respectively. Cardiac evaluation confirmed the prenatal findings, including aortic coarctation, and in addition revealed a bicuspid aortic valve and large atrial septal defect type II. Furthermore, he presented with multiple skeletal abnormalities, hypothyroidism (treated with levothyroxine), feeding difficulties, and a left bronchomalacia. He did not have hearing loss. Over time, he underwent several cardiac surgeries (see Table 1 for details) and experienced recurrent infections.

The patient was treated with NAM from the age of three weeks in a dosage of 25 mg two times daily. Two weeks later pyridoxine 25 mg once daily was started, which was increased to 25 mg two times daily when he was two months old. NAM and pyridoxine were administered intravenously, when oral intake was not possible. No evident side effects were noted.

Currently, at the age of eight months the patient is doing well with a normal motor and cognitive development. He is receiving ongoing supplementation with NAM and vitamin B6.

2.2. Biochemical measurements

In order to investigate the biochemical effects of the *KYNU* variants analysis of organic acids, purines/pyrimidines and analogues, metabolomics and measurements of kynurenine metabolites, NAD^+ and its degradation product *N*-methyl-2-pyridone-5-carboxamide (2PY) were performed prior to treatment. Samples were taken at the age of five days (patient 1) and twenty days (patient 2).

In both patients, excretion of the upstream metabolite xanthurenic acid was clearly increased in the urinary organic acids and purines/pyrimidines profiles (see Fig. 3A and B). 3-hydroxykynurenine, the main substrate of the KYNU enzyme, was also increased in both profiles of the first patient, but only detectable in the purines/pyrimidines profile of the second patient. Both metabolites were absent in control profiles. Metabolomics in plasma demonstrated significant elevations of 3-hydroxykynurenine and xanthurenic acid as well as of 8-methoxykynurenate, together with moderate increases of kynurenine and kynurenic acid as compared to within and between batch controls (see Fig. 3C; z-scores are depicted in supplementary table 1). Measurement of kynurenine metabolites in plasma showed that tryptophan was in the same range as parents, while levels of 3-hydroxykynurenine, xanthurenic acid, kynurenine and kynurenic acid were clearly increased in the patients (Fig. 4A; means and ranges are included in Table 2). Unexpectedly, NAD^+ and 2PY, as measured in whole-blood samples, were not decreased in patient 2 compared to controls and parents (Fig. 4A). There even seemed to be an increase in 2PY, although the low numbers prevent statistical comparison. In summary, these results obtained by different analysis methods showed clear accumulation of upstream kynurenine metabolites, supporting the pathogenicity of the different *KYNU* variants.

We conducted biochemical monitoring of patient 2 during treatment with NAM and pyridoxine. After initiation of NAM, a clear decrease in tryptophan, 3-hydroxykynurenine, kynurenine and xanthurenic acid levels was observed, whereas kynurenic acid and NAD^+ levels remained essentially the same (Fig. 4B). Pyridoxine did not have an additional effect. 2-PY levels increased under NAM supplementation without an additional effect of pyridoxine.

Since elevation of kynurenine metabolites was previously described in two siblings [10] with, presumably, mildly decreased kynureninase activity of around 64 % [3], we also performed the urinary analyses in the four parents of the two patients, each of them heterozygous for one *KYNU* variant. In these samples, xanthurenic acid and 3-hydroxykynurenine were undetectable (data not shown). In addition, plasma kynurenine metabolites (Fig. 4A) did not differ notably between parents and controls.

3. Discussion

3.1. Clinical features

In this paper, we describe the first two patients with KYNU deficiency diagnosed in The Netherlands. So far, ten patients with KYNU deficiency have been published worldwide [3,6–8]. The clinical features of our patients correspond to the phenotype of the patients described in literature. All patients with KYNU deficiency present with skeletal abnormalities and almost all with a congenital heart defect. The majority have facial dysmorphisms and developmental delay. Sensorineural hearing loss, renal defects, airway malacia and vocal cord paresis are seen in a minority of the patients.

In addition, one of our patients developed hypothyroidism, only described in one other patient with KYNU deficiency [3], which may expand the phenotype of KYNU deficiency. This is especially interesting because of the suggested connection between tryptophan and thyroid hormone metabolism through the enzyme kynurenine/2-aminoadipate aminotransferase (AADAT). AADAT has both a role in the conversion of kynurenine and 3-OH-kynurenine to kynurenic acid and xanthurenic acid respectively, and in the transamination of the bioactive thyroid hormone 3,3′,5-triiodothyronine (T3) and its prohormone thyroxine (T4) to their pyruvic acid analogues TK3 and TK4 respectively [11].

Our patients suffered from numerous opportunistic infections, not previously described in patients with KYNU deficiency, potentially hinting at impaired immunity. Post-surgical complications may partly explain the infections. However, tryptophan catabolites play a regulatory role in immune response, and the potential association of recurrent infections with disrupted NAD^+ *de novo* biosynthesis is a highly interesting topic demanding further research [12]. Our first patient developed necrotizing enterocolitis, which may be an additional link between KYNU deficiency and immunity. His severe illness at that moment may be the explanation, but it could alternatively be related to his defect in NAD^+ biosynthesis. Shortage of tryptophan (and subsequently NAD^+) was found to induce structural and functional intestinal changes in mouse models and organoids, reminiscent of changes seen in the intestine of children with severe malnutrition [13]. Interestingly, this pathology was associated with decreased activity of SIRT1, an NAD^+-consuming enzyme [13], while another study demonstrated a link between deficiency of NAD^+ consuming enzyme SIRT2, and impaired intestinal proliferation and differentiation [14].

Our data underline the association of KYNU deficiency with several congenital anomalies. It is therefore important to include KYNU deficiency in the differential diagnosis of congenital anomalies like VACTERL association (vertebral defects, anal atresia, congenital heart defects, trachea-oesophageal fistula, renal anomalies and limb abnormalities).

3.2. Genetics

In patient 1, KYNU deficiency was caused by compound heterozygosity of two novel variants, expanding the genotype of KYNU deficiency. Patient 2 was homozygous for the c.989G > A (p.Arg330Gln) variant in *KYNU*, previously reported in only one other patient by Ehmke and coworkers [6] (second patient). Both our patient and the previously described patient with the homozygous c.989G > A genotype share similarities in phenotype, including facial dysmorphisms, a congenital heart defect, butterfly vertebrae, feeding difficulties, and the absence of renal malformations. However, the type of the congenital heart defect differs: our patient had a small left ventricle, bicuspid aortic valve with hypoplastic aortic arch and aortic coarctation, while the other patient had tetralogy of Fallot and an anomalous origin of the left coronary artery from the pulmonary artery (ALCAPA). Furthermore, the patient described by Ehmke suffered from severe hypotonia, sensorineural hearing loss and clinodactyly, while our patient did not (clinodactyly excluded by X-ray) [6]. These differences suggest a partial

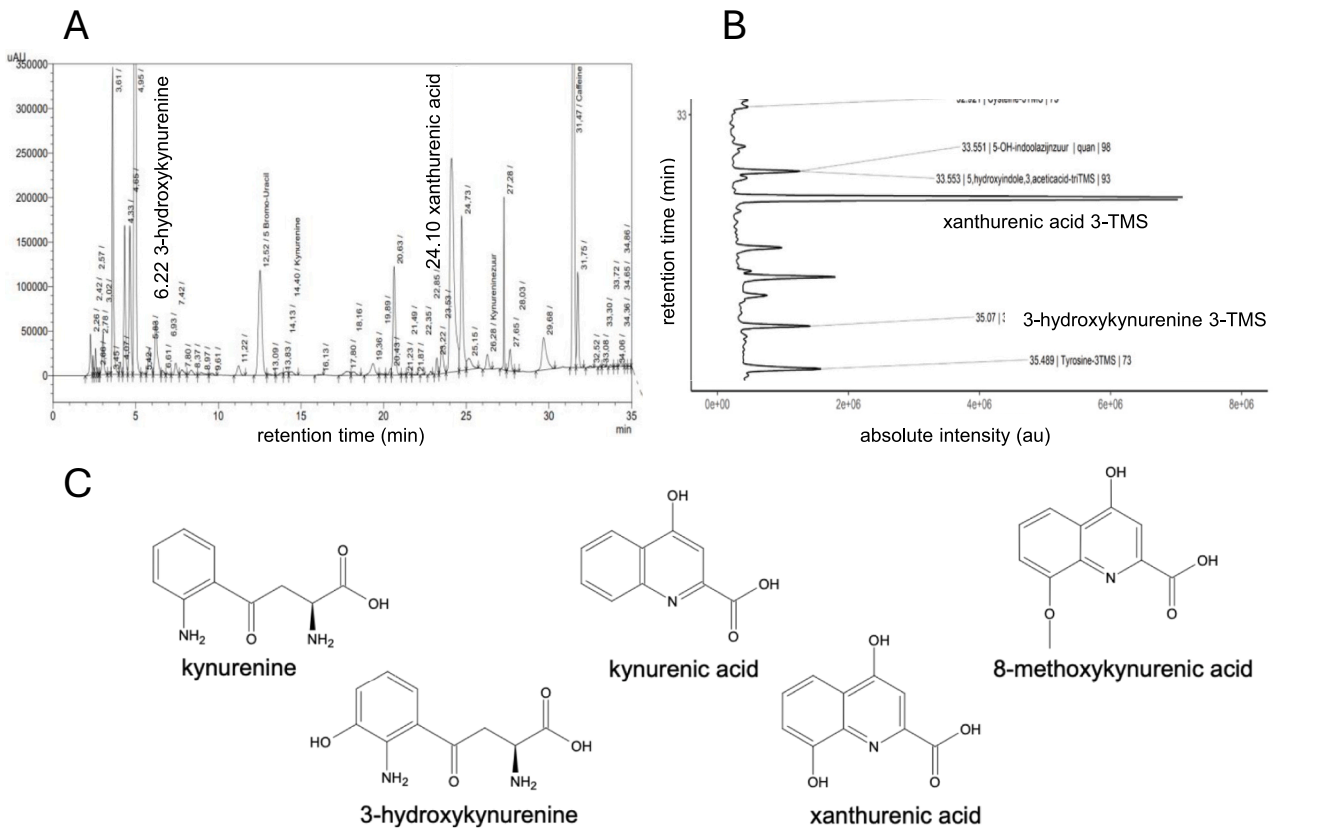

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Fig. 3. Biochemical results.
Analytical results and chemical structures of (unusual) kynurenine pathway metabolites. A: Reversed-phase HPLC-UV chromatogram of purine and pyrimidine analysis showing peaks of 3-hydroxykynurenine and xanthurenic acid. B: GC–MS chromatogram showing silyl-derivatives 3-hydroxykynurenine and xanthurenic acid. C: structures of kynurenine pathway metabolites detected with HPLC-UV (panel A), GC–MS (panel B), and metabolomics using ultra high-performance liquid chromatography mass spectrometry (UHPLC-MS(/MS)) analysis (panel D). D: *Z*-score plots of UHPLC-MS(/MS) metabolomics analysis for patients 1 and 2, in positive and negative ionization mode. Metabolites from the kynurenine pathway are underlined. Metabolites with z-score greater than 2 are aberrant.

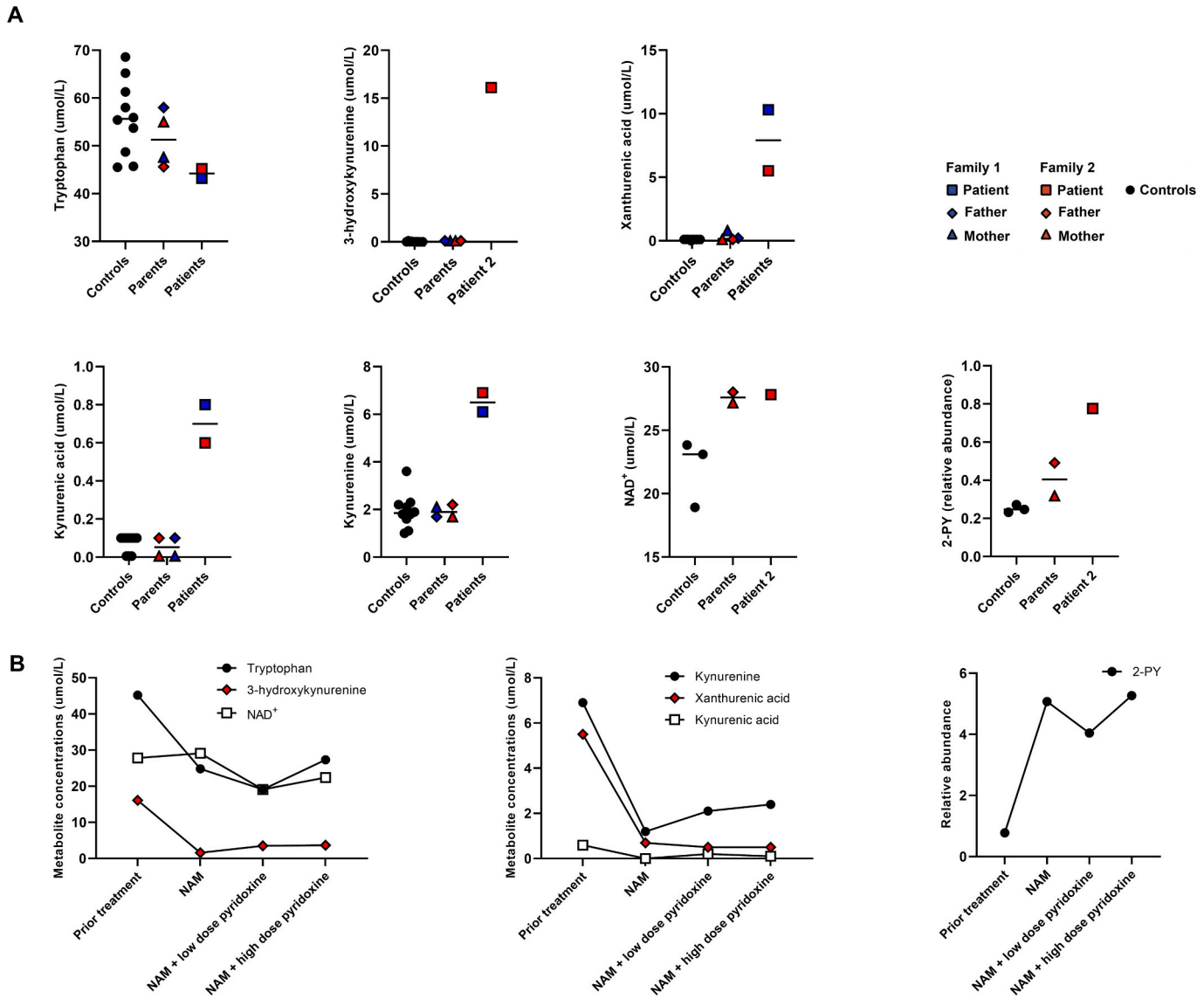

Fig. 4. Measurements of kynurenine metabolites, NAD^+ and *N*-methyl-2-pyridone-5-carboxamide (2PY) prior and during treatment.
A. Measurements of kynurenine metabolites, NAD^+ and *N*-methyl-2-pyridone-5-carboxamide (2PY) in the two KYNU patients and their parents prior to treatment. Kynurenine metabolites were measured in plasma and NAD^+ and 2PY in whole blood samples. Control values are depicted in filled circles. Values of the first KYNU family are depicted in blue and values of the second KYNU family in red (values of the patient as square; mother as triangle and the father as diamond). The line in the graphs represent the mean. Measurement of 3-hydroxykynurenine was not reliable in the first patient and is therefore omitted. For 2PY only relative abundance could be determined, not actual concentrations. For kynurenine metabolites 2 patients, 4 parents (2 of each patient) and 10 controls were measured. For NAD^+ and 2PY only patient 2 was measured alongside his 2 parents and 3 control samples. LOQ for kynurenine metabolites is 0.01 μmol/L. Values below LOQ are replaced by 0.005 μmol/L (0.5*LOQ) for graphical representation.
B. Measurements of kynurenine metabolites, NAD^+ and 2PY in the second patient during treatment. Metabolites are clustered over different graphs based on measurement unit/range.
Fasting status was not controlled for the different individuals.
NAM = nicotinamide. Low dose pyridoxine: 25 mg once daily; high dose pyridoxine: 25 mg twice daily.

phenotype-genotype correlation, but it should be noted that both patients have consanguineous parents, which increases the risk of additional diagnoses. In addition, Ehmke's patient required extracorporeal membrane oxygenation which can also affect the neurological phenotype [6]. Finally, also maternal NAD^+ levels during pregnancy may influence clinical outcome among patients carrying the same mutation(s).

The variants in patient 2 were not identified during pregnancy even

Table 2
Levels of kynurenine metabolites, NAD^+ and 2PY, prior to treatment.

Metabolite	Patients: range P1–P2	Parents: mean (range)	Controls: mean (range)
Tryptophan	43–45	52 (46–58)	56 (46–69)
3-hydroxykynurenine	n.a.–16	0.10 (0.10–0.10)	0.02 (<0.01–0.10)
Xanthurenic acid	10–5.5	0.30 (0.10–0.80)	0.09 (<0.01–0.10)
Kynurenine	6.1–6.9	1.9 (1.7–2.2)	1.9 (1.0–3.6)
Kynurenic acid	0.8–0.6	0.05 (<0.01–0.10)	0.07 (<0.01–0.10)
NAD^+	n.a.–27.8	27.6 (27.2–28.0)	22.0 (18.9–23.8)
2PY	n.a.–0.8	0.4 (0.3–0.5)	0.3 (0.2–0.3)

For kynurenine metabolites 2 patients, 4 parents (2 of each patient) and 10 controls were measured. For NAD^+ and 2PY only patient 2 was measured alongside his 2 parents and 3 control samples.
LOQ for kynurenine metabolites is 0.01 μmol/L. Values below LOQ are replaced by 0.005 μmol/L (0.5*LOQ) for calculations.
n.a. = not applicable: measurement of 3-hydroxykynurenine was not reliable in the first patient and is therefore omitted; measurements of NAD^+ and 2PY were only performed in the second patient.
P1 = patient 1; P2 = patient 2; 2PY=*N*-methyl-2-pyridone-5-carboxamide.
Values are depicted in umol/L, except of 2PY values which are depicted in relative abundance.

though exome sequencing was performed. The reason for this is that parents opted for a restricted analysis, which was performed using the HPO-terms 'Persistent left superior vena cava (HP:0005301)', 'Abnormal aortic morphology (HP:0001679)' and 'Coarctation of aorta (HP:0001680)', as well as a gene panel 'congenital anomalies' based on the panel 'Fetal anomalies' in the PanelApp, which did not include the *KYNU* gene at the time. We propose to add *KYNU* to relevant diagnostic Next Generation Sequencing gene panels, *e.g.* multiple congenital anomalies, congenital heart defects, and skeletal abnormalities.

3.3. Biochemistry

To study the biochemical effects our patients' *KYNU* variants, we applied multiple methods and consistently found elevations of upstream kynurenine metabolites. This is in line with previously published results [3,6–8] and supports pathogenicity of the two novel *KYNU* variants found in our first patient. Of note, 8-methoxykynurate, actually a methylated derivate of xanthurenic acid, was only recently modelled to associate with KYNU deficiency [15] and subsequently found to accumulate in urine of the patient described in [7]. We now verified in our two patients that this is a reliable marker of KYNU deficiency. Kynurenine metabolites in plasma and urine samples of the parents were found to be similar to control samples, indicating that heterozygosity for nonsense or missense mutations in the *KYNU* gene does not lead to biochemical aberrations. It cannot be excluded though that variants in *KYNU* give rise to a wide range of biochemical and biological effects, ranging from asymptomatic individuals with normal biochemistry and asymptomatic individuals excreting KYNU metabolites on one side of the spectrum, to severe disease as we describe here [10].

Surprisingly, our patients' NAD^+ levels were not decreased prior to treatment, in contrast to an earlier reported patient [3]. In this patient, the plasma NAD^+ concentration was 9.4 times lower than in unaffected family members. One explanation may be that this patient carried two heterozygous nonsense mutations while our patient carried a homozygous missense mutation, possibly retaining some residual enzyme activity. Indeed, in several NADSYN1 deficient patients with established residual enzyme activity, NAD^+ levels were not lower compared to parents [16]. Alternatively, measurement of NAD^+ in whole blood (our study) *versus* plasma [3], sampling conditions, and dietary intake may explain these differences.

We measured 2PY since this may be an indirect reflection of the NAD^+ pool. In case of an excess, NAM is degraded to 1-methylnicotinamide (MeNAM), and its derivatives 2PY and N-Methyl-4-pyridone-3-carboxamide (4PY) [16,17]. Since NAM is one of the main NAD^+ precursors in circulation, low levels of its degradation products may indicate NAM preservation when supply of NAD^+ precursors *via de novo* biosynthesis and/or Preiss-Handler pathway is limited [16,17]. However, contrary to our expectation, 2PY was not decreased in patient 2 prior to treatment with NAM (Fig. 4A). 2PY was not measured before in other KYNU deficient patients but was decreased in three NADSYN1 deficient patients [16].

3.4. Treatment

KYNU deficiency and the other CNDD have a potential advantage of antenatal treatment with NAD^+ precursors, since clinical manifestations mainly develop *in utero*. Early antenatal diagnosis and timely initiation of treatment would presumably be most effective. This is corroborated by preclinical evidence in mouse models [3,16], but no clinical experience has been published, yet (reviewed by [5]). Patient 1 developed congenital anomalies despite daily maternal ingestion of 33 mg NAM from early pregnancy onwards, which suggests that the potential effect of antenatal treatment with NAD^+ precursors should be evaluated with higher dosages. Furthermore, some of the manifestations (*e.g.* developmental delay, muscular hypotonia and failure to thrive) may still be amenable to treatment with NAD^+ precursors started after birth. In addition, if left untreated, patients with KYNU deficiency, as well as other CNDD, are at risk of developing the triad of diarrhoea, dermatitis and dementia (referred to as pellagra), historically associated with nutritional B3 deficiency. Life-threatening episodes of pellagra-like dermatitis have been reported in two NADSYN1 deficient patients [16]. CNDD-patients are expected to be especially vulnerable at times when oral intake of niacin is limited, for example during illness and fasting, and they would mostly rely on *de novo* NAD^+ biosynthesis. In those instances, intravenous administration of vitamin B3 and B6 is indicated.

There are several different NAD^+ precursors available for clinical use, including NA, NAM, NMN and NR. Most experience has been gained with NA and NAM, since these are historically used for the treatment of pellagra and the pellagra-like disorder Hartnup disease (reviewed by [1]). Nevertheless, NA is considered less favourable because of painful skin flushing being reported as an adverse effect. There is increasing awareness that long term and high-dose NAM treatment may result in end-product inhibition of NAD^+-consuming enzymes [18,19], intensifying the interest in NMN and NR as alternative NAD^+ precursors. It has to be noted though, that NMN and NR have limited bioavailability due to substantial degradation in the gut and circulation [20–22]. Moreover, pharmacological grade precursors are not universally obtainable and are currently not supplied in the Netherlands. Since vitamin B6 is a cofactor of KYNU, we later added pyridoxine to the treatment regime in an attempt to boost any residual activity. We based our dosing of NAM and pyridoxine on Dutch guidelines for paediatric dosing [23].

To the best of our knowledge treatment of KYNU deficient patients was not described in literature before. In the broader context of CNDD, treatment of four NADSYN1 deficient patients with NAM was reported. These patients were treated for short periods (6–10 weeks) with NAM

doses between 100 and 400 mg/day [16,24]. In general, these treatments had a minor effect on NAD^+ and NAM levels but clearly increased levels of degradation products (if measured), without reports of adverse effects. For the second patient we followed metabolite levels while on treatment. This was not possible for the first patient due to multiple surgeries and complications in his short life. We observed a clear but unexpected decrease in tryptophan, 3-hydroxykynurenine, kynurenine and xanthurenic acid levels when NAM was started. This may suggest a form of feedback inhibition when the NAD^+ pool is increased by supplementation of its precursors. It has indeed been described that *in vitro*, NAD(*P*)H exerts negative feedback on tryptophan dioxygenase (TDO, the first liver and CNS specific enzyme of the kynurenine pathway) in rat liver by means of allosteric modulation. Additionally, hormonal and transcriptional changes also play a role (reviewed by [2]). Although we did not find an effect of NAM treatment on NAD^+ whole blood levels, the increase of 2PY levels suggests that it indeed enlarged the total NAD^+ pool. The decrease in tryptophan is in discordance with decreased TDO activity as a result of feedback inhibition. Alternatively, the decrease in tryptophan concentration may reflect incorporation of tryptophan into proteins during growth in the early postnatal period. A longer follow-up period may shed more light on this unexpected finding. Adding pyridoxine to the treatment regimen did not lead to a further decrease in tryptophan and kynurenine metabolites or a further increase in 2PY. Therefore, it needs further investigation whether pyridoxine may have any added (clinical) beneficial effects alongside NAD^+ precursors in cases of KYNU deficiency with (expected) residual enzyme activity.

In our patients no evident side effects of NAM and pyridoxine supplementation were noted. Also, maternal NR and NAM supplementation during pregnancy with the first patient's sister (who was later identified as not affected by KYNU deficiency) seemed safe.

To determine the long-term biochemical and clinical effects of treatment with vitamin B3 and B6 we propose a standardized follow-up for all patients with KYNU deficiency on therapy. We advise to evaluate motor and cognitive function, growth, and bone development at baseline and yearly thereafter. In addition, we suggest regular monitoring of blood and urine kynurenine metabolite levels, and screening for potential toxicity of long-term supplementation of vitamin B3 and B6.

3.5. Conclusion

In conclusion, we describe two new cases of KYNU deficiency, thereby expanding the phenotype and genotype of this very rare disorder. Metabolic profiling is indispensable for studying new *KYNU* variants and understanding pathophysiology. Supplementation of NAD^+ precursors and vitamin B6 may be a promising novel treatment for patients with KYNU deficiency, however standardized follow-up is necessary to determine the long-term benefits.

4. Material and methods

4.1. Ethics approval

This article describes the individualized treatment of a few patients with an ultrarare disease in the clinical care setting. Medical ethics committee approval was not required in our institution. The parents of the two patients have given informed consent for treatment and monitoring as well as publication of experimental and clinical data, and use of photographs.

4.2. Sample collection

Genomic DNA was isolated from amniocytes for the fetus and from whole blood for both parents. For organic acid and purine/pyrimidine measurements, urine samples were stored at −20 °C prior to analysis. For metabolomics and kynurenine metabolites, lithium heparin-whole blood samples were centrifugated to obtain plasma shortly after collection. Plasma samples were stored at −20 °C prior to analysis. For measurement of NAD^+ and *N*-methyl-2-pyridone-5-carboxamide (2PY) 1 microtube EDTA-whole-blood sample was collected on ice. 10 μL of fresh whole blood sample was aliquoted (in duplicate) and 0.5 mL of methanol was added (to prevent change due to freeze-thaw cycles). The samples were stored at −20 °C prior to analysis.

4.3. Exome sequencing analysis

Exome sequencing analysis is part of the routine diagnostic workup in patients with congenital anomalies suspected of an underlying Mendelian disorder. Exome sequencing was performed as described earlier [25].

4.4. Organic acids analysis in urine

Organic acid analysis in urine was performed using a well-established ISO15189-validated gas chromatography mass spectrometry (GC–MS) method. In short, samples were mixed with internal standard (3,3-dimethylglutaric acid) and after oximation with hydroxylammoniumchloride, organic acids were extracted using solid phase extraction (SPE) columns. Elution was performed with acid ethylacetate, acidic butanol and methanol. Subsequently, the eluate was evaporated and derivatised using *N*,*O*-Bis(trimethylsilyl)trifluoroacetamide (BSTFA). GC–MS of silyl-derivates was performed with a DB-1701 column (Agilent, Santa Clara, CA, USA) coupled to a QP2020 mass spectrometer (Shimadzu, Kyoto, Japan). Data was processed in GCMS Postrun Analysis software (Shimadzu), using an in-house developed database.

4.5. Purine and pyrimidine analysis in urine

Purine and pyrimidine analysis in urine was performed using a well-established ISO15189-validated high-performance liquid chromatography photo diode array detector (HPLC-PDA) method. In short, samples were mixed with internal standard mix (5-Bromouracil and caffeine) and filtered with centrifugal filter units. HPLC separation was performed using an HiChrom C18 column and HiChrom C18 guard column (Avantor, USA) and detection with PDA system from Shimadzu.

4.6. Metabolomics in plasma

Metabolomics was performed as described previously [26], using ultra high-performance liquid chromatography mass spectrometry (UHPLC-MS(/MS)) analysis. A detailed description of the method is included in the supplemental information.

4.7. Measurement of tryptophan and kynurenine metabolites in plasma

Control samples used for this analysis were pseudo-anonymized samples of healthy persons (5 male/5 female) aged between 23 and 35 years. All samples were measured once, and control samples were processed identically alongside patient samples.

Analysis of tryptophan metabolites was performed using ultra-performance liquid chromatography hyphenated to tandem mass spectrometry (UPLC-MS/MS). A detailed description of the method is included in the supplemental information.

4.8. Measurement of NAD^+ and N-methyl-2-pyridone-5-carboxamide (2PY) in whole blood

Control samples used for this analysis were anonymized samples for which age and sex were not traceable ($n = 3$). All samples were measured once, and control samples were processed identically alongside patient samples.

NAD^+ and 2PY were analysed using UPLC coupled to an Ultra-High

Resolution Q-Time-Of-Flight mass spectrometer. A detailed description of the method is included in the supplemental information.

CRediT authorship contribution statement

Susanna M.I. Goorden: Writing – original draft, Conceptualization. **Désirée Y. van Haaften-Visser:** Writing – original draft, Conceptualization. **Maria M. Trętowicz:** Writing – review & editing, Investigation, Formal analysis. **Ramon Bonte:** Investigation, Formal analysis. **Elly Bogaerts:** Investigation, Formal analysis. **Youssra Jamal:** Investigation, Formal analysis. **Sandrien Vrieswijk:** Investigation, Formal analysis. **Erika Huijser:** Investigation, Formal analysis. **Regina Bökenkamp:** Writing – review & editing. **Roel L.F. van der Palen:** Writing – review & editing. **Mariette J.V. Hoffer:** Writing – review & editing. **Riekelt H. Houtkooper:** Writing – review & editing. **Frédéric M. Vaz:** Writing – review & editing. **Gijs W.E. Santen:** Writing – review & editing. **Jörgen Bierau:** Writing – original draft, Conceptualization. **Esmeralda Oussoren:** Writing – original draft, Conceptualization.

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Declaration of competing interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ymgme.2025.109194.

Data availability

Data will be made available on request.

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