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INHERITED METABOLIC DISORDERS AND NUTRITION

Editorial

Dear Colleagues,

We are delighted to announce the launch of the inaugural issue of Inherited Metabolic Disorders and Nutrition (IMDN), the official journal of the Child Nutrition and Metabolism Association. The journal provides a platform for academics to advance scientific research, report rare case studies, and share innovative ideas - all contributing to the growing body of knowledge in our field.

Inherited metabolic disorders represent a critical area of research, with an increasing number of researchers and publications dedicated to this field worldwide. IMDN was established in response to the ongoing need for additional avenues to publish high-quality research in this area. The journal aspires to make a lasting contribution to the fields of paediatric nutrition and inherited metabolic disorders. With the release of the first issue, a strong commitment is renewed to position IMDN as a leading resource within the international scientific community. Over time, we hope that IMDN will become an essential tool for sharing information and experiences related to metabolic disorders and nutrition.

IMDN will be published quarterly and will feature peer-reviewed research articles, case reports, reviews, original studies, and other relevant content. Our editorial board comprises experts from around the globe, and we welcome contributions from our international colleagues.

For more information about IMDN, please visit our website at www.imdn.org. I encourage you to read the first issue with great interest, and I extend my sincere thanks to the authors whose work is featured in it. As a newly established journal, IMDN will undoubtedly thrive with your continued support and contributions.

We look forward to receiving your submissions for future issues.

Best regards,

Nur Arslan
Editor-in-Chief

ORIGINAL ARTICLE

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Development and Validation of a Multiplex LC-MS/MS Assay for Lysosphingolipid Quantification in the Diagnosis of Gaucher and Fabry Diseases

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Abstract

Objectives: Lysosphingolipids (LysoSLs), the N-deacylated forms of sphingolipids, are increasingly recognized as toxic metabolites that accumulate in various lysosomal storage disorders (LSDs), collectively known as sphingolipidoses. These compounds, due to their amphiphilic nature and bioactive properties, can contribute to cellular dysfunction and disease pathogenesis. Recent studies have demonstrated that specific LysoSLs, such as hexosylsphingosine (HexSph), globotriaosylsphingosine (LysoGb3), and lysosphingomyelin (LysoSM), are elevated in plasma, serum, or dried blood spots from patients with Gaucher, Fabry, Krabbe, or Niemann-Pick diseases. These findings have paved the way for the use of LysoSLs as sensitive and specific biomarkers in the diagnosis, prognosis, and therapeutic monitoring of sphingolipidoses. This study aimed to develop and validate a method for the simultaneous quantification of three LysoSLs—HexSph, LysoGb3, and LysoSM—using liquid chromatography–tandem mass spectrometry (LC-MS/MS), for use in the diagnosis and follow-up of sphingolipidoses.

Materials and Methods: A multiplex analytical method was optimized for quantifying HexSph, LysoGb3, and LysoSM by ultra-fast LC coupled to tandem MS (UFLC-MS/MS) in positive-ion mode with electrospray ionization (LC-20 AD UFLC XR; Shimadzu 8040, Japan), employing multiple reaction monitoring. The method was validated for linearity, accuracy, precision, limit of detection, limit of quantification, and recovery rates. Following method validation, plasma samples were collected from 14 patients diagnosed with Gaucher disease or Fabry disease at Akdeniz University Hospital and from 20 healthy adult volunteers. Sample preparation involved methanol/acetone/water extraction prior to analysis of LysoSL concentrations.

Results: The total analysis time for all three analytes was determined to be 10 minutes. Calibration curves demonstrated strong linearity, with coefficients of determination (r^2) of 0.995 for LysoSM, 0.982 for HexSph, and 0.961 for LysoGb3. Plasma concentrations of LysoSLs were measured in both the patient and control groups. Significantly elevated levels of LysoSM and HexSph were observed in Gaucher patients compared to healthy controls. While LysoGb3 levels were higher in Fabry patients than in controls, the difference did not reach statistical significance.

Conclusion: This validated multiplex LC-MS/MS assay enables rapid and reliable simultaneous quantification of key LysoSLs in plasma. The assay holds promise as a diagnostic and monitoring tool for sphingolipidoses and may be further expanded to include additional biomarkers relevant to other LSDs. Quantification of LysoSLs can support clinical decision-making, including therapeutic selection and monitoring of patients undergoing enzyme replacement therapy.

Keywords: Lysosphingolipid, Hexosylsphingosine, Globotriaosylsphingosine, Lysosphingomyelin, LC-MS/MS

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INTRODUCTION

Sphingolipidoses are a subgroup of lysosomal storage disorders (LSDs) caused by inherited defects in lysosomal degradation pathways of sphingolipids.[1,2] This group of inborn metabolic diseases includes primary sphingolipidoses, such as Niemann-Pick types A, B, and A/B (NPA/B), Fabry disease (FD), Krabbe disease (KD), Gaucher disease (GD), and Tay-Sachs disease, all of which are caused by mutations in genes encoding specific lysosomal enzymes. Secondary sphingolipidoses, such as NPC, as well as disorders related to saposin deficiencies, are included.

Due to overlapping clinical manifestations among various sphingolipidoses, accurate clinical diagnosis is often challenging. Therefore, identification and analysis of molecular biomarkers that can facilitate differential diagnoses have become increasingly important. Liquid chromatography–tandem mass spectrometry (LC-MS/MS) is a widely used, highly sensitive, and highly specific analytical technique for biomarker quantification. One of its key advantages is the ability to simultaneously measure multiple analytes within a single run.

In recent years, the metabolism and biological functions of sphingolipids have been the focus of extensive research, and LC-MS/MS has become an essential tool in sphingolipid profiling. Although increased levels of glycosphingolipids have been reported in several sphingolipidoses, their relatively low sensitivity and specificity limit their utility as diagnostic biomarkers.[3] In contrast, lysosphingolipids (LysoSLs)—the N-deacylated derivatives of glycosphingolipids—have been found to accumulate markedly in the plasma and urine of patients with sphingolipidoses. These findings support their potential as sensitive and specific biomarkers for both diagnosis and therapeutic monitoring.[4]

Various forms of LysoSLs (LysoSLs) have been found to be selectively elevated in specific sphingolipidoses. For instance, significantly increased levels of globotriaosylsphingosine (LysoGb3) have been detected in both plasma and urine of male and female patients with FD.[5-7] In Krabbe disease, galactosylsphingosine (GalSph) is markedly elevated,[8,9] while glucosylsphingosine (GlcSph) shows a significant increase in GD.[3] Additionally, lysosphingomyelin (LysoSM), the N-deacylated derivative of sphingomyelin, has been reported to accumulate in patients with NPA, NPB, and NPC.[10,11]

The aim of this study was to establish and validate a robust analytical method for the simultaneous quantification of hexosylsphingosine (HexSph), LysoGb3, and LysoSM using LC-MS/MS. This method was optimized for use in clinical laboratories to support the diagnostic workflow for suspected sphingolipidoses and to monitor biochemical responses in patients receiving specific therapies, such as enzyme replacement therapy or substrate reduction therapy. The development of a multiplex assay for LysoSLs not only improves diagnostic efficiency but also facilitates the early detection and longitudinal assessment of disease activity, potentially leading to better patient outcomes. Our study introduces a locally optimized, rapid multiplex LC–MS/MS workflow for simultaneous quantification of LysoGb3, LysoSM, and HexSph with a 10-minute run time, which is shorter than that of comparable published methods.[12]

MATERIALS AND METHODS

Reagents and Chemicals

The following chemicals and reagents were used for LysoSL analyses: GlcSph (bovine buttermilk), N-omega-CD3-octadecanoyl-ceramide trihexoside (internal standard 1537; Matreya Chemicals), GalSph, LysoSM, LysoGb3, acetonitrile, methanol, acetone, chloroform, formic acid, and ultrapure water.

Sample Collection

Ethical approval was obtained from the Ethics Committee of Akdeniz University Faculty of Medicine (decision number: 373, dated: 30.05.2018). After obtaining informed consent, venous blood samples were collected into K2-EDTA tubes from 14 patients diagnosed with Fabry or GD and from 20 healthy adult volunteers at Akdeniz University Hospital. Plasma samples were separated and stored for analysis. Diagnoses were confirmed in all patients through demonstration of significant lysosomal enzyme deficiencies and/or mutation analysis.

Preparation of LysoSL Calibration Standards

Stock standard solutions were prepared by dissolving purified standards in a chloroform:methanol (2:1, v/v) mixture at the following concentrations: GlcSph, 0.5 mg/mL; LysoSM, 1 mg/mL; and LysoGb3, 0.1 mg/mL. GlcSph was used as the standard for HexSph. Working solutions at a concentration of 10 µmol/L for each analyte were prepared in the same solvent mixture and stored at -20 °C. These working solutions were diluted with methanol/acetone/water (45:45:10, v/v) to a final concentration of 10 nmol/L to prepare the precipitation solution containing the internal standard. A deuterium-labeled internal standard, N-omega-CD3-octadecanoyl-ceramide trihexoside (C18-CD3-Gb3), was used for quantification.

Calibration curves were constructed using six concentrations prepared by serial dilution of the stock solutions. The calibration ranges were 0-200 nmol/L for LysoGb3 and LysoSM, and 0-1000 nmol/L for HexSph. The accuracy and precision of the assay were evaluated using QC samples prepared at two concentration levels.

Sample Preparation

To 100 µL of plasma, calibrator, or QC sample, 500 µL of working precipitation solution was added. After vortexing for 30 seconds, the mixture was sonicated for 1 minute and subsequently centrifuged at 16,200 × g for 10 minutes. The clear supernatant was transferred to a new tube and evaporated under nitrogen. The dried residue was reconstituted in 100 µL of acetonitrile/water (1:1, v/v) containing 0.1% formic acid and centrifuged again at 16,200 × g for 10 minutes prior to LC-MS/MS analysis.

LC-MS/MS Method Optimization

Chromatographic separation of LysoGb3, HexSph, and LysoSM was achieved using a reverse-phase Raptor acid-resistant C18 column (2.1 × 100 mm, 2.7 µm particle size; Restek, USA). Because GlcSph and GalSph co-elute on this column, the two analytes were quantified together as HexSph (GlcSph + GalSph). Unlike most published studies focusing on a single analyte–disease pair, we provide a multiplexed approach applicable to multiple sphingolipidoses.

Mass spectrometric detection was performed on a UFLC-MS/MS system (LC-20 AD UFLC XR, Shimadzu 8040, Japan) equipped with a positive-mode electrospray ionization source. For each analyte, the precursor ions and optimized transitions were selected and monitored in multiple reaction monitoring mode. Detailed LC-MS/MS parameters are provided in Table 1.

Method Validation

The accuracy of the method was evaluated using quality control (QC) samples. Accuracy was calculated as the relative error (%), which was obtained by subtracting the nominal value from the mean measured value, dividing the difference by the nominal value, and multiplying by 100. Intra-day and inter-day precision were assessed by analyzing each standard in triplicate over consecutive days. Linearity was evaluated using six-point calibration curves for each biomarker. The limit of detection (LOD) and limit of quantification (LOQ) were determined for all analytes. Carryover effects were assessed by injecting blank samples after the highest calibration standard.

Table 1. Detailed LC-MS/MS parameters.

Parameter	Description
Mass spectrometer	Shimadzu 8040
Ionization mode	ESI, positive mode (+)
Column	Raptor ARC-18, 2.1 × 100 mm, 2.7 µm particle size
Column temperature	45 °C
Mobile phase A	H_2O + 0.1% FA
Mobile phase B	ACN + 0.1% FA
Gradient program (Pump B)	Time (min) %B 0–4 80 4–6 100 6–10 40
Flow rate	0.5 mL/min
Injection volume	2 µL
Total run time	10 minutes

H_2O 0.1% FA; water containing 0.1 percent FA, ACN 0.1% FA; acetonitrile containing 0.1 percent FA. ARC-18, acid-resistant C18; ESI, electrospray ionization; FA, formic acid.

Statistical Analysis

Statistical analyses were performed using SPSS version 20.0. Differences between groups were assessed using a two-tailed Student's t-test. A p-value ≤ 0.05 was considered statistically significant.

RESULTS

Chromatographic Analysis of LysoSLs

Chromatographic separation of LysoSLs was achieved with retention times of 1.57 minutes for LysoGb3, 1.72 minutes for LysoSM, and 1.98 minutes for HexSph. However, to ensure complete column elution and prevent carryover, the total run time was set to 10 minutes (Figure 1).

Linearity, Detection, and Quantification Limits

Repeated calibration curves were generated to assess linearity for LysoGb3, LysoSM, and HexSph, and the r^2 values were 0.995 for LysoSM, 0.982 for HexSph, and 0.961 for LysoGb3. A blank sample was injected after the highest calibration standard, and no carry-over was observed. The LOD and LOQ for LysoSLs were calculated from the mean and standard deviation of blank samples, which were run to obtain those statistics. The LOD and LOQ for LysoSLs were 0.012 and 0.036 nmol/L for LysoSM, 0.01 and 0.03 nmol/L for HexSph, and 7.18 and 21.54 nmol/L for LysoGb3, respectively (Table 2).

Precision and Accuracy

Precision and accuracy for each analyte were determined intraday (n = 5) and interday (n = 5). The % CV was below 20% for LysoSM and HexSph at all concentrations tested, whereas for LysoGb3 it was below 20% at 25 nmol/L and above.

LysoSL Levels in Healthy Controls and Lysosomal Disease (LD) Patients

Demographic characteristics of the study groups are summarized in Table 3. All Gaucher patients had Type 1 disease. Our study cohort consisted of seven male patients with the classic Fabry phenotype and three female patients with a stroke-associated Fabry phenotype. All patients were genetically confirmed. None of the patients were on enzyme replacement therapy at the time

of sampling. Plasma concentrations of LysoGb3, LysoSM, and HexSph in patients and controls are shown in Table 4.

Patients with GD exhibited significantly higher mean plasma levels of LysoSM and HexSph than those in healthy individuals. Although LysoGb3 levels were numerically higher in Fabry patients than in controls, the difference did not reach statistical significance ($p = 0.08$). The lack of statistical significance is likely due to the limited sample size (n=10) and the considerable

Table 2. Mass shifts and retention times of LysoSLs in LC-MS/MS.

Compound	Precursor ion (m/z)	Product ion (m/z)	Retention time (min)
LysoGb3	786.20	282.20	1.57
LysoSM	465.20	184.10	1.72
HexSph	462.20	282.30	1.98
LysoGb3-IS	843.20	264.30	1.40

Table 3. Demographic characteristics of patients and healthy controls.

Group	Controls	FD	GD
Sample size	20	10	4
Sex (M/F)	10/10	7/3	4/0
Age (mean, range)	37 (21–77)	37 (22–51)	40 (38–42)

Table 4. Plasma LysoSL levels in Gaucher and Fabry patients vs. healthy controls.

LysoSL	Group	Mean ± SEM (nmol/L)	p-value
LysoSM	Gaucher (n=4)	0.87 ± 0.28	0.001
	Control (n=20)	0.25 ± 0.02	
HexSph	Gaucher (n=4)	0.83 ± 0.39	0.001
	Control (n=20)	0.02 ± 0.002	
LysoGb3	Fabry (n=10)	35.63 ± 15.89	0.08
	Control (n=20)	10.03 ± 0.28	

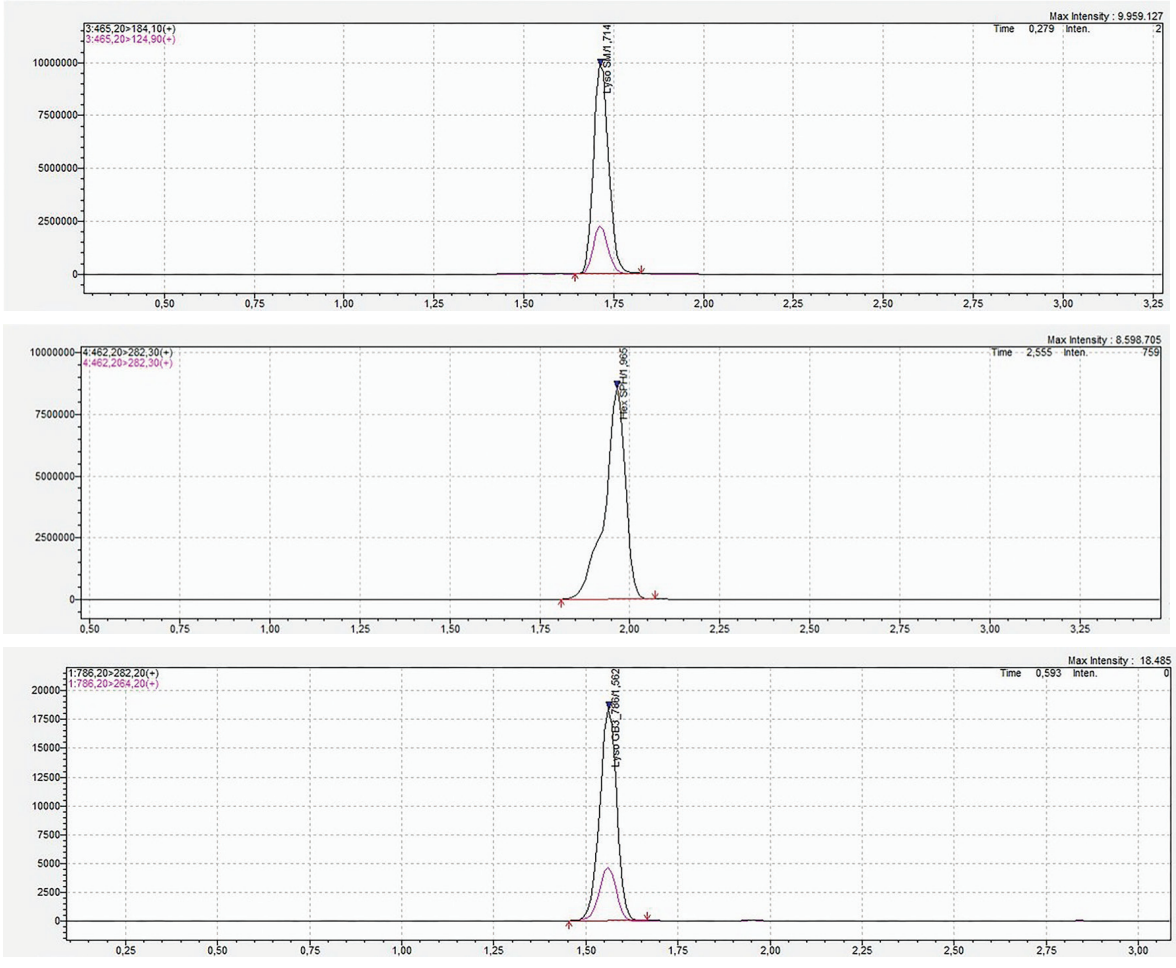


Figure 1. Chromatographic separation of LysoSLs with ARC-18 column in the 100 nmol/L calibrator (A. LysoSM 1.72, B. HexSph 1.98, C. LysoGb3 has an elution time (retention time) of 1.57 min.

variability between patients. In addition, differences in matrix and calibration, which affect absolute concentrations, may have masked numerical differences, even though they do exist.

DISCUSSION

LysoSLs have emerged as reliable and informative biomarkers for the diagnosis, prognosis, and therapeutic monitoring of sphingolipidoses, a group of inherited LSDs characterized by the accumulation of sphingolipid substrates due to enzyme deficiencies.[3,5] Numerous LC-MS/MS methods have been developed for the quantification of LysoSLs in various biological matrices, including plasma, urine, and dried blood spots.[6,9,11–15] However, a significant limitation of many previously published assays is their focus on a single analyte associated with a specific disease. While such approaches are effective for targeted diagnostics, they may fail to capture the broader metabolic alterations observed in LDs with overlapping or non-specific symptoms.

Recent studies have highlighted the value of multiplex panels that allow for the simultaneous measurement of multiple LysoSLs, suggesting that such panels can improve diagnostic yield, reduce turnaround time, and enhance cost-efficiency, particularly in high-throughput clinical settings.[14] The ability to evaluate multiple biomarkers from a single sample can also be particularly advantageous in early screening programs or when patient sample volume is limited. Moreover, multiplexing supports a more comprehensive understanding of disease pathology and may help differentiate between related disorders or phenotypic variants.

In this study, we developed and validated a rapid and accurate UFLC-MS/MS method for the simultaneous quantification of three key LysoSLs—HexSph (a combination of GlcSph and GalSph), LysoGb3, and LysoSM—in human plasma. The method is based on simple protein precipitation, avoiding the need for complex and time-consuming liquid–liquid extraction protocols. This streamlined sample preparation significantly reduces overall analysis time and enables high-throughput testing without compromising sensitivity or accuracy. The developed method is rapid, reproducible, and suitable for clinical laboratories. It simplifies sample preparation, reduces run time, and provides robust quantification.

Chromatographic separation was performed on a reversed-phase C18 column, which enabled efficient retention and resolution of LysoSLs. Due to the structural similarity between GlcSph and GalSph, these analytes were not resolved and were therefore reported collectively as HexSph. The use of a deuterated LysoGb3 internal standard ensured analytical robustness and reproducibility. The method demonstrated excellent linearity, precision, and accuracy, with detection and quantification limits comparable to or better than those reported in prior literature.

The analysis enabled the quantification of plasma LysoSL levels in healthy controls and in subjects with confirmed Gaucher and FD. Among Gaucher patients, LysoSM and HexSph were significantly elevated compared with controls, suggesting their relevance as disease-specific biomarkers. These findings are consistent with previous studies reporting elevated levels of LysoSM and GlcSph in GD, which is caused by β-glucocerebrosidase deficiency. In Fabry patients, plasma LysoGb3 levels were increased; however, this difference did not reach statistical significance, potentially owing to the limited sample size and the cross-sectional design of the study. Larger cohort studies may be needed to fully validate the diagnostic sensitivity and specificity of LysoGb3 in this population. During the study period (2018-2019), commercial control materials were not yet available in our laboratory.

Importantly, our method's short run time (10 minutes per sample) and compatibility with standard LC-MS/MS instrumentation make it well-suited for routine clinical application. The proposed panel could be expanded in future studies to include additional LysoSLs or disease-specific lipid species, thereby increasing its diagnostic utility. Furthermore, monitoring LysoSL levels over time may provide insights into disease progression and response to treatment, particularly in patients receiving enzyme replacement therapy or substrate reduction therapy.[16]

CONCLUSION

This study presents a robust, high-throughput UFLC-MS/MS method for the simultaneous quantification of clinically relevant LysoSLs in plasma. The approach holds promise for improving the efficiency and diagnostic accuracy of sphingolipidosis screening and may serve as a valuable tool in both clinical and research settings. Further validation in larger and more diverse patient populations is warranted to fully establish its diagnostic and prognostic utility.

Ethics

Ethics Committee Approval: Ethical approval was obtained from the Ethics Committee of Akdeniz University Faculty of Medicine (decision number: 373, dated: 30.05.2018).

Informed Consent: Informed consent was obtained from the parents of all patients included in this paper.

Footnotes

Authorship Contributions

Surgical and Medical Practices: H.A., E.S., Concept: H.A., Data Collection or Processing: H.A., B.K.E., N.Ö., Analysis or

Interpretation: H.A., E.S., Literature Search: H.A., A.E.B., B.K.E., Writing: H.A., A.E.B., B.K.E.

Conflict of Interest: The authors have no conflicts of interest to declare.

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ORIGINAL ARTICLE

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Confirmation and Determination of Urinary Ethylmalonic Acid by Capillary Electrophoresis with Indirect UV Detection

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Abstract

Objectives: Ethylmalonic acid (EMA) is a key urinary biomarker of ethylmalonic encephalopathy (EE), but routine gas chromatography–mass spectrometry (GC–MS) is time- and resource-intensive. This study aimed to develop and validate a simple, rapid capillary electrophoresis (CE) method with indirect ultraviolet (UV) detection for the quantitative determination of EMA in urine.

Materials and Methods: A fused-silica capillary and a background electrolyte containing 5 mmol/L 2,6-pyridinedicarboxylic acid and 0.1 mmol/L cetyltrimethylammonium bromide (pH 5.74) were used. Separation was performed at 28 kV and monitored at 200 nm. Fresh human urine was diluted 1:20, filtered, and used to prepare matrix-matched calibrators (10–750 µmol/L). Linearity, precision, sensitivity, and accuracy were assessed according to International Council for Harmonisation validation guidelines, using spiked urine samples. Electropherograms from healthy control urine, EMA-spiked urine, and urine from a patient with EE illustrated the applicability.

Results: Under optimized conditions, EMA migrated at approximately 2.0 min with baseline resolution from endogenous components. Calibration was linear between 10 and 750 µmol/L (R^2 = 0.99920). Within-run precision, expressed as relative standard deviation (%), was 0.17% for migration time and 0.89% for corrected peak area, while between-day precision for peak area was 1.91%. The limits of detection and quantification were 11.78 and 39.3 µmol/L, respectively. Recovery in spiked urine ranged from 96 ± 2% to 98 ± 2%.

Conclusion: The CE–UV method enables rapid, reliable quantification of urinary EMA with minimal sample preparation. Its short analysis time and modest instrumentation requirements support its use for first-line screening and monitoring of EE, with GC–MS reserved for confirmatory analyses.

Keywords: Ethylmalonic Acid, Electrophoresis, Capillary, Urine, Biomarkers, Metabolism, Inborn Errors

INTRODUCTION

Ethylmalonic encephalopathy (EE) is an autosomal recessive mitochondrial disorder caused by pathogenic variants in the *ETHE1* gene.[1] Clinically, EE is characterized by developmental delay, relapsing petechiae, orthostatic acrocyanosis, and chronic diarrhea, with a heterogeneous phenotype that can vary widely among patients.[2] The prognosis is poor. Approximately half of affected children die before the age of two due to metabolic decompensation.[3] Biochemical findings typically include persistently elevated lactate and increased butyryl- and isovalerylcarnitine in blood, together with urinary excretion of ethylmalonic acid (EMA), methylsuccinic acid, butyrylglycine, and isovalerylglycine.[1] Combined treatment with coenzyme Q10, N-acetyl cysteine, and decontamination of bowel with metronidazole results in marked neurological improvement, reduction of acrocyanosis and petechial lesions, and disappearance of diarrhea, especially in early treatment.[4]

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For disorders like EE, in which urinary organic acid analysis is integral to diagnosis, gas chromatography–mass spectrometry (GC–MS) remains the reference method for comprehensive analysis.[5] However, capillary electrophoresis (CE) offers an accessible and operationally simple alternative for targeted analytes. It enables rapid separations with minimal sample preparation and instrument time.[6,7] Indirect ultraviolet (UV) detection by CE has previously been applied to biologically relevant short-chain organic acids, including EMA.[8] More recently, urinary EMA has also been determined using CE with capacitively coupled contactless conductivity detection (CE–C^4D), illustrating detector-dependent trade-offs between sensitivity and instrumentation requirements.[9] These attributes make CE an attractive option for clinical laboratories performing high-throughput screening and follow-up quantification.

The objective of this study was to develop an simple, rapid, and accurate CE method to determine EMA in urine.

MATERIALS AND METHODS

Materials

EMA, cetyltrimethylammonium bromide (CTAB), and 2,6-pyridinedicarboxylic acid (PDCA) were obtained from Sigma-Aldrich, Merck (Darmstadt, Germany), and Fluka (Buchs, Switzerland), respectively. Solutions were prepared with purified, deionized water (Elgacam C114, Elga, England filtration system).

Urine samples were prepared daily by diluting fresh human urine in purified water. The dilution ratio was 1/20. EMA stock solution was dissolved in purified water and stored at +4 °C. Solutions prepared daily were filtered before analysis using a microfilter with a 0.45-µm pore size. The background electrolyte (BGE) used for separations consisted of 5 mmol/L PDCA and 0.1 mmol/L CTAB, adjusted to pH 5.74.

Apparatus and Operating Condition

Agilent CE system (Germany) equipped with a diode-array detector was used for EMA seperations. Agilent ChemStation software was used for data processing. 75-µm i.d. fused-silica capillaries for the separation experiment were purchased from Agilent. The total lengths of the capillary and the detector were 57 cm and 49 cm, respectively. The separations were performed at 28 kV with a wavelength set at 200 nm in indirect-UV mode.

The new fused-silica capillary adjustment was performed by rinsing with 1 mol/L NaOH for 30 min, followed by purified water for 10 min. The capillary was rinsed with 0.1 mmol/L NaOH and water for 3 min each, and was buffered for 3 min on each working day. A three-min flush with buffer was performed between runs. Injections were administered at 5 × 10−3 MPa for 5 s.

pH measurements were performed using Metrohm 654 Digital pH Meter (Metrohm, Switzerland) equipped with a pH glass electrode. The Addition of CTAB provided a positively charged inner surface, slowing or reversing electroosmotic flow (EOF) toward the anode, so that anionic analytes co-migrated with the bulk flow under the applied field.

Sample Preparation

Fresh urine was diluted 1/20 (urine/water), mixed, and filtered (0.45 µm) immediately prior to analysis. The 1:20 dilution was selected to minimize urine matrix effects (e.g., high ionic strength or conductivity and viscosity), which can compromise peak shape, current stability, and the migration-time repeatability in CE, while still enabling reliable detection and quantification of EMA within the validated range. This sample-handling approach is consistent with prior CE methods for urinary organic acids, where urine is commonly analyzed after centrifugation, with dilution as needed; a 1:20 final dilution has been reported for urine matrices in CE–based organic acid profiling.[10] Matrix-matched calibrators and recovery samples were prepared by spiking aliquots of 1/20-diluted pooled urine with EMA at specified concentrations (see Sections 2.4–2.5).

Calibration and Linearity

Method validation was performed according to the International Council for Harmonisation (ICH) Q2 (R^2) guideline.[11] Linearity was evaluated in 1:20-diluted human urine over the range 10–750 µmol/L EMA using matrix-matched external calibration. Calibration points across this range were injected ($n \geq 3$ per level), and peak quantification was performed using corrected peak areas (A/t). The calibration model was fitted using ordinary least squares linear regression.

Precision

Within-run precision was assessed by six successive injections ($n = 6$) of a 50 µmol/L EMA standard in urine diluted 1:20; precision was expressed as relative standard deviation (RSD%) for migration time and the corrected peak area (A/t). Between-day precision was determined as RSD% of peak areas from measurements obtained on three different days ($n = 3$).

Sensitivity Limit of Detection/Limit of Quantification (LOD/LOQ)

The LOD was defined as the EMA concentration producing a signal with peak height three times the baseline noise ($S/N \approx 3$), and the LOQ as the concentration corresponding to $S/N \approx 10$.

Accuracy (Recovery)

Accuracy was assessed by recovery experiments in urine diluted 1/20 at three EMA concentrations (25, 50, and 100 µmol/L). Each level was measured six times ($n = 6$), and recoveries were calculated as (measured/added) × 100%.

Data Processing

Electropherograms were integrated using ChemStation. Quantification employed corrected peak areas (A/t) to minimize run-to-run variability associated with migration time shifts. Quality metrics (linearity, precision, recovery, LOD/LOQ) were summarized according to ICH-style analytical validation conventions.

RESULTS

Validation Results

Method performance characteristics are summarized in Table 1. Linearity was demonstrated over 10–750 µmol/L using matrix-matched calibration in 1:20-diluted urine; the regression equation was $y = 2.25045 \times 10^{-3} x + 1.59091 \times 10^{-2}$ with $R^2 = 0.99920$ (Figure 1). Within-run precision, expressed as RSD%, was 0.17 RSD% for migration time and 0.89 RSD% for the corrected peak area (A/t) (n = 6). Between-day precision for corrected peak area was 1.91% RSD (three different days).

Sensitivity, assessed using signal-to-noise criteria, yielded an LOD of 11.78 µmol/L and an LOQ of 39.3 µmol/L. Accuracy assessed by recovery experiments at 25, 50, and 100 µmol/L (n = 6 each) showed recoveries ranging from 96 ± 2% to 98 ± 2% (Table 1).

EMA Electropherograms and Urinary EMA Levels

Under the separation conditions described above, EMA migrated at approximately 2.0 min. Electropherograms of a healthy control urine sample, a control urine sample spiked with EMA, and a urine sample from a patient diagnosed with EE are shown in Figure 2A–C, respectively. In the healthy control urine (Figure 1), no distinct peak was observed at the EMA migration time, indicating that EMA < LOQ (39.3 µmol/L) under the applied conditions. In the matrix-matched spiked urine (Figure 2B), pooled urine (1:20 dilution) fortified with EMA at 50 µmol/L produced a well-defined peak at the same migration time, confirming the peak identity and demonstrating baseline resolution from endogenous components. In the patient sample (Figure 2C), a prominent peak at ≈ 2.0 min was detected and quantified as 78.4 µmol/L EMA in the analyzed (1:20 diluted) urine, which is above the LOQ. Because indirect UV detection yields negative peaks, electropherograms were displayed after polarity inversion to facilitate visualization and integration. Urinary EMA levels for the illustrative samples are summarized in Table 2.

Table 1. Validation values.

	Parameter	Result
Precision	Within-run precision (corrected peak area, A/t), RSD% (n = 6)	0.89
	Within-run precision (migration time), RSD% (n = 6)	0.17
	Between-day precision (corrected peak area, A/t), RSD% (n = 3 days)	1.91
Sensitivity	LOD (µmol/L)	11.78
	LOQ (µmol/L)	39.30
Accuracy (recovery)	EMA-spiked urine samples (25 µmol/L), recovery (%)	96 ± 2
	EMA-spiked urine samples (50 µmol/L), recovery (%)	96 ± 2
	EMA-spiked urine samples (100 µmol/L), recovery (%)	98 ± 2

EMA, ethylmalonic acid; LOD, limit of detection; LOQ, limit of quantification; RSD, relative standard deviation.

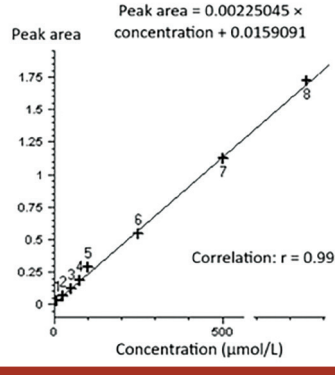


Figure 1. Calibration curve for EMA ethylmalonic acid.
EMA, ethylmalonic acid.

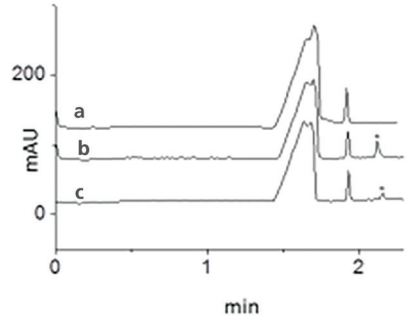


Figure 2. (a) Shows the electropherogram of healthy urine; (b) shows the electropherogram of a healthy urine spiked with EMA; (c) shows the electropherogram of a patient diagnosed with EE.
EE, ethylmalonic encephalopathy; EMA, ethylmalonic acid.

Table 2. Urinary EMA levels in illustrative human urine samples analyzed by CE–UV.

Sample (Figure 1)	Matrix/dilution	EMA status	Reported urinary EMA (µmol/L)	Interpretation
Healthy control	Urine, 1:20 diluted	Unspiked	<LOQ (39.3)*	Not quantifiable under conditions
Spiked control	Pooled urine, 1:20 diluted	Spiked	50	Peak identity confirmed at ≈ 2.0 min
EE patient	Urine, 1:20 diluted	Clinical sample	78.4	Quantifiable; above LOQ

*LOQ, 39.3 µmol/L. For the spiked sample, the concentration indicates the nominal added EMA level.

CE, capillary electrophoresis; EE, ethylmalonic encephalopathy; EMA, ethylmalonic acid; LOQ, limit of quantification; 2,6-pyridinedicarboxylic acid; UV, ultraviolet.

DISCUSSION

This study optimized a CE–UV method for the rapid quantification of urinary EMA. The method achieved a migration time of ~ 2 minutes in urine diluted 1:20, demonstrated excellent linearity across 10–750 µmol/L (r = 0.99920), exhibited tight intra- and interday precision, and showed high recovery. The LOD and LOQ were 11.78 and 39.3 µmol/L, respectively. EMA is a clinically significant urinary biomarker in EE and may be elevated in short-chain acyl-CoA dehydrogenase deficiency. Therefore, fast, reproducible first-line quantification of EMA has practical value for triage and monitoring.[1,12]

Traditionally, urine organic acid analysis is performed by GC–MS, which remains the confirmatory reference method. However, GC–MS requires derivatization and extraction as well as batch processing, which together extend turnaround times and increase resource use.[13] Faster preliminary results are clinically useful in disorders where EMA abnormalities inform acute and longitudinal management. This enables earlier decision-making while MS confirmation proceeds.[1] In this context, our CE–UV assay shortens the analytical step to minutes while maintaining the quantitative performance necessary for routine screening and monitoring. This enables a more efficient workflow in which cases that are clearly abnormal or ambiguous proceed to MS confirmation.[14]

The separation strategy employed in this study established principles for the separation of anionic analytes. Cationic surfactants, such as CTAB, dynamically modify the capillary wall, thereby reversing EOF. This promotes the rapid and reproducible migration of anions to the detector.[15,16] Because EMA lacks a strong native UV absorbance, indirect UV detection is appropriate. A Prior study has demonstrated indirect CE–UV of biologically relevant short-chain organic acids, including EMA, by using UV-absorbing co-ions and mobility matching.[8] The use of PDCA as the absorbing co-ion in the BGE during method implementations further supports robust indirect UV detection of carboxylic acids.[17] Our data expand upon existing literature by demonstrating the quantification of short-term EMA directly from minimally processed urine using a widely available UV/diode-array detector platform. Alternative CE implementations, such as CE–C^4D, have also achieved sensitive EMA detection, albeit with different detector trade-offs.[9]

Two methodological aspects merit emphasis. First, stable approximately 2-minute separations are achieved using low-level CTAB (0.1 mmol/L) with PDCA (5 mmol/L), a balanced EOF control, and indirect UV detection.[15] Second, our LOQ of 39.3 µmol/L is adequate for routine urine EMA screening within a reflex workflow, while CE–C^4D and MS methods achieve lower limits—an LOQ of ~ 3–4 µmol/L and an LOD of ~ 1 µmol/L—reflecting a trade-off between accessibility and sensitivity.[9] Given that EMA elevations in EE and related phenotypes are typically above normal, borderline results can be reflexed to MS for confirmation.[1]

From an interpretive standpoint, the method's analytical limitations should be considered when reporting results for healthy subjects and patients. In the healthy control (Figure 1), the absence of a distinct EMA peak at ≈ 2.0 min should be interpreted as EMA < LOQ (39.3 µmol/L) rather than complete absence. By contrast, the EE patient sample (Figure 1) showed an EMA concentration of 78.4 µmol/L in 1:20-diluted urine, which was readily quantifiable by CE–UV. Compared with indirect UV detection, CE–C^4D generally provides lower limits of detection for small ionic acids, which can improve interpretation of low-level or borderline EMA elevations (9).Therefore, samples near the LOQ or with strong clinical suspicion should be reflexed to CE–C^4D or MS-based confirmatory testing

This study has limitations. Indirect UV lacks the molecular specificity of MS; therefore, rare co-migrating matrix anions could interfere. This warrants orthogonal confirmation when measurements are near the LOQ or resolution is suboptimal. Also, the absolute concentrations in spot urine were reported without creatinine normalization. Laboratories that adopt this method should validate reporting in µmol/L with paired creatinine measurement or in mmol/mol creatinine, according to local practice standards. Finally, a head-to-head comparison against GC–MS and LC–MS in the same cohort was not performed to establish clinical concordance and decision thresholds for reflex testing.

In summary, we present a fast, precise, and accessible CE–UV assay for urinary EMA that can separate samples in approximately two minutes each with excellent linearity, low imprecision, and high recovery. In a reflex workflow, CE–UV can be used for same-

day screening. Samples with borderline concentrations can then be referred to GC–MS for confirmation or further analysis. This strategy could reduce turnaround time, allow GC–MS resources to focus on cases requiring greater specificity, reduce workload, and support longitudinal monitoring in EE.

CONCLUSION

A rapid and accessible CE–UV method for urinary EMA quantification was developed and validated. The assay provides an approximately 2-minute separation and demonstrates excellent linearity, low imprecision, and high recovery in urine diluted 1:20. This approach can support same-day screening and follow-up, with reflex MS confirmation recommended for borderline concentrations or when analytical specificity is required.

Ethics

Ethics Committee Approval: This study was approved by Clinical Research Ethical Committee of Cerrahpaşa Faculty of Medicine (approval number: E-29430533-604.01-01-273163, dated: 08.10.2015).

Informed Consent: All urine specimens were de-identified residual samples obtained during routine laboratory procedures, and no identifiable patient information was accessed. Therefore, the requirement for informed consent was waived by the Local Ethics Committee.

Footnotes

Authorship Contributions

Concept: M.Ş.C., E.K., T.Z., A.Ç.A.Z., F.B.E.B., Design: M.Ş.C., E.K., T.Z., A.Ç.A.Z., F.B.E.B., Data Collection or Processing: M.Ş.C., K.Ç., E.K., T.Z., A.Ç.A.Z., F.B.E.B., Analysis or Interpretation: M.Ş.C., K.Ç., E.K., T.Z., A.Ç.A.Z., F.B.E.B., Literature Search: M.Ş.C., K.Ç., E.K., T.Z., A.Ç.A.Z., F.B.E.B., Writing: M.Ş.C., K.Ç., E.K., T.Z., A.Ç.A.Z., F.B.E.B.

Conflict of Interest: One author of this article, Ertuğrul Kıykım, is a member of the editorial board of the Inherited Metabolic Disorders and Nutrition. However, he did not take part in any stage of the editorial decision of the manuscript. The editors who evaluated this manuscript are from different institutions. The other authors declared no conflict of interest.

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CASE REPORT

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A Novel *CLN1* Variant in a Turkish Patient with Infantile Neuronal Ceroid Lipofuscinoses

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Abstract

The neuronal ceroid lipofuscinoses (NCLs) are a group of progressive neurodegenerative disorders characterised by abnormal accumulation of ceroid and lipofuscin in lysosomes and the cytoplasm. The progressive accumulation of ceroid and lipofuscin in the central nervous system leads to psychomotor retardation, vision loss, and epilepsy. NCLs are the most common neurodegenerative diseases of childhood, and a diagnosis of NCLs should be considered in individuals presenting with characteristic clinical symptoms and magnetic resonance imaging findings. A definitive diagnosis should be confirmed by an enzyme activity assay, skin biopsy or variant analysis. We present a case of infantile NCL with a novel variant.

Keywords: *CLN1*, Infantile neuronal ceroid lipofuscinoses, Novel variant

INTRODUCTION

The neuronal ceroid lipofuscinoses (NCLs) are a group of inherited, progressive neurodegenerative disorders that primarily affect children. They are among the most common neurodegenerative diseases of childhood. The age at which symptoms first appear can range from birth to adulthood. The main clinical symptoms are vision loss, dementia, loss of motor skills, epilepsy and, eventually, premature death.[1] NCLs are characterised by total brain atrophy and the abnormal accumulation of autofluorescent pigment in neuronal tissues.[2]

NCLs are classified both clinically and genetically. They are categorised by the age at which the disease manifests (congenital, infantile, late-infantile, juvenile, or adult) and by the defective gene. To date, fourteen different NCL forms have been described based on genetic analysis.[3] However, NCLs are a genetically heterogeneous group, and variants in the same gene can lead to different clinical courses.[4] A definitive diagnosis should be confirmed by both an enzyme activity assay and variant analysis. Here, we present a case of infantile NCL with a novel variant.

CASE REPORT

A two-year-old girl born to parents who were first-degree cousins presented to our clinic with restlessness. The prenatal, natal, and postnatal periods were uneventful, and she developed age-appropriate physical skills by one year of age. The patient had approximately 10–15 meaningful words at 12 months of age; however, her speech development did not progress further and subsequently regressed. She gradually lost previously acquired words and developed an articulation impairment. After the age of one year, her parents noticed a significant deterioration in her speech and other cognitive functions. The patient achieved independent walking at 13 months of age; however, her gait became ataxic after one year of age and progressively

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deteriorated, such that by 18 months she had completely lost her ability to walk. She developed myoclonic jerks, both spontaneous and stimulus-sensitive. There was no history of prolonged epileptic seizures or status epilepticus.

On physical examination, she was conscious and had significant hypotonia in her trunk and severe hypertonia in her limbs. Her deep tendon reflexes were increased. No visual tracking response was observed during the examination. Fundoscopic examination could not be performed at the time of evaluation; therefore, retinal findings could not be assessed. Examinations of the cardiac and respiratory systems were normal. There was no evidence of organomegaly. Inborn errors of metabolism, such as congenital defects of glycosylation, leukodystrophies, mitochondrial cytopathies, and NCL, were considered in the differential diagnosis. The complete blood count and biochemical parameters were within normal limits. Metabolic screening tests, such as Tandem MS and urinary organic acid analysis, revealed no pathological findings; however, nonspecific changes were observed in quantitative blood amino acid levels. Magnetic resonance imaging (MRI) of the brain revealed diffuse cerebral atrophy and corpus callosum agenesis. A skin biopsy revealed the presence of loose, membrane-bound granular osmiophilic deposits in sweat gland epithelial cells, blood vessel smooth muscle cells, endothelial cells, and fibroblasts. Examination by electron microscopy revealed buffy coat lymphocytes, which, together with the storage inclusions seen in the skin biopsy, were typical of the classic form of infantile NCL.

CLN1 gene sequencing was performed using the MiSeq next-generation sequencing platform (Illumina, San Diego, California, USA) according the manufacturer's instructions. The sequences were then aligned to the hg19 genome using the MiSeq Reporter software (Illumina Inc.). The data were visualised using IGV 2.3 (Broad Institute). A homozygous p.P238Cfs*56 (c.712_713delCC) variant was identified. This frameshift variant is predicted to result in a premature stop codon, leading to a truncated and likely non-functional protein. According to the American College of Medical Genetics and Genomics/Association for Molecular Pathology guidelines, the variant was classified as likely pathogenic. The frameshift nature supports the PVS1 criterion, and its absence from population databases and an in-house cohort of approximately 300 Turkish exome datasets supports PM2. In addition, *in silico* prediction tools provided supporting evidence, although conflicting results were also noted.The variant was further evaluated using standard bioinformatics pipelines in an in-house exome sequencing database comprising approximately 300 individuals of Turkish origin and was not detected in this cohort. Enzyme analysis was requested; however, the results were not available at the time of writing. Segregation analysis in the family was requested; however, the results were not available at the time of writing.

DISCUSSION

Here, we present a Turkish patient with infantile NCL carrying a novel *CLN1* variant. To date, fourteen different NCLs have been described, although some individuals with NCL were described as having a classical disease presentation despite no mutation being detected in the *NCL* gene.[1] *CLN1*, *CLN2*, *CLN10*, and *CLN13* are caused by defects in *lysosomal enzymes*, while *CLN3*, *CLN6*, *CLN7*, and *CLN8* are caused by defects in transmembrane proteins. *CLN12* and *CLN14*, on the other hand, are caused by mutations in the ATPase and potassium channel genes, respectively.[1,5,6] A newly described *CLN4* gene plays an important role in putative synaptic functions.[7]

Infantile NCL is caused by defects in palmitoyl-protein thioesterase-1 (PPT-1). PPT-1 cleaves long-chain fatty acid moieties from cysteine residues on a multitude of protein targets.[8]

NCLs present with similar clinical manifestations, including cognitive decline, motor impairment, myoclonus, seizures, deteriorating visual acuity, and other visual impairments. Although there are no specific laboratory tests available for NCL, MRI findings such as diffuse atrophy, cerebral atrophy, cerebellar atrophy and thalamic T2-weighted hypointensity, as well as diffuse T2-weighted hyperintensity in the periventricular area and centrum semiovale, can aid the differential diagnosis of NCL.[1,2]

In our patient, agenesis of the corpus callosum was also observed on MRI. This finding is not commonly reported in classical infantile *CLN1*-related NCL and may represent either an additional feature or a coincidental anomaly.

In infantile NCL, a previously healthy, normally developing child begins to lose developmental milestones. Additional symptoms include myoclonus, visual impairment and refractory seizures. Infantile NCL is a rapidly progressive lysosomal storage disorder, with patients experiencing fulminant brain atrophy and progressive microcephaly.[8] Electron microscopy of affected tissues demonstrates the accumulation of granular osmiophilic dense bodies. Our patient exhibits the same clinical features as those reported in the literature.[2] Although the identified variant is compatible with the clinical and pathological findings, the absence of enzyme activity analysis, segregation data, and fundoscopic examination is an important limitation. Biochemical and functional validation would further strengthen the diagnostic accuracy and interpretation of the variant.

To date, 64 pathogenic variants have been identified in the *CLN1* gene. The novel variant detected in our patient expands the mutational spectrum of *CLN1*-related disease. NCLs may present in congenital, infantile, late-infantile, juvenile, or adult

forms. However, this variant did not produce any additional or atypical phenotypic features. The patient exhibited findings fully consistent with the classical infantile form, indicating that the newly identified genotype does not appear to alter the expected clinical presentation. Further cases and functional studies will be necessary to confirm its pathogenicity and to determine whether this variant has any subtle effects on disease progression.

CONCLUSION

We present the case of a Turkish patient with classic infantile NCL who carries a novel *CLN1* variant. While this variant expands the known *CLN1* mutational spectrum, it does not appear to alter the expected clinical phenotype. The patient's symptoms were fully consistent with the classical infantile form. Further cases and functional studies are required to confirm the pathogenicity of this variant.

Ethics

Informed Consent: Informed consent was obtained from the parents of all patients included in this paper.

Footnotes

Authorship Contributions

Concept: T.A.Ç., S.G., Design: T.A.Ç., S.G., E.İ.Ş., A.Ç.A.Z., Data Collection or Processing: T.A.Ç., S.G., E.İ.Ş., K.Ç., T.Z., M.Ş.C., S.C., A.Ç.A.Z., Analysis or Interpretation: T.A.Ç., S.G., K.Ç., H.B.A., T.Z., M.Ş.C., S.C., A.Ç.A.Z., Literature Search: T.A.Ç., S.G., H.B.A., T.Z., S.C., A.Ç.A.Z., Writing: T.A.Ç., S.G.

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REVIEW

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A Brief Overview of Fatty Acid Oxidation Disorders

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Abstract

A number of genetic disorders affecting every stage of fatty acid oxidation (FAO) have been identified. The most common manifestations are fasting-induced hypoglycemia and, less commonly, life-threatening coma. The research findings indicate chronic cardiomyopathy and muscle weakness. Less frequently, evidence of rhabdomyolysis is observed. The initial symptoms may manifest at various stages, ranging from the neonatal period to adulthood. The diagnosis of this condition is facilitated by detecting elevated levels of abnormal acylcarnitines, which can be measured by tandem mass spectrometry. During periods of acute illness, initiating intravenous fluid therapy containing dextrose is recommended to suppress lipolysis. In long-term treatment, the most significant aspect is the prevention of prolonged fasting. This review aims to provide information on the clinical findings, diagnosis, and treatment methods of FAO disorders.

Keywords: Fatty acid oxidation, Reye-like syndrome, Hypoketotic hypoglycemia, Cardiomyopathy, Rhabdomyolysis

INTRODUCTION

All cells, with the exception of adult erythrocytes, use mitochondrial fatty acid oxidation (FAO) as the primary mechanism for the breakdown of long-chain fatty acids.[1] The mechanism of FAO was identified by German chemist Georg Franz Knoop in the 1900s.[2] His seminal research on odd- and even-chain ω-phenyl fatty acids demonstrated that fatty acid metabolism proceeded via removal of two-carbon units sequentially. In addition to promoting oxidative phosphorylation and the tricarboxylic acid (TCA) cycle, FAO increases the production of ketone bodies in the liver. This pathway is made up of 25 enzymes and specific transport proteins.[3] Furthermore, the FAO has been found to play a significant role in the pathophysiology of common disorders such as insulin resistance, diabetes, obesity, kidney fibrosis and heart failure.[2]

CLINICAL MANIFESTATIONS

Numerous clinical diseases are caused by disorders of FAO. Patients may exhibit completely diverse symptoms even if they have the same mutation.[4] Hypoketotic hypoglycemia during catabolic states such as illness, fever, fasting, and exercise may be a clinical sign in patients with FAO disorders (FAODs). Hypoketotic hypoglycemia can progress to Reye-like syndrome, which may lead to coma or death. Carnitine transporter deficiency (CTD), very long-chain acyl-CoA dehydrogenase (VLCAD), trifunctional protein (TFP), long-chain hydroxyacyl-CoA dehydrogenase (LCHAD), carnitine acyl-carnitine translocase (CACT), and carnitine palmitoyltransferase-II (CPT-II) deficiencies may manifest as hypertrophic or dilated cardiomyopathy.[5,6] Although there have been documented exceptions to this trend, patients with CPT-IA and medium-chain acyl-CoA dehydrogenase (MCAD) deficiencies typically do not have cardiomyopathy.[5-7] Patients with FAOD disorders may also present with arrhythmias and conduction deficits, but they often do not have cardiomyopathy.[5,6] The presence of these symptoms in CTD, CPT-IA, and MCAD deficiencies (MCADDs) is also controversial.[6]

Furthermore, skeletal myopathy, which is characterized by rhabdomyolysis, myalgia, and muscle weakness, may manifest or develop in patients with an FAOD.[5,6] These symptoms are the most common in patients who present at a later age. Moderate lipid accumulation may be seen in muscle biopsies, primarily in type 1 fibers.[8] VLCAD-deficient patients had proximal myopathy

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whereas similar findings appeared in distal muscle groups in patients with LCHAD deficiency.[2]

Hepatomegaly likely reflects fatty liver.[6] Hepatic steatosis is primarily associated with MCADD, VLCAD deficiency (VLCADD), LCHAD/mitochondrial TFP deficiency, and multiple acyl-CoA dehydrogenase deficiency (MADD). Lipid accumulation in the liver results from inhibition of mitochondrial β-oxidation, thereby preventing hepatocytes from adequately using fatty acids as fuel during fasting, illness, or catabolic stress. Fatty acids that should be oxidized are instead re-esterified into triglycerides and retained in hepatocytes. Patients with LCHAD and MTP deficiencies may experience retinopathy and polyneuropathy (peripheral neuropathy).[9,10]

Hepatic, cardiac, and skeletal muscle signs and symptoms of early-onset multisystem failure are particularly common in long chain FAOD.[11] The heart has a continuously high energy demand to sustain contractile function. To secure continuous ATP production, the heart is a metabolic "omnivore" that can use many different substrates, depending on their availability.[12] FAO, however, is the preferred pathway for energy production in the heart, with more than half of ATP production derived from fatty acids. In contrast, the prenatal heart relies on glucose and lactate, but in the immediate postnatal period, when dietary fat is abundant, the heart switches to FAO as a source of energy.[13]

FAO is also crucial for ATP production in muscle; in particular, during exercise, FAO increases from rest to low-intensity exercise but does not increase further as exercise intensity increases. This pattern is in contrast to that seen for glucose and glycogen utilization, which always increase with exercise intensity. The sources of fatty acids also differ depending on the exercise intensity, with the contribution of plasma free fatty acids increasing with exercise intensity.[14]

In some patients, renal tubular disease may also be evident. Failure to supply the brain with glucose or ketones leads to neurological symptoms. Neonatal presentations usually feature undetectable protein expression or enzyme activity and are strongly associated with the severity of the mutation.[15]

The major manifestations of the late-onset forms of these disorders usually involve skeletal muscle or the heart. In these cases, residual enzyme activity and ketogenesis appear sufficient to preserve the liver and protect the brain from major involvement. Many patients with later-onset disease do not exhibit severe fasting intolerance but have exercise-induced rhabdomyolysis. In all long-chain FAOD that exhibit rhabdomyolysis, release of myoglobin may result in glomerular injury and acute renal failure.[11]

Many individuals are compound heterozygotes for a severe and a mild mutation, and intermediate forms of these long-chain FAODs exist. During fasting, these individuals are at risk of metabolic decompensation, hepatic failure, and potentially life-threatening hypertrophic cardiomyopathy. Additionally, some patients experience skeletal myopathy and are susceptible to acute muscle complaints, such as rhabdomyolysis, while under stress.[9,15,16]

Although the precise mechanisms causing heterogeneous presentations are still not completely known, as with many other enzyme deficiencies, the amount of residual activity of the enzyme is thought to define the clinical phenotype.[17] In addition to residual enzyme activity, other genetic and environmental factors have been found to be significant in the manifestation of disease. Heterogeneous clinical presentations within the same family provide particular evidence for this.[18] The marked intrafamilial phenotypic variability observed in FAODs suggests that the clinical course cannot be explained solely by the underlying pathogenic genotype. Even among relatives carrying the same disease-causing variant, presentations may range from severe neonatal or infantile disease to relatively mild, late-onset, or even apparently asymptomatic forms. This variability supports the concept that additional factors, including modifier genes, epigenetic mechanisms, residual enzyme activity, and environmental triggers such as fasting, intercurrent illness, dietary habits, and metabolic stress, contribute substantially to disease expression.

Carnitine Transporter Deficiency

Early childhood onset cardiomyopathy, with or without weakness and hypotonia, recurrent hypoglycemic hypoketotic seizures and/or coma, failure to thrive, and exceptionally low plasma and tissue carnitine concentrations are the hallmarks of CTD, a potentially fatal autosomal recessive disorder.[19] This condition exhibits phenotypic diversity; many patients develop symptomatic cardiomyopathy or recurrent hypoketotic hypoglycemia (Reye-like episodes) in early years of life.[4] Muscle hypotonia, modest developmental delay, failure to thrive, delayed walking, and poor growth are symptoms that newborns may initially exhibit. Some patients may have isolated cardiomyopathy, which can present with sudden death later in childhood. Within the same pedigree, both a symptomatic and symptomatic individuals have been reported.[18] Evidence suggests that compared to those who appear clinically, asymptomatic patients typically have slightly higher residual activity.[20] It is suggested carriers of a single *OCTN2* mutation may be susceptible to clinical illness if they experience enough stress.[19]

Carnitine Palmitoyltransferase-IA Deficiency

Related carnitine acyltransferases are encoded by 3 genes in the human genome. The liver isoform of CPT-I is encoded by *CPT-IA*, muscle-type CPT-I is encoded by CPT-IB, and CPT-IC encodes brain isoform.[21] The liver, kidney, lung, spleen, gut, pancreas, ovary, and fibroblasts all express CPT-IA isoform.[4]

The hepatic (and renal) CPT-IA deficiency is currently the sole known human CPT-I defect.[11] Patients with CPT-IA deficiency typically exhibit recurrent episodes of fasting-induced hepatic failure (Reye-like hepatic encephalopathy) with hypoketotic hypoglycemia, metabolic acidosis with elevated transaminases, hepatomegaly, hepatosteatosis, and mild to moderate hyperammonemia in infancy, reflecting the tissue localization of this isoform of CPT-IA.[11] Hypoglycemia stems from increased glucose utilization due to the inability to produce ketone bodies, and from decreased gluconeogenic capacity because of the absence of acetyl-CoA produced by FAO, a crucial activator of gluconeogenesis. The expression of CPT-IA in the renal tubular epithelium, which depends on FAO to sustain the high energy transport mechanisms, explains why renal tubular acidosis during catabolic periods has also been documented in a number of patients.[4]

Neonates have been documented to exhibit a variety of cardiac abnormalities, such as tachycardia, bradycardia, arrhythmias, right bundle branch block, and abrupt cardiac arrest.[4] One possible explanation is the expression of CPT-IA in the fetal heart, which persists until the neonatal stage.[22] Although hyperlipidemia is uncommon in other FAOD, hyperlipidemia with elevated triglycerides and/or cholesterol might be an additional feature of CPT-IA deficiency.[23] Hepatic VLDL production may be particularly favored by blockade of the outer mitochondrial membrane, leading to hyperlipidemia.

Carnitine Palmitoyltransferase-II Deficiency

There is one widely expressed isoform of the CPT-II enzyme.[4] There are three phenotypes of CPT-II deficiency: a lethal neonatal-onset form with congenital anomalies, a severe neonatal hepatocardiomuscular form, and a mild myopathic adult form.[4] Non-ketotic hypoglycemia, liver disease, hypotonia, cardiomyopathy, and congenital anomalies are the hallmarks of the fatal newborn phenotype.[24] The infantile form, whether there is cardiac disease or not, manifests as periods of decompensation with liver and skeletal muscle involvement, mainly as non-ketotic hypoglycemia caused by fasting and/or concurrent infection.[25] After childhood, cardiac symptoms are uncommon.[2]

About 50% of myopathic diseases are caused by the prevalent S113L mutation.[4] Because the frequent and high residual activity S113L mutation, causes the production of a thermolabile enzyme, the enzyme activity is further decreased by protein degradation during intense exercise when muscle temperature rises, aggravating the injury to the muscles.[4] The S113L variant, particularly in the homozygous state, is typically associated with the late-onset muscular form rather than with the severe neonatal or infantile forms. For infantile type CPT-II deficiency, there is no reliable genotype–phenotype correlation exists.[17]

Carnitine Acyl-Carnitine Translocase Deficiency

CACT deficiency causes heterogenous clinical phenotype.[26] Attenuated cases may manifest in the first few months of life, but severe neonatal-onset disease is most common, with symptoms emerging within two days of delivery.[26] Early-onset illness is characterized by hyperammonemia, cardiac arrhythmias, and an increased incidence of cardiac arrest. Clinical manifestations also include poor feeding, lethargy, hypoketotic hypoglycemia, hypotonia, transaminitis, liver dysfunction with hepatomegaly, and rhabdomyolysis. Cases of univentricular or biventricular hypertrophic cardiomyopathy, ranging from mild to severe, may improve with proper nutritional and medical interventions. Arrhythmia, especially ventricular tachycardia, is the most prominent cardiac manifestation during the neonatal period. This is followed by various tachyarrhythmias and bradyarrhythmias. Individuals with the early-onset form of the disease typically present with brain damage due to hyperammonemia. Patients with later-onset disease have milder symptoms and are less likely to experience recurrent hyperammonemia, leading to better developmental outcomes. Typically, carnitine levels are quite low (<5 μM).[2] In the plasma acylcarnitine profile, long-chain acylcarnitines are significantly elevated, whereas free carnitine levels are significantly reduced. Excess unsaturated species in urine organic acids can indicate severe dicarboxylic aciduria.[27] Differential diagnosis with genetic testing is necessary as this abnormal profile cannot be distinguished from that of neonatal CPT-2 deficiency.[27]

Very Long-chain Acyl-CoA Dehydrogenase Deficiency

The first rate-limiting stage of mitochondrial long-chain FAO is catalyzed by VLCAD.[11] Like other FAO enzymes, human VLCAD is highly active in the heart, liver, and skeletal muscle.[28] VLCADD is the most prevalent long-chain FAOD in the majority of populations.[29] The three main phenotypes of VLCADD are a late onset myopathic form, a milder childhood form that typically presents with hypoketotic hypoglycemia, but frequently has exercise intolerance and rhabdomyolysis as a significant feature, especially in older children and young adults.[4] A severe infantile phenotype manifests early in life with hypoglycemia, hepatic dysfunction, acidosis, and cardiomyopathy. Within the first few months, 75% of patients who survive early onset symptoms succumb to death.[30] Lower incidences of cardiomyopathy and hypoketotic hypoglycemia are features of childhood-onset

VLCADD, which is milder than early-onset. There has also been evidence of an association to rhabdomyolytic episodes in later childhood.[31]

Muscle pain and stiffness driven by exercise, fasting, temperature extremes, and occasionally infection are the hallmarks of the late-onset myopathic variants of both CPT-II and VLCADD.[4] Although about 20% of individuals will exhibit some lipid accumulation, muscle biopsies are often normal. When combined, these two conditions are the most prevalent inherited metabolic causes of rhabdomyolysis and myalgia in both adults and children.

Numerous *ACADVL* mutations have been identified, the most prevalent of which is c.848T>C.[32] The V243A mutation in the *VLCAD* gene has been identified in a large percentage of asymptomatic patients since newborn screening was implemented.[17] Which individuals, if any, may remain asymptomatic is unknown. There has also been evidence of a relationship between residual activity and clinical phenotype for VLCADD, with residual activities >10% indicating a milder phenotype.[33]

Long-Chain Hydroxyacyl-CoA Dehydrogenase Deficiency and Trifunctional Protein Deficiency

Isolated LCHADD is more common than TFP deficiency in relation to TFP functions.[4] Patients with TFP deficiency display a broad range of clinical symptoms, from mild to severe infantile presentation with hepatic indications to severe newborn manifestations such as cardiomyopathy and mortality.[18] Irreversible peripheral neuropathy and retinopathy presenting in TFP and LCHADD, have not been documented in any of the other long-chain FAOD.[17] Peripheral neuropathy develops in up to 80% of TFP deficient cases during long-term follow-up, according to various studies,[9,34] whereas in LCHADD, it is reported to occur only in 5–10% of cases.[35] In comparison, only 5–13% of patients with TFP deficiency and 30% to >50% of LCHADD patients are known to experience retinopathy.[9,34] These diseases typically manifest as episodic rhabdomyolysis during physical activity, illness, or fasting, however this may not show up for years following the initial neurological manifestation.[34,36] It has been suggested that the particular toxicity of the 3-hydroxacyl metabolites is the cause of the neuropathy and rhabdomyolysis observed in mild TFP deficiency.[37] One uncommon morbidity of both LCHADD and TFP deficiency is hypoparathyroidism.[38]

Mild TFP deficiency is uncommon and has a peculiar phenotype that shares characteristics with spinal muscular atrophy and inherited sensory-motor neuropathies.[34,36] In addition to symmetric weakness in the wrist and finger extensors, there is a gradual peripheral polyneuropathy that primarily affects the lower limbs starting in infancy or early childhood.[4] Exercise intolerance, planovalgus deformities, bilateral Achilles tendon contractures, and loss of vibratory sensation are potential manifestations.

Acute fatty liver of pregnancy and hemolysis, elevated liver enzymes, and low platelets (HELLP) syndrome can occur in heterozygous pregnant female carriers of TFP mutations carrying a homozygously affected offspring.[11] The pathophysiology of all these extra issues with TFP defects is unknown, but it could be caused by the hazardous accumulation of 3-hydroxy fatty acid intermediates or by unidentified FAO pathway requirements in the retinal, placental, and neurological tissues.[11] Remarkably, none of the other FAO abnormalities have been linked to maternal HELLP syndrome.[17]

While some mutations in the *HADHA* or *HADHB* cause deficiencies in all three enzymes, the prevalent c.1528G>C *HADHA* mutation causes solely LCHADD.[34,37] Patients with mutations in both the *HADHA* and *HADHB* genes exhibit comparably diverse clinical symptoms, making clinical distinction between them impossible. Patients with LCHADD exhibit a variety of phenotypes despite having the same genotype, indicating the significance of other genetic and environmental variables.[35]

Medium-chain Acyl-CoA Dehydrogenase Deficiency

The most prevalent FAO condition is MCADD.[17] MCADD presents as primary hepatic failure accompanied by encephalopathy (Reye-like syndrome) characterized by moderately hypoketotic hypoglycemia.[39] The initial attack is fatal in 20% of cases.[40] According to reports, the death rate within the first 72 hours is 4%.[41] Patients with homozygous c.985A>G mutation have a severe genotype.[3] It should be noted that patients with MCADD may have ketones in their urine when tested with dipsticks during catabolic episodes.[11] This is hypothesised to result from the partial oxidation of long-chain acyl-CoAs to medium-chain acyl-CoAs, producing acetyl-CoA. Although there have been a few anecdotal reports of both cardiac and muscle complications, cardiac, renal, and skeletal muscle signs and symptoms have not yet been demonstrated to contribute to the MCADD phenotype.[40] It is possible that partial oxidation of long-chain fatty acids supplies enough acetyl-CoA to fulfill the requirements of skeletal and cardiac tissues in the great majority of MCADD patients.

Multiple Acyl-CoA Dehydrogenase Deficiency

Deficiencies of either electron transfer flavoprotein (ETF) or ETF-ubiquinone oxidoreductase (ETF-QO), result in MADD, a disorder of fatty acid, amino acid, and choline oxidation.[4] In addition to leg weakness and exercise intolerance with sporadic rhabdomyolysis, patients may exhibit cyclical vomiting, loss of appetite, and proximal muscle weakness affecting the neck, shoulders, hips, and/or respiratory muscles. Acute encephalopathy may be observed in certain patients.[4]

Three clinical phenotypes have been attributed to MADD; a mild or late-onset form, a neonatal form with congenital anomalies, and a neonatal form without congenital malformations.[17] Within the first 24 to 48 hours following a preterm birth, the first group of patients typically exhibits symptoms such as severe non-ketotic hypoglycemia, hypotonia, hepatomegaly, and deep metabolic acidosis. Numerous congenital anomalies, such as multicystic dysplastic kidney, sandal-foot deformity, external genital anomalies, and facial anomalies including low-set ears, large foreheads, hypertelorism, and hypoplastic facial features, are associated with this phenotype. It is believed that the accumulation of metabolites during pregnancy causes dysmorphic characteristics. Within the first twenty-four hours, the second group presents with hypotonia, tachypnea, hepatomegaly, metabolic acidosis, and hypoketotic hypoglycemia. Within the first week, the majority of these individuals die. Patients with severe cardiomyopathy survive only a few months despite prompt diagnosis and meticulous therapy. The third group of patients exhibits various clinical manifestations. In the initial months, these patients may exhibit hypoketotic hypoglycemia, metabolic acidosis, and sporadic vomiting episodes; in adulthood, they may develop Reye-like syndrome and proximal myopathy.[42] High-dose riboflavin treatment has been shown to be beneficial for patients with a moderate clinical presentation.

Riboflavin, also known as vitamin B2, is a precursor of flavin adenine dinucleotide (FAD), a cofactor of numerous dehydrogenases involved in cellular metabolism, including acyl-CoA dehydrogenases in FAO. Notably, riboflavin cannot be synthesized in the human body. Therefore, riboflavin is absorbed by the intestines from the diet, transported through the bloodstream, and taken up by tissue-specific riboflavin transporters. Inside the cell, riboflavin kinase converts riboflavin into flavin mononucleotide (FMN). FMN is adenylated to FAD-by-FAD synthase, encoded by FLAD1. Recently, mutations in FLAD1 were reported to cause a novel form of MADD.

Riboflavin transporter deficiency, comprising *RFVT1* and *RFVT2* (caused by biallelic pathogenic variants in *SLC52A2* and *SLC52A3*, respectively), is a rare neurologic condition characterized by progressive peripheral and cranial neuronopathy causing muscle weakness, with consequent respiratory compromise, vision loss, deafness, and sensory ataxia. The acylcarnitine profile in the blood is abnormal, with accumulation of short- and medium-chain (and sometimes long-chain) acylcarnitines. This can lead to patients being misdiagnosed with MADD. The diagnosis of *RFVT1* and *RFVT2* is established in an individual who has suggestive findings and biallelic pathogenic variants in either *SLC52A2* or *SLC52A3*.

The majority of these riboflavin-responsive MADD patients have been shown to have *ETF-DH* gene mutations, with the majority of these mutations occurring close to the ubiquinone binding pocket.[43] The precursor of FAD is riboflavin, and the capacity of FAD to function as a chemical chaperone that encourages the folding of specific misfolded ETF-QO proteins leads to riboflavin responsiveness.[36] Supplementing with riboflavin aids patients with specific mutations in the *ETF-DH* gene restore residual activity to a greater degree.[43] When riboflavin is administered, the clinical effect is typically rapid and apparent. The majority of patients are young adults or teens.[36]

Short Chain Acyl-CoA Dehydrogenase Deficiency

Short chain acyl-CoA dehydrogenase (SCAD) deficiency is now considered as a biochemical phenotype of uncertain clinical importance, despite the fact that it causes disruptions in certain metabolites consistent with an FAOD.[44] The pathogenesis of SCAD deficiency is poorly understood, and the condition's signs and symptoms have been incredibly variable.[45] Neurological, myopathic, and hepatic signs and symptoms were included in the very first description. Short-chain fatty acid intermediates were thought to cause neurological symptoms because they are volatile and more likely to cross the blood–brain barrier, but this hasn't been proven despite the availability of an animal model.[46] The majority of detected neonates with SCAD deficiency do not develop a clinical phenotype, according to current experience with diagnosis based on newborn screening.[47] Concerns have been raised about patients with SCAD deficiency being mistakenly classified as having a metabolic disorder and that using this as a diagnostic endpoint could lead to inadequate research into the underlying reasons of any symptoms[11].

DIAGNOSIS

Clinicians examining a patient with a preliminary diagnosis of FAOD should be aware of potential pitfalls that can lead to misdiagnosis. Since the FAO pathway is not active when the patient is clinically stable and normoglycaemic, the accumulation of pathological intermediate metabolites will not be significant. To obtain the most useful information about the patient's metabolic status, samples should be collected during metabolic decompensation, for example, at the time of presentation to the emergency department. Samples should be collected as soon as possible after the patient's presentation, before biochemical stabilisation is achieved. With correction of hypoglycemia, abnormal levels of intermediate metabolites will also rapidly return to normal.

The investigation should begin with tests that identify which tissues are affected during the attack and indicate which approach should be taken. These tests include blood gas

analysis, plasma or serum electrolyte measurements, glucose, lactate, and ammonia levels, liver function tests, creatine kinase levels, and urine ketone measurements.

Due to defective FAO, ketone synthesis is impaired. Consequently, even in cases of hypoglycemia resulting from catabolic processes in these patients, urinary ketone levels remain abnormally low (hypoketotic hypoglycemia). In the urine organic acid analysis, an increase in medium-chain dicarboxylic acid levels is observed alongside low ketone levels. Ketone positivity may be present in the urine of patients with MCADD owing to the metabolism of acetyl-CoA, which is produced by the partial oxidation of long-chain fatty acids.

When samples are collected for routine laboratory tests, it is appropriate to also collect samples for metabolic disease diagnostic tests to be performed in the second stage. These include measurements of carnitine (total and free), acylcarnitines, free fatty acids, beta-hydroxybutyrate, and acetoacetate in plasma and serum. Findings that may be observed in plasma acylcarnitine and urine organic acid analyses of FAODs are summarised in Table 1.

It is generally assumed that an abnormal acylcarnitine profile reflects the intramitochondrial accumulation of acyl-CoAs and, as such, indicates the substrate of the deficient enzyme in vivo. The accumulating acyl-CoAs are exported from the mitochondria as acylcarnitines via CPT-II, carnitine acetyltransferase, and CACT. The molecular mechanism of acylcarnitine export across the cell membrane is unresolved.

Rapid confirmation of a particular suspected enzyme deficiency can be performed in lymphocytes, as these cells express all enzymes involved in FAO.

Mutations causing certain diseases have been identified in FAODs. A variety of methodologies have been developed to facilitate mutation analysis using diverse biological samples. Mutation screening can be performed on samples obtained from whole blood or newborn screening cards. The identification of disease-causing mutations through genetic analysis serves to confirm the biochemical diagnosis. Furthermore, in the context of disease forms characterised by a genotype-phenotype relationship, it can provide prognostic insights and inform treatment decisions. This information is of particular significance for genetic counselling and preimplantation genetics.

TREATMENT

The extent to which FAO occurs is primarily determined by the rate of lipolysis in adipose tissue. Preventing prolonged starvation and administering emergency treatment regimens during intercurrent infections reduce FAO. Even in the fed state, cardiac and skeletal muscles use long-chain fatty acids as an energy

Table 1. Laboratory findings in fatty acid oxidation disorders.

	Plasma				Urine	
	Carnitine		Acyl-carnitine	C_{8}-C_{18} FFA	Organic acids	Acyl-glycine
	Co	AC/Co				
Membrane bound enzymes						
Carnitine transporter defect	↓	N	↓	N	N	N
CPT-I deficiency	N/↑	N	N	N	N	N
CACT deficiency	N/↓	↑	+	N	N	N
CPT-II deficiency (neonatal)	N/↓	↑	+	N	N	N
CPT-II deficiency (late onset)	N/↓	↑	+	N	N	N
VLCAD deficiency	N/↓	↑	+	+	+ (acute)	N
ETF-DH (MADD)	N/↓	↑	+	+	+	+
TFP deficiency	N/↓	↑	+ (acute)	+	+ (acute)	N
Mitochondrial matrix enzymes						
MCAD deficiency	N/↓	↑	+	+	+ (acute)	+
SCAD deficiency	N/↓	↑	+	N	+ (acute)	+
ETF-A (MADD)	N/↓	↑	+	+	+	+
ETF-B (MADD)	N/↓	↑	+	+	+	+

AC, acyl-carnitines; CACT, carnitine acylcarnitine translocase; CPT-I, carnitine palmitoyltransferase-I; CPT-II, carnitine palmitoyltransferase-II; ETF-A, electron transfer flavoprotein α subunit; ETF-B, electron transfer flavoprotein β subunit; ETF-DH, electron transfer flavoprotein dehydrogenase; FFA, free fatty acid; MADD, multiple acyl-CoA dehydrogenase; MCAD, medium-chain acyl-CoA dehydrogenase; SCAD, short-chain acyl-CoA dehydrogenase; TFP, trifunctional protein; VLCAD, very long-chain acyl-CoA dehydrogenase.

source. To minimise this, diets containing low amounts of long-chain fatty acids are implemented. When FAOD is suspected, the primary goal is to provide sufficient glucose to prevent lipolysis in target tissues. The main principles of nutritional therapy are as follows: calorie intake in the form of carbohydrates should be provided at regular intervals to prevent both hypoglycemia and the mobilisation of long-chain fatty acids from stores. When the proportion of long-chain fatty acids in the diet is reduced, essential fatty acid supplementation should be provided to prevent deficiency.

For patients with long-chain FAO defects, high-carbohydrate diets are insufficient to ensure adequate caloric intake. To solve this issue, medium-chain triglycerides (MCTs) offer a significant amount of the energy in the diet.[2] Many patients with a defect in one of the long-chain-specific enzymes receive dietary long-chain triglyceride restriction and MCT supplementation, which, in theory, would limit the supply of long-chain fatty acids whose degradation is impaired and provide, as an alternative, a medium-chain fatty acid substrate that can bypass the enzymatic defect. The assumption that 8-, 10-, and 12-carbon fatty acids can enter the mitochondria independently of the carnitine cycle underlies the recommendation to use MCT in the diet. MCTs' well-established ketogenic properties should be beneficial in long-chain FAODs. However, it is unclear whether MCTs should be provided continuously, or only immediately before or during periods of elevated energy demand. In fact, research on *VLCAD* KO mice indicates that while long-term MCT administration caused tissue fat buildup, MCTs are advantageous before exercise.[48] Furthermore, MCT supplementation before exercise enhanced heart function and substrate oxidation in patients with long-chain FAODs.[49]

One theory is that catalytic intermediate leakage from the TCA cycle is the cause of the heart and muscular disease in FAODs.[50] This theory led to the production of triheptanoin, a novel MCT that produces both acetyl-CoA and anaplerotic propionyl-CoA when the heptanoate is oxidized. Ketone bodies like beta-hydroxybutyrate and betahydroxypentoate can be produced from heptanoate.[51] The liver produces ketone bodies, which are then released into the bloodstream and absorbed by various tissues where they can be utilized as TCA cycle substrates and intermediates.[52] Remarkably, a fasting-induced impairment in anaplerosis was validated by recent research in the *LCAD* KO mice, and patients with an FAOD showed clinical improvement after receiving triheptanoin.[50,53]

Although the main treatment for carnitine transporter insufficiency is oral l-carnitine supplementation, it also helps remove harmful metabolites and prevents carnitine shortage in other FAODs. Heart and skeletal muscle tissues contain about 98% of the body's carnitine.[54] Carnitine transporters mediate the uptake of orally administered carnitine into cardiac and skeletal muscle. A single oral dose of l-carnitine has a half-life of 60.3±15 minutes, and regular oral ingestion is necessary to maintain carnitine levels.[54] Although patients with CTD take carnitine supplements, their plasma carnitine levels increase but do not fully normalize. For people with CTD, high-dose carnitine supplementation (up to 200–250 mg/kg/day) is typically necessary. Tissue carnitine levels are not reflected in plasma levels during use of oral l-carnitine supplements. It has been demonstrated that people with CTD have reduced beta oxidation capacity during exercise, which is somewhat recovered by carnitine supplementation.[55] Regrettably, excessive doses of carnitine supplements cause sweat, urine, and breath to smell fishy.

Patients with the *ETF-DH* mutation who have late-onset type MADD typically response favorably to coenzyme Q_{10} and riboflavin.[56]

Carnitine supplementation is frequently used to treat secondary carnitine deficiency in patients with other FAODs. The use of carnitine has been controversial due to the link between acylcarnitines and ventricular fibrillation in a cat model of acute ischemia.[57] Studies on animals reveal both negative and positive effects of carnitine.[58] Studies on humans that are currently available have not demonstrated positive effects.[57]

Footnotes

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REVIEW

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Mucopolysaccharidosis Type II (Hunter Syndrome): A Brief Review of Pathophysiology, Clinical Spectrum, and Current Therapeutic Strategies

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Abstract

Mucopolysaccharidosis type II (MPS II, Hunter syndrome) is a rare, X-linked lysosomal storage disorder caused by a deficiency of iduronate-2-sulfatase, resulting in progressive accumulation of heparan sulfate and dermatan sulfate in multiple tissues. The disease presents as a broad clinical spectrum ranging from attenuated forms with preserved cognitive function to severe neuronopathic forms characterized by progressive neurodegeneration. Common clinical manifestations include coarse facial features, hepatosplenomegaly, skeletal abnormalities, cardiac valve disease, airway obstruction, hearing loss, and neurological impairment. The diagnosis is established by demonstrating deficient enzyme activity and by molecular confirmation of pathogenic variants in the *IDS* gene. Intravenous enzyme replacement therapy has significantly improved somatic manifestations and the quality of life; however, its inability to cross the blood–brain barrier limits its effectiveness in preventing neurological disease progression. Recent advances, including hematopoietic stem cell transplantation, blood–brain barrier-penetrating enzyme replacement therapies, intrathecal and intracerebroventricular enzyme administration, and gene therapy, offer promising approaches to address central nervous system involvement. This review summarizes the current understanding of the pathophysiology, clinical spectrum, diagnosis, and evolving therapeutic strategies for MPS II, with particular emphasis on emerging treatments targeting neurological disease.

Keywords: Mucopolysaccharidosis type II; Hunter syndrome; Lysosomal storage disorder; Enzyme replacement therapy; Gene therapy; Newborn screening

INTRODUCTION

Mucopolysaccharidosis type II (MPS II, Hunter syndrome) is a rare, X-linked recessive, multisystemic, and progressive lysosomal storage disorder.[1] The disease was first described in 1917 by Canadian physician Charles A. Hunter in two male siblings. Hunter characterized them as having severe somatic manifestations, including macrocephaly, dysmorphic facial features, hepatosplenomegaly, joint contractures, hearing impairment, noisy breathing, and cardiomegaly, despite having normal intelligence. The term mucopolysaccharidosis was introduced by Brante in 1952, and the biochemical basis of the disease was established in 1968 when Fratantoni et al. described the *in vitro* phenomenon of cross-correction, demonstrating that enzyme-deficient cells could be functionally corrected by enzyme-producing cells.[2] The core biochemical defect is a deficiency of the enzyme iduronate-2- sulfatase (I2S) (EC 3.1.6.13), which plays a vital role in the lysosomal catabolism of glycosaminoglycans (GAGs). Specifically, I2S catalyzes the hydrolysis of the C-2 sulfate ester bond at the non-reducing terminal L-iduronic acid residues of dermatan sulfate (DS) and heparan sulfate (HS). The reduction or complete absence of I2S activity leads to the massive, progressive accumulation of partially degraded HS and DS within the lysosomes of virtually every cell type in the body, as well as within the extracellular matrix.[2]

Although the progressive lysosomal accumulation of HS and DS is the primary biochemical hallmark of MPS II, the

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pathological consequences of this storage process extend considerably beyond simple substrate deposition.[3] Increasing evidence suggests that GAG accumulation initiates a complex network of secondary cellular disturbances, including lysosomal dysfunction, impaired autophagy, oxidative stress, and chronic inflammation, which collectively drive tissue injury and multisystem disease progression. The pathophysiology extends far beyond the mere mechanical engorgement of cells by HS and DS, encompassing a destructive cascade of cellular dysfunction and inflammatory signaling. Primary GAG accumulation impairs lysosomal function, thereby inhibiting other acid hydrolases and leading to secondary lysosomal accumulation of neurotoxic and pro-inflammatory molecules, including GM2 and GM3 gangliosides, globotriaosylsphingosine, unesterified cholesterol, and tau proteins.[4] Furthermore, undegraded HS fragments structurally mimic the lipopolysaccharides of Gram-negative bacteria and act as potent ligands for toll-like receptor 4 (TLR4) on the surface of microglia and peripheral macrophages. Activation of the TLR4-MyD88 pathway induces NF-κB signaling and activates the NLRP3 inflammasome, leading to excessive release of pro-inflammatory mediators, including cytokines such as interleukin-1β and tumor necrosis factor-alpha, and matrix metalloproteinases, thereby promoting chronic systemic inflammation and neurodegeneration. In parallel, the massive intracellular GAG burden compromises lysosomal membrane integrity, allowing destructive hydrolases, such as cathepsin B, to leak into the cytosol and accelerate cell death. Simultaneously, disruption of autophagic and mitophagic flux markedly increases intracellular oxidative stress, further exacerbating cellular dysfunction and tissue damage.[5]

This enzyme is encoded by the *IDS* gene, linking the biochemical defect directly to the disease's underlying genetic cause. MPS II is caused by pathogenic variants in the *IDS* gene located at *Xq28* on the X chromosome. The *IDS* gene spans approximately 24 kb, contains nine exons, and encodes a 550-amino acid polypeptide.[6] To date, over 800 distinct pathogenic variants have been identified, including nonsense and missense mutations, splice-site mutations, and major structural alterations. A significant challenge in understanding the genetic architecture is the presence of the IDSP1 pseudogene, located approximately 20-25 kb telomeric to the active *IDS* gene. Sharing up to 100% sequence homology with specific exons of the *IDS* gene, this pseudogene predisposes the locus to unequal homologous recombination events. These events frequently result in complex genetic rearrangements, such as inversions and large deletions, which are characteristically associated with severe disease phenotypes. Although MPS II almost exclusively affects males due to its X-linked recessive inheritance, rare symptomatic female cases have been documented, primarily resulting from skewed X-chromosome inactivation or structural chromosomal abnormalities.[3,7]

Reflecting these underlying genetic drivers, the epidemiological landscape of MPS II demonstrates significant global variation.[7,8] Worldwide, it is recognized as an ultra-rare lysosomal storage disorder, with a reported birth prevalence ranging from 0.13 to 2.16 per 100,000 live births.[9] Striking geographic heterogeneity and ethnicity-driven variations are documented worldwide.[8,10] In East Asian populations—such as Taiwan (1.07/100,000), Japan (0.84/100,000), and South Korea (0.74/100,000)—MPS II represents the predominant subtype, accounting for 50% to 55% of all diagnosed MPS cases.[11-13] This high relative frequency is primarily driven by founder variants, particularly by high allele frequencies of p.R468 alterations and by recurrent IDS–IDSP1 genomic recombinations.[14,15] Conversely, MPS II represents only 4% to 27% of all MPS diagnoses in European and North American populations, with birth prevalence reported as 0.13 per 100,000 in Norway, 0.64 per 100,000 in Germany, and 0.26 per 100,000 in the United States.[12,16] Real-world data from high-throughput newborn screening (NBS) panels indicate that traditional clinical ascertainment has underestimated disease incidence; for example, US screening initiatives revealed a prevalence of 1 in 73,290, higher than historical registry estimates.[17] Owing to its X-linked recessive inheritance pattern, MPS II almost exclusively affects hemizygous males and has a male-specific birth prevalence ranging from 2.05 per 100,000 male live births in Taiwan to 4.2 per 100,000 male live births in Estonia.[11,18] Symptomatic cases in females remain extraordinarily rare, with approximately 24 confirmed cases documented in the global literature. Disease expression in females requires a total loss of functional enzyme activity, which predominantly arises from extremely skewed X-chromosome inactivation (silencing the normal allele), structural X-chromosome rearrangements (e.g., monosomy X/Turner syndrome or X-autosome translocations), or exceptionally rare homozygous inheritance resulting from parental consanguinity.[9]

CLINICAL FINDINGS

MPS II presents as a continuous phenotypic spectrum, driven by the progressive accumulation of GAGs at the cellular and tissue levels. Clinically, the disease is categorized into two main phenotypes based on the presence of neurological involvement and the rate of progression: the severe (neuronopathic) and the attenuated (non-neuronopathic) phenotypes. The neuronopathic form, representing approximately two-thirds of the patient population, typically manifests between 18 and 36 months of age. The severe phenotype is dominated by progressive cognitive and neurobehavioral decline. Conversely, patients with the attenuated form do not experience significant cognitive regression and often survive into adulthood.[19] However, data from the Hunter Outcome Survey (HOS), the largest global registry for patients with MPS II, strongly indicate that patients with the attenuated form experience a somatic disease burden as severe

as that of their neuronopathic counterparts and require frequent surgical interventions for manifestations such as hernias and carpal tunnel syndrome during the first two decades of life.[20] The clinical features of the severe and attenuated phenotypes of MPS II are summarized in Table 1.

The clinical distinction among phenotypic forms is most evident in the extent of neurological involvement, the principal determinant of disease severity and long-term prognosis. Primary neurological involvement is the hallmark of the severe phenotype, presenting as developmental arrest followed by regression, typically beginning at 18–36 months.[7] Elevated levels of neurofilament light chain in cerebrospinal fluid (CSF) and serum have recently been established as robust biomarkers of progressive axonal damage and neurodegeneration. Clinically, this manifests as extreme hyperactivity, aggression, sleep disturbances, and ultimately intractable seizures. Secondary neurological complications arise from GAG deposition in the meninges and neural vasculature, leading to communicating hydrocephalus, cervical spinal stenosis (cervical myelopathy), and carpal tunnel syndrome.[21]

Skeletal dysplasia is largely driven by the accumulation of DS. DS interferes with small leucine-rich proteoglycans, disrupting collagen fibril assembly in the extracellular matrix. Furthermore, accumulated HS competitively inhibits the signaling pathways of fibroblast growth factor and bone morphogenetic protein, both of which are critical for bone morphogenesis. Patients develop restricted joint mobility, claw-hand deformities, short stature, kyphoscoliosis, thickened ribs, and the characteristic skeletal presentation, dysostosis multiplex.[3]

DS is a major component of normal cardiac valves. In MPS II, massive DS deposition within valve leaflets and the myocardium triggers fibrosis and inflammation, leading to severe valvular disease, particularly mitral and aortic regurgitation or stenosis. Left ventricular hypertrophy, arrhythmias, and eventual heart failure follow and together constitute one of the leading causes of mortality.[3,22]

GAG deposition in mucosal tissue causes macroglossia, adenotonsillar hypertrophy, and vocal cord thickening. More critically, tracheobronchomalacia, combined with highly viscous secretions, drastically narrows the upper and lower airways. This leads to progressive obstructive sleep apnea, noisy breathing, difficult intubation, and life-threatening recurrent respiratory infections. Mixed conductive and sensorineural hearing loss is nearly universal, resulting from chronic otitis media, ossicular dysostosis, and cochlear nerve compression.[7]

GAG storage in the reticuloendothelial system results in almost universal hepatosplenomegaly and associated inguinal or umbilical hernias. Pebble-like, ivory-colored skin lesions on the back and upper arms have been reported in MPS II. Notably, unlike MPS I or VI, significant corneal clouding is extremely rare in MPS II.[23] This is hypothesized to result from the protective effect of an extra sulfate group on the DS molecules that accumulate in MPS II.[24]

DIAGNOSIS

Establishing a prompt and precise diagnosis of MPS II requires an integrated approach that combines clinical awareness with targeted biochemical screening, enzymatic confirmation, and molecular characterization.[23] Initial diagnostic evaluation is commonly prompted by overt physical features or an abnormal NBS result. Quantitative assessment of total urinary GAGs (uGAGs) via dye-binding assays offers a useful initial indicator; however, it lacks specificity and may yield false-negative results. To avoid false negatives, qualitative GAG profiling by liquid chromatography–tandem mass spectrometry or urine GAG electrophoresis is preferred for specifically detecting elevated levels of DS and HS.[15,25]

In male patients, definitive biochemical diagnosis relies on demonstrating deficient or completely absent I2S activity in

Table 1. Clinical features of the severe and attenuated phenotypes in mucopolysaccharidosis type II.

Clinical feature/system	Neuronopathic (severe) phenotype	Attenuated phenotype
Prevalence and onset	2/3 of cases; typical onset at 18–36 months	1/3 of cases; typically later onset
Primary CNS disease	Progressive cognitive decline, developmental arrest, hyperactivity, aggression, and intractable seizures	Absent or minimal; preserved cognitive functions and normal neurodevelopmental trajectory
Secondary CNS disease	Communicating hydrocephalus, cervical spinal stenosis, and carpal tunnel syndrome	High risk for hydrocephalus, spinal stenosis, and carpal tunnel syndrome (similar to severe form)
Somatic burden	Severe: Dysostosis multiplex, restricted joint mobility, valvular heart disease, macroglossia, and upper airway obstruction	Marked somatic burden; early surgical interventions are frequently required for hernias and carpal tunnel syndrome
Prognosis	Shortened survival; premature death is typically driven by neurodegeneration and cardiorespiratory failure	Prolonged survival into adulthood; prognosis depends on somatic and valvular complications

CNS, central nervous system.

leukocytes, plasma, or cultured fibroblasts. Dried blood spot enzyme assays have revolutionized early detection by providing a rapid, minimal-volume substrate suitable for both high-throughput NBS and primary diagnostic triage. Notably, it is clinically mandatory to measure the normal activity of at least one other sulfatase to rule out multiple sulfatase deficiency.[15]

Identifying pathogenic variants in the *IDS* gene provides essential genetic confirmation, enables genotype–phenotype predictions, and guides family counseling. Standard Sanger sequencing or targeted next-generation sequencing (NGS) panels effectively detect single- nucleotide variants and small insertions or deletions. Nonetheless, molecular confirmation is often complicated by IDSP1, a *pseudogene* located ~20–25 kb telomeric to IDS and sharing *extensive* sequence identity with IDS. Non-allelic homologous recombination between these loci accounts for 10-15% of pathogenic alleles, producing complex inversions and structural deletions that can easily escape detection by conventional Polymerase Chain Reaction (PCR) or short-read NGS. Resolving these complex genomic rearrangements often requires dedicated techniques such as long-read sequencing, MLPA, or PCR-RFLP analysis.[14,23,26]

Detection of female carriers is a critical component of genetic counseling, as MPS II is inherited in an X-linked recessive pattern in which approximately 92% of mothers of affected males are carriers and ~8% carry de novo mutations.[27] Measurement of I2S enzyme activity or uGAGs in females is clinically unreliable for carrier identification because random X-chromosome inactivation causes significant overlap with normal control ranges. Although most carriers are clinically asymptomatic, skewed X-chromosome inactivation can occasionally result in mild, progressive somatic manifestations—such as joint stiffness, carpal tunnel syndrome, hypoacusia, and skeletal anomalies—warranting longitudinal clinical monitoring.[28] Consequently, targeted molecular genetic testing for the familial IDS variant is the only reliable approach to identify female carriers.[15] Once the proband's mutation is established, direct DNA-based methodologies—including Sanger sequencing, restriction fragment digestion, specialized PCR assays for gene–pseudogene recombinations, and high-sensitivity NGS, capable of detecting low-level somatic or germline mosaicism—enable precise cascade testing of at-risk female relatives and provide essential options for prenatal and preimplantation genetic diagnosis.[14,23,25]

CURRENT TREATMENTS

The therapeutic management of MPS II has historically shifted from palliative, symptom-focused care to disease-specific therapy.[20] Although current treatments significantly alter the somatic course of the disease, addressing the profound neurological burden remains a critical challenge.

Intravenous (IV) administration of recombinant human I2S is the current first-line treatment for MPS II. When administered weekly at a dose of 0.5 mg/kg, IV enzyme replacement therapy (ERT) effectively targets somatic macrophages via mannose-6-phosphate receptor-mediated endocytosis. Clinical trials and extensive real-world registry data, including findings from the HOS, consistently show that ERT rapidly reduces uGAG excretion and normalizes liver and spleen volumes. It also provides measurable improvements or stabilization in functional endurance [e.g., the 6-minute walk test (6MWT)] and pulmonary capacity.[20] While IV ERT effectively manages systemic disease, its inability to penetrate avascular tissues (such as cardiac valves and cartilage) and the central nervous system limits its overall therapeutic efficacy.[29] In addition, avascular or poorly vascularized tissues, such as cardiac valves, bone growth plates, and cartilage, exhibit poor enzyme penetration. Consequently, valvulopathy, airway obstruction, and skeletal dysplasia often continue to progress despite long-term enzymatic treatment.[30] Another important therapeutic limitation is the development of anti-drug antibodies (ADAs), which occur in approximately 50% of treated patients. Although neutralizing ADAs can attenuate biochemical responses, including slower reductions in uGAG levels, their direct impact on functional clinical outcomes, such as the 6MWT, remains variable. Nevertheless, they continue to pose a concern due to infusion-related hypersensitivity reactions.[20]

The role of allogeneic hematopoietic stem cell transplantation (HSCT) in MPS II has historically been controversial. Unlike in MPS I (Hurler syndrome), early HSCT attempts in MPS II were associated with high morbidity and limited preservation of neurocognitive function.[31] However, modern advancements in preconditioning regimens—including targeted busulfan and fludarabine—and improvements in donor matching have significantly reduced procedural mortality.[32] Current evidence suggests that if HSCT is performed very early in the disease course, before the onset of symptoms or before 2 years of age, donor-derived engrafted microglia may provide a continuous source of I2S to the brain parenchyma, potentially stabilizing cognitive decline. Thus, very early HSCT is increasingly recognized as a viable option for carefully selected patients with neuronopathic disease.[33]

CURRENT CHALLENGES, EMERGING FRONTIERS, AND FUTURE DIRECTIONS

Driven by the urgent need to address neuronopathic manifestations, recent therapeutic advances focus on pre-symptomatic detection and drug delivery strategies capable of bypassing the blood–brain barrier (BBB). A major historical hurdle in MPS II has been the diagnostic odyssey during which irreversible CNS and somatic damage occur before treatment is initiated. The inclusion of MPS II in NBS panels, such as the Recommended Uniform Screening Panel in the United States,

represents a monumental shift.[8] However, NBS introduces a complex prognostic challenge: accurately predicting the clinical phenotype from the identified genotype in a pre-symptomatic neonate. While large structural deletions or complex rearrangements reliably predict the severe phenotype, private missense variants or variants of unknown significance complicate clinical decision-making. Accurate phenotypic prediction is imperative, as it dictates the risk-benefit justification for aggressive CNS–targeted early interventions, such as HSCT or experimental gene therapies.[25]

Direct enzyme administration into the CSF through intrathecal or intracerebroventricular (ICV) delivery has emerged as a promising strategy for circumventing BBB limitations. Clinical studies evaluating ICV idursulfase beta, delivered via surgically implanted reservoirs, have demonstrated substantial reductions in CSF HS concentrations, accompanied by stabilization of neurodevelopmental decline, ultimately leading to regulatory approval in selected regions, including Japan.[34] A complementary non-invasive strategy relies on receptor-mediated transcytosis, often described as a "molecular Trojan horse" approach. In this method, the therapeutic I2S enzyme is fused to monoclonal antibodies that target BBB transcytosis receptors, particularly the human transferrin receptor. Following IV administration, receptor binding initiates transcytosis across the cerebrovascular endothelium, facilitating enzyme delivery into the brain parenchyma.[35] Pabinafusp alfa, currently approved in Japan, represents the first systemic ERT to demonstrate dual efficacy by reducing both somatic disease burden and CSF HS levels while stabilizing neurocognitive function. Similar investigational compounds, including tividenofusp alfa, continue to advance through late-stage global clinical development.[8]

Beyond enzyme replacement strategies, next-generation therapeutic modalities increasingly focus on achieving sustained endogenous production of I2S through gene-based approaches. *In vivo* gene therapy using adeno-associated viral (AAV) vectors, particularly AAV9-based platforms such as RGX-121, has demonstrated encouraging early clinical results following intracisternal CNS delivery, including marked reductions in CSF biomarkers and sustained neurodevelopmental acquisition.[34] Alternatively, *ex vivo* lentiviral *hematopoietic stem cell* gene therapy involves harvesting autologous CD34+ stem cells, genetically modifying them to overexpress the *IDS* gene, and subsequently reinfusing them into the patient. Following engraftment, these cells may differentiate into brain microglia and provide continuous CNS enzyme production without the graft-versus-host disease risks associated with allogeneic HSCT. Genome-editing strategies also represent an emerging frontier. Early clinical investigations utilizing zinc finger nucleases, such as SB-913, have explored targeted insertion of the *IDS* transgene into the highly active albumin locus of hepatocytes to facilitate durable systemic enzyme production.[8] Although initial studies have demonstrated favorable safety profiles, achieving therapeutically meaningful and sustained enzyme expression remains an important challenge requiring further optimization.

CONCLUSION

MPS II is a relentlessly progressive, multisystem disorder. The introduction of IV ERT marked a historic milestone, fundamentally altering the natural history of somatic manifestations. While standard IV ERT has transformed systemic management, addressing neurological progression remains the cornerstone of modern research. Today, the management of Hunter syndrome is at a transformative crossroads. The synergistic integration of NBS programs with breakthrough technologies—such as BBB-crossing molecular Trojan horses, ICV enzyme administration, and advanced gene therapies—holds unprecedented promise. The ultimate goal of MPS II management is shifting from mere somatic palliation toward comprehensive disease correction, aiming to preserve neurocognitive capacity and restore normal quality of life for all affected individuals.

Footnotes

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CASE REPORT

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A Novel *FBXL4* Variant Presenting with Shunt-Dependent Hydrocephalus

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Abstract

FBXL4-associated encephalomyopathic mitochondrial depletion syndrome type 13 is a very rare mitochondrial disorder. To date, more than 110 cases have been reported in the literature, and the disease presents with multisystem involvement accompanied by lactic acidosis. We aimed to expand the phenotypic spectrum by reporting a case with a previously undescribed c.1390-2A>G variant in the *FBXL4* gene and a previously unreported clinical presentation of severe hydrocephalus. The patient presented with severe lactic acidosis, developmental delay, moderate hyperammonemia, hypotonia, dysmorphic features, seizures, intrauterine growth restriction, hypertrophic cardiomyopathy, severe hydrocephalus, peripapillary atrophy, and encephalopathy. We aimed to contribute to the literature by identifying a new splice-site variant and the severe clinical presentation associated with it, thereby expanding the phenotypic spectrum, and by deepening understanding of the genotype-phenotype relationship of splice-site mutations.

Keywords: *FBXL4* gene, Mitochondrial depletion syndromes, Lactic acidosis, Hydrocephalus

INTRODUCTION

Mitochondria are organelles involved in cellular energy production and apoptosis. Mitochondrial proteins are encoded by both nuclear and mitochondrial DNA. Most of these proteins are encoded by nuclear DNA, which is thought to contain between 1,000 and 1,500 genes. *F-box and leucine-rich repeat protein 4* (*FBXL4*) is a mitochondrial protein localized to the intermembrane space, encoded by the nuclear *FBXL4* (MIM#605654) gene, and is a member of the F-box protein family. The F-box portion of the protein interacts with the Skp1 adaptor to assemble the Skp1-Cullin1-F-box E3 ubiquitin ligase complex, which ubiquitinates the mitophagy receptors BNIP3 and NIX, leading to their degradation and suppression of mitophagy.[1-3]

FBXL4-associated disease was first described in 2013. In these studies, it was noted that the *FBXL4* protein was localized between the inner and outer mitochondrial membranes, and that impairment of its function resulted in mtDNA depletion, mitochondrial structural abnormalities, and reduced activity of oxidative phosphorylation enzymes.[2,3] Common clinical manifestations of the disease, defined as *FBXL4*-associated encephalomyopathic mitochondrial depletion syndrome type 13, include hypotonia, lactic acidosis, growth retardation, facial dysmorphism, malnutrition, and cerebral atrophy. Although less frequent, the following manifestations are also observed: microcephaly, hyperammonemia, nystagmus, strabismus, optic atrophy, cardiac involvement, and seizures.[4] While hydrocephalus has previously been reported, no case of severe hydrocephalus necessitating ventriculoperitoneal (VP) shunt placement has been described to date.

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CASE REPORT

The patient, a 34-month-old boy, was born at term by cesarean section to a 36-year-old mother from a 1st cousin marriage. His four siblings do not have any health problems. Elevated lactate levels were detected at 6 months of age. Thiamine 100 mg/day, riboflavin 100 mg/day, coenzyme Q10 100 mg/day, biotin 10 mg/day, and sodium benzoate 250 mg/kg/day were started on suspicion of mitochondrial disease.

The patient's body weight was 10 kg [standard deviation score (SDS)≤−3.0], height was 92 cm (SDS −1.88), and head circumference was 51 cm (SDS +0.28). He had growth retardation, developmental delay, hypotonia, and dysmorphic features. Dysmorphic features included a prominent forehead, a long, smooth philtrum, retrognathia, micrognathia, thick eyebrows, upslanting palpebrae, long eyelashes, and a thin upper lip (Figure 1).

Laboratory examination revealed a mild elevation of aspartate aminotransferase (116 U/L; maximum level of 225 U/L). The acylcarnitine profile was normal. Analysis of plasma amino acid levels revealed an elevated alanine concentration of 1302 µmol/L [reference range (RR): 157-481]. Urine organic acid analysis revealed elevated levels of pyruvic acid (108 Mmol/molcre; RR: 0-32), lactic acid (364 Mmol/molcre; RR: 0-60), 2-ketoglutaric acid (209 Mmol/molcre; RR: 0-136), 3-hydroxybutyric acid (108 Mmol/molcre; RR: 0-63), 3-hydroxyisobutyric acid (108 Mmol/molcre; RR: 0-28), and 2-hydroxybutyric acid (6.4 Mmol/molcre; RR: 0.03-1.8). Lactate levels ranged from 3.2 to 17.1 Mmol/L (RR: 0-2). Ammonia levels were between 57 and 243 mcg/dL (RR: 31-123). Eye examination revealed peripapillary atrophy, whereas echocardiography showed left ventricular hypertrophy.

Imaging performed at 3 months of age showed dilatation of both lateral ventricles (left, 25 mm; right, 23 mm) and mild dilatation of the 3rd and 4th ventricles. Because the diagnosis remained unclear, the ventricular dilation identified on previous cranial magnetic resonance imaging (MRI) did not fully explain the clinical picture; hypotonia and developmental delay persisted, and lactic acidosis raised suspicion for an underlying mitochondrial disorder. A repeat cranial MRI was performed at 2 years and 10 months of age. Cranial MRI showed severe tetraventricular hydrocephalus. Bilateral cortical sulci were partially effaced. The corpus callosum was hypoplastic. Genetic analysis revealed a homozygous c.1390-2A>G variant in the *FBXL4* gene (rs1263785322) (Figure 2). The variant has not been reported in the homozygous state in gnomAD Exomes, 1000 Genomes, ESP6500, and ExAC databases. The variant was classified as likely pathogenic because it met the PVS1 and PM2 criteria of the ACMG variant classification.

The patient underwent VP shunting for hydrocephalus at the age of 39 months. A postoperative cranial computed tomography scan showed severely dilated ventricles and a VP shunt (Figure 3). Phenytoin was started preoperatively as prophylaxis. After the shunt operation, we admitted the patient because of malnutrition and metabolic acidosis. The patient received nasogastric tube feeding. Sodium alginate and pantoprazole were initiated for vomiting in a patient with FBXL4-associated encephalomyopathic mitochondrial depletion syndrome type 13. Electroencephalography performed at the 3rd postoperative month was compatible with both a highly active, multifocal epileptic disorder and cerebral dysfunction.



Figure 1. (a, b) Prominent forehead, long and smooth philtrum, micro-retrognathia, thick eyebrows, upslanting palpebra, long eyelashes, thin upper lip.

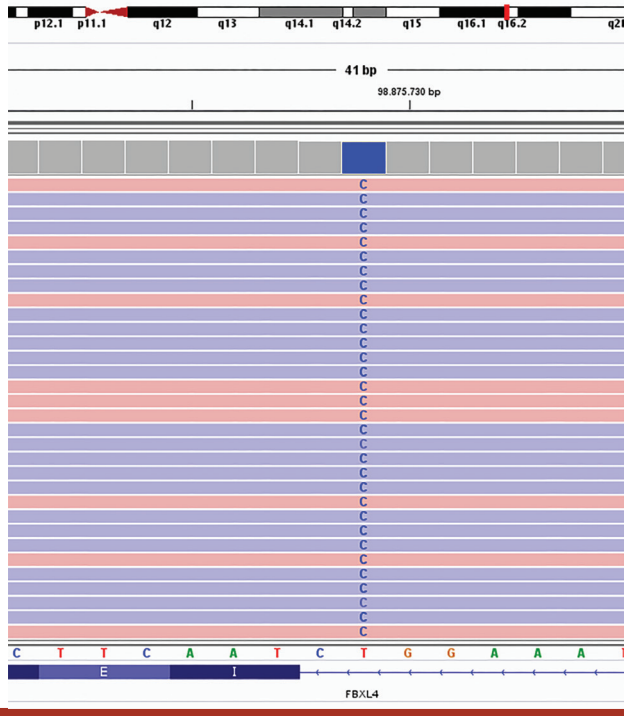


Figure 2. Integrative Genomics Viewer visualization of c.1390-2A>G variant.

MR spectroscopy performed at the 3rd postoperative month showed no lactate peak in a single-voxel view with a medium-length TE in the thalamic region of the deep gray matter; no peak was detected in other regions (Figure 4). MRI performed at 6 months postoperatively showed enlarged ventricles and diffuse, symmetric, mild signal enhancement in the thalamus, nucleus caudatus, and lentiformis on the T2 series (Figure 5). In the same imaging series, signal enhancement was observed in the brainstem at the level of the middle cerebellar peduncles (Figure 6).



Figure 3. Enlarged lateral ventricules and shunt catheter after VP shunt operation.

VP, ventriculoperitoneal.

A cerebrospinal fluid culture performed because of encephalopathy revealed growth of vancomycin-sensitive *Staphylococcus epidermidis*. Appropriate antibiotic therapy was initiated. An external ventricular drain was placed, antibiotic therapy was initiated, and topiramate, levetiracetam, and clonazepam were added to the treatment regimen for seizures and dystonic contractions. Proteinuria was detected. Blood gas analysis revealed a pH of 6.89, a pCO_2 of 88 mmHg, an HCO_3 of 16.6 mg/dL, and a lactate of 14.5 Mmol/L; sodium dichloroacetate was administered at 50 mg/kg/day, and hemodialysis was therefore performed. He died of sepsis and encephalopathy at 45 months of age (Table 1).

Method

From the patient, 2 mL of peripheral blood was collected in EDTA tubes and stored at −20 °C. Genomic DNA was isolated from peripheral blood leukocytes using QIAamp DNA Blood Mini QIAcube Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocols. All coding exons and exon-intron boundaries of 4493 genes were amplified using the Clinical Exome Solution v2 kit (SOPHiA Genetics, Boston, USA).

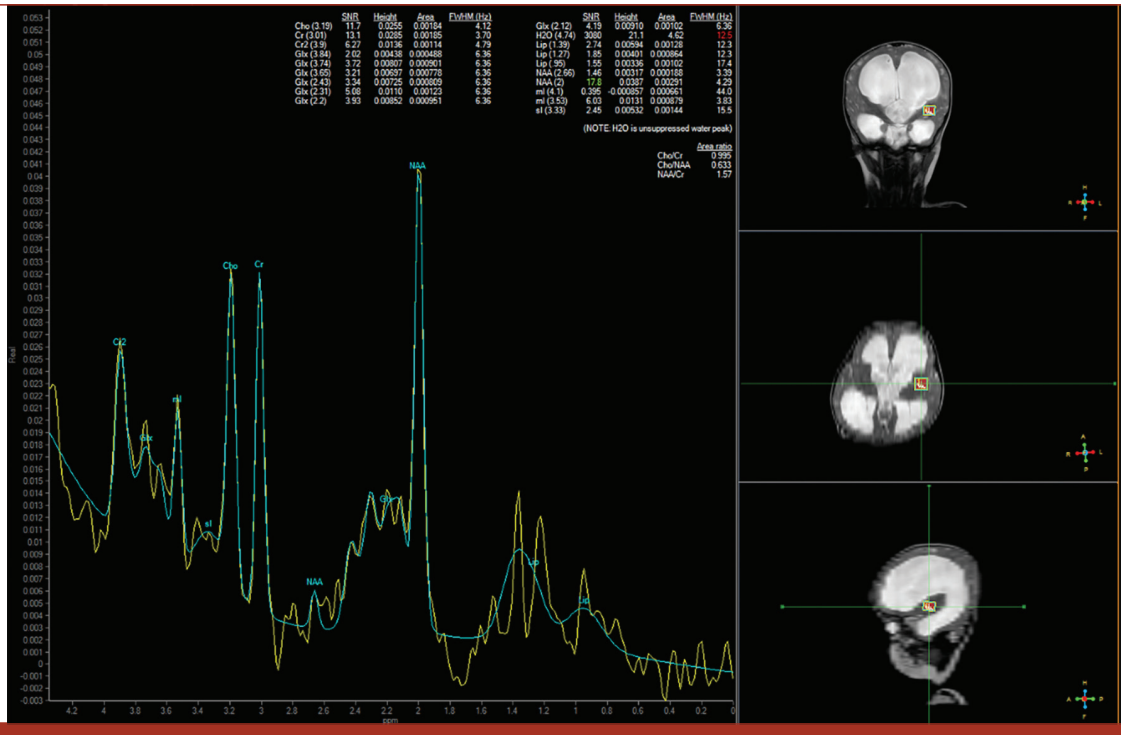


Figure 4. Brain MR spectroscopic imaging of the patient at the 3rd postoperative month revealed lactate peak on single-voxel MR spectroscopy with a medium-length TE in the thalamic region of the deep gray matter.

MR, magnetic resonance.

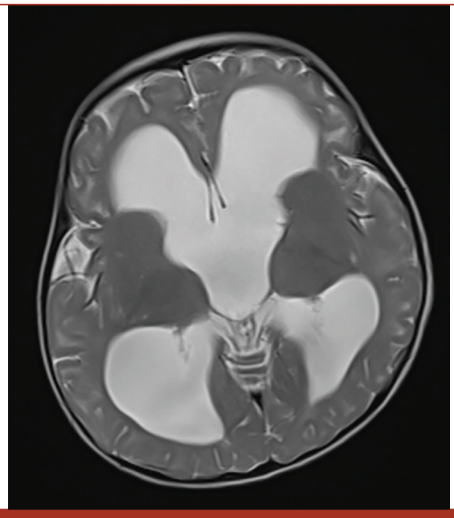


Figure 5. Enlarged ventricles and diffuse, symmetric mild signal enhancement in the thalamus, nucleus caudatus and lentiformis in T2 series.



Figure 6. Signal enhancement in the brain stem at the level of the middle cerebellar peduncles.

Table 1. Chronological timeline of the patient's clinical course.

Age	Clinical findings/investigations	Management
3 months	Cranial MRI: Dilatation of both lateral ventricles and mild dilatation of the third and fourth ventricles.	
6 months	Persistent hyperlactatemia and hyperammonemia detected; mitochondrial disease suspected.	Mitochondrial support and sodium benzoate treatment was initiated.
34 months	Growth retardation, developmental delay, hypotonia, dysmorphic features, persistent lactic acidosis, hyperalaninemia, intermittent hyperammonemia, peripapillary atrophy, and left ventricular hypertrophy. Repeat cranial MRI: severe tetraventricular hydrocephalus and hypoplasia of the corpus callosum. Genetic test: Homozygous *FBXL4* c.1390-2A>G likely pathogenic variant.	Metabolic investigations performed. Molecular diagnosis of *FBXL4*-associated encephalomyopathic mitochondrial DNA depletion syndrome established.
39 months	Progressive hydrocephalus.	Ventriculoperitoneal (VP) shunt placement; prophylactic phenytoin initiated.
42 months (3 months after VP shunt)	EEG showed highly active multifocal epileptic disorder with cerebral dysfunction. MR spectroscopy demonstrated a mild lipid-lactate peak in the bilateral thalami.	Antiepileptic treatment continued.
45 months (6 months after VP shunt)	Follow-up MRI showed persistent ventricular enlargement and new bilateral symmetric T2 hyperintensities involving the thalami, caudate nuclei, lentiform nuclei, and brainstem (middle cerebellar peduncles).	
45 months	Encephalopathy; CSF analysis: *Staphylococcus epidermidis*. Proteinuria, severe metabolic acidosis (pH 6.89, lactate 14.5 mmol/L), sepsis, seizures, and dystonic contractions developed.	External ventricular drainage, targeted antibiotic therapy, topiramate, levetiracetam, clonazepam, sodium dichloroacetate, and hemodialysis were administered. The patient died at 45 months of age.

MRI, magnetic resonance imaging; CSF: cerebrospinal fluid.

The prepared library was sequenced on the Illumina NextSeq platform (Illumina Inc., San Diego, CA, USA). The data were analyzed using SOPHiA DDM software (SOPHiA Genetics, Boston, USA) in conjunction with clinical findings. Informed consent for diagnostic and research studies was obtained from the patients' parents in accordance with the Declaration of Helsinki protocols.

DISCUSSION

Dysfunction of *FBXL4* leads to defects in mitochondrial fusion, mitophagy, and mitochondrial DNA maintenance, resulting in encephalomyopathic- type mitochondrial DNA depletion syndrome 13. The disease was first described in 2013 in two distinct patient groups, and more than 110 cases have been reported so far.[5-22] In addition to the typical clinical presentation, findings, including small for gestational age, short stature, and feeding difficulties, have been described in the literature.[4]

Although microcephaly is present in approximately half of the patients reported in the literature, our patient has relative macrocephaly. Antenatal hydrocephalus was reported in only 1 patient, whereas dilated ventricles are more frequently reported in the literature.[4,12,18,19,20] No patients have been reported in the literature with an indication for VP shunt due to severe hydrocephalus. Postoperatively, the patient experienced seizures and dystonia. It is unclear whether this was operation-related or related to the disease course.

Although the age of disease onset has been reported to range from the intrauterine period to 3 months of age, our patient's delayed presentation may be attributable to compensated lactic acidosis. Additionally, the impact of socioeconomic factors on delayed admission cannot be overlooked. However, it is possible that he did not experience an episode of lactic acidosis or hyperammonemia prior to his first admission, or that any episode was not severe enough to be noticed.

To date, survival in this disease is variable: the median age at death is two years (range: 2 days-75 months), although individuals surviving to 36 years of age have been reported.[23] It has also been reported that survival time is longer in individuals with missense variants than in those with null variants.[4]

To date, 6 splice-site mutations have been reported[4,24,25,26] In a case with a homozygous splice-site pathogenic variant, symptoms appeared on the first day of life; hyperammonemia, hearing loss, and cardiac findings were observed, findings that are reported in less than 50% of patients in reviews, and the patient died at 23 months of age.[26] In another case in which a compound-heterozygous splice-site pathogenic variant was reported, the age of onset was in the neonatal period, as in the previous case, and the patient was 4 years old and alive at the time of publication.[25] Detailed clinical information about patients with other splice-site variants could not be obtained.[4,24] Therefore, the relationship between splice-site variants and phenotypes could not be evaluated. However, upon examining patients with the described clinical findings, we do not have sufficient data to conclude that variants in the joint region are associated with early-onset disease. The c.1390-2A>G homozygous variant in the *FBXL4* gene identified in our patient represents a novel splice-site alteration between exons 7 and 8 that has not been reported previously. The more severe clinical findings observed in patients with splice-site variants may be due to these variants exerting a more deleterious effect on proteins through exon skipping, intron retention, and activation of cryptic splice sites. The c.1390-2A>G variant detected in this case can directly disrupt a critical splice site in the gene, leading to complete inhibition of protein synthesis or defective protein synthesis. This changes the acceptor splice site from the consensus sequence, thereby interfering with splicing.

Cranial MR imaging of the patient showed cerebral atrophy, involvement of the basal ganglia and white matter,a thin corpus callosum, and hydrocephalus, findings that are consistent with the literature. The finding that distinguishes the patient from the literature is that he had hydrocephalus severe enough to require shunt insertion. In addition, although less commonly reported in the literature, our patient had brainstem involvement, and no lactate peak was detected on MR spectroscopy despite high lactate levels.[4,24]

During follow-up, the patient's encephalopathic periods were not always accompanied by lactic acidosis. This suggests that central nervous system manifestations of the disease are risk factors independent of its prognosis. Moreover, clinical deterioration associated with elevated lactic acid levels worsened during catabolic processes such as dehydration, infection, and surgery, making the patient's externalization difficult.

Furthermore, the history of SGA birth and dysmorphic features, observed in most patients described in the literature, may indicate that the *FBXL4* gene is also active during the intrauterine period.[17,18,24]

Study Limitations

A key limitation of this report is that genetic testing was limited to a targeted panel of 4,493 genes rather than whole-exome or whole-genome sequencing. An additional genetic diagnosis contributing to the unusually severe hydrocephalus phenotype cannot be ruled out; a dual diagnosis remains possible. This limitation is explicitly acknowledged because further genomic studies are unavailable.

CONCLUSION

We aimed to contribute to phenotyping by reporting a patient with a novel variant in the *FBXL4* gene and a previously unreported clinical finding. Further studies and additional case reports are needed to clarify the function of FBXL4 and to improve phenotyping.

Ethics

Informed Consent: Informed consent for diagnostic and research studies was obtained from the patients' parents in accordance with the Declaration of Helsinki protocols.

Footnotes

Authorship Contributions

Surgical and Medical Practices: İ.T., M.S.K., Concept: A.N.A., Design: A.N.A., Data Collection or Processing: A.N.A., H.P., M.S.K., Analysis or Interpretation: A.N.A., H.P., Literature Search: A.N.A., Writing: A.N.A.

Conflict of Interest: The author(s) have no conflicts of interest to declare.

Financial Disclosure: The author(s) declared that this study received no financial support.

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REVIEW

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Inherit Metab Disord Nutr 2026;2:38–52

Current Perspectives on Non-ketotic Hyperglycinemia

Hatice Güneş

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Abstract

Non-ketotic hyperglycinemia (NKH) is a rare autosomal recessive neurometabolic disorder caused by defects in the glycine cleavage system, leading to glycine accumulation in plasma and cerebrospinal fluid (CSF) and a severe early-onset encephalopathy. This review summarizes current knowledge of NKH pathophysiology, clinical spectrum, diagnostic strategies, treatment options, prognostic factors, differential diagnosis, and future perspectives. Diagnostic evaluation relies on detecting elevated CSF and plasma glycine with an increased CSF/plasma glycine ratio, confirming genetic variants in *GLDC* and *AMT*, and, when available, functional studies. Therapeutic strategies remain largely symptomatic and palliative. Current treatments aim to reduce glycine levels and modulate N-methyl-D-aspartate (NMDA) receptor overactivation, mainly using sodium benzoate, dextromethorphan, and other NMDA receptor antagonists, combined with a ketogenic diet and intensive supportive care and rehabilitation. The prognosis of classic NKH is generally poor.

Keywords: AMT, glycine encephalopathy, glycine cleavage system, GLDC, ketogenic diet, non-ketotic hyperglycinemia, neonatal encephalopathy

INTRODUCTION

Non-ketotic hyperglycinemia (NKH) is a rare autosomal recessive neurometabolic disorder caused by defective activity of the mitochondrial glycine cleavage system (GCS), resulting in accumulation of glycine in plasma and cerebrospinal fluid (CSF) without ketosis. Elevated glycine levels lead to profound central nervous system dysfunction, particularly during the neonatal period.[1]

The estimated incidence of NKH varies geographically, but is generally reported as 1:76,000 live births.[2-4] It is more prevalent among individuals of Finnish descent.[5] The classic neonatal form is the most severe phenotype, characterized by hypotonia, lethargy, intractable seizures, and respiratory failure. Attenuated forms of NKH, presenting with later onset and milder neurological symptoms, have also been reported, underscoring the considerable phenotypic variability observed in this disorder.

Despite advances in molecular diagnostics and supportive care, the prognosis remains poor for most patients with classic NKH. Current therapies are largely symptomatic and aim to reduce glycine levels or attenuate modulate N-methyl-D-aspartate (NMDA) receptor-mediated excitotoxicity. However, emerging molecular approaches are under investigation and may offer future therapeutic potential.

This review provides a comprehensive overview of the pathophysiology, genetic basis, clinical spectrum, diagnostic evaluation, and management strategies for NKH.

GLYCINE METABOLISM AND PATHOPHYSIOLOGY

Glycine is a non-essential amino acid synthesized in the body from serine and threonine. It participates in numerous physiological reactions and primarily serves as a neurotransmitter in the brain. In the brain, glycine acts as an excitatory neurotransmitter via the NMDA receptor, which is involved in learning and memory, and as an inhibitory neurotransmitter via the glycine receptor (GlyR), which helps regulate motor and sensory functions in the brainstem and spinal cord. The GCS catabolizes glycine.[6] The dual role of glycine as an excitatory and inhibitory neurotransmitter is crucial for maintaining the balance of neural activity, and

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disruptions in glycine signaling have been linked to neurological disorders such as epilepsy and schizophrenia.[7,8]

The pathophysiological process of the disease likely begins early in utero; therefore, irreversible glycine-induced brain damage has probably already occurred by the time the patient presents with symptoms. GlyRs regulate distinct processes during both prenatal and postnatal brain development. During embryonic development, studies indicate that GlyRs help regulate neurotransmitter release and cell migration. Postnatally, GlyRs initially mediate excitation and promote neurotransmitter release and synaptic communication. Later, GlyRs function as inhibitory receptors and contribute to synaptic inhibition. The importance of GCS in neurogenesis and morphogenesis is supported by the presence of congenital malformations such as corpus callosum dysgenesis in NKH.[6,7]

Recent NKH models, including mouse and zebrafish models and human induced pluripotent stem cell (iPSC) systems, have elucidated complementary aspects of disease pathophysiology. Hypomorphic and *GLDC-deficient* mouse models reproduce glycine accumulation, impaired folate-mediated one-carbon metabolism, altered serine balance, hydrocephalus, electroencephalogram (EEG) abnormalities, and energy-metabolism defects and demonstrate that AAV9-mediated *GLDC* gene therapy can normalize glycine levels and restore folate profiles. Zebrafish models extend the phenotypic spectrum to include craniofacial and renal developmental abnormalities, and human iPSC-derived astrocytes reveal metabolic rewiring accompanied by altered glial differentiation. Additional rat and oligodendroglial models link glycine exposure to myelin disruption, glutamatergic imbalance, and oxidative stress. Together, these model systems show that NKH involves glycine toxicity, disrupted one-carbon and serine metabolism, impaired energy regulation, and glial-white matter vulnerability.[9-14]

GCS and One-Carbon Metabolism

The GCS is a four-protein complex consisting of the P-, T-, H-, and L-proteins, with the H-protein bearing a lipoyl group that shuttles reaction intermediates.[7,8] The P-protein, encoded by *GLDC*, initiates glycine decarboxylation and transfers the aminomethyl group to the H-protein. The P-protein, encoded by *GLDC*, initiates glycine decarboxylation and transfers the aminomethyl group to the H-protein. The T-protein, encoded by *AMT*, transfers the resulting one-carbon unit to tetrahydrofolate, generating 5,10-methylenetetrahydrofolate, whereas the L-protein regenerates the oxidized form of the H-protein. Thus, rather than functioning solely as a glycine-degrading pathway, the GCS supplies one-carbon units that contribute to folate-dependent cellular metabolism.[7]

The interaction among glycine degradation, serine metabolism, and folate-mediated one-carbon metabolism is summarized in Figure 1. GCS-mediated glycine cleavage generates 5,10-methylenetetrahydrofolate, whereas serine hydroxymethyltransferase catalyzes the reversible interconversion of serine and glycine using this one-carbon pool. Consequently, impaired GCS activity may affect not only glycine

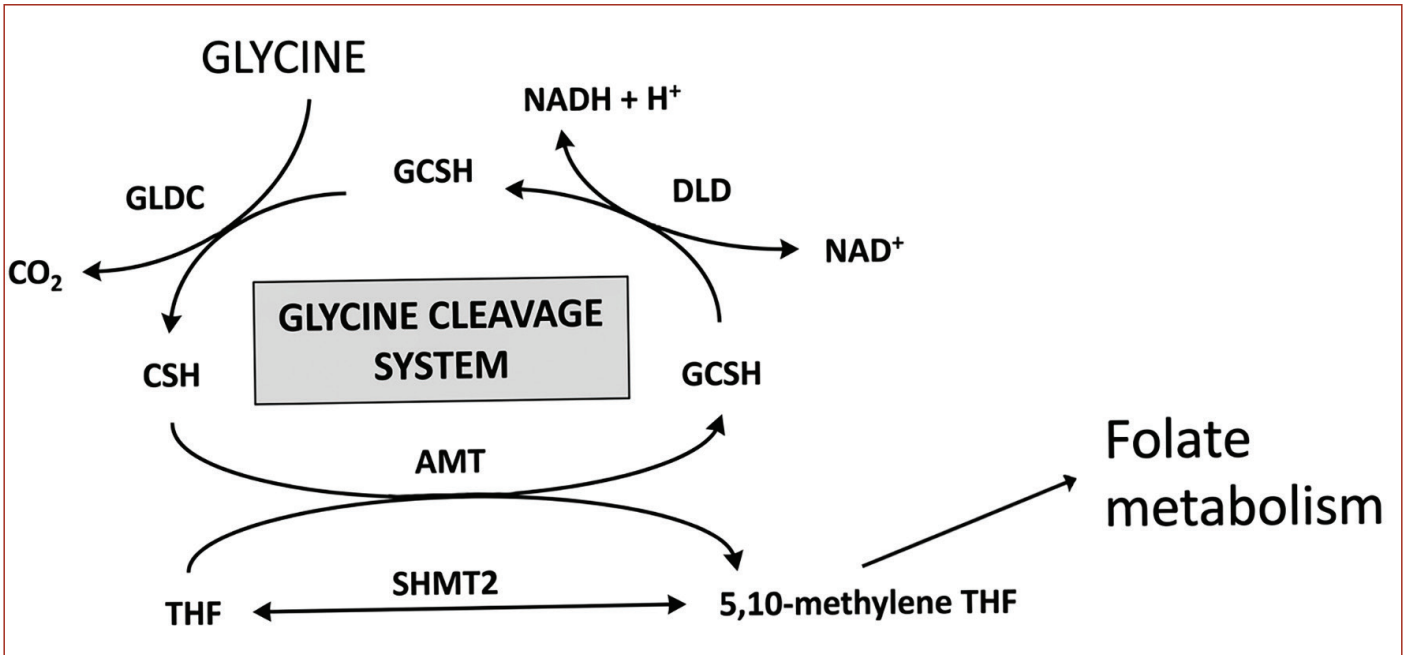


Figure 1. Glycine cleavage system.

homeostasis but also the availability of one-carbon units and serine.[15-17]

Metabolic Consequences of GCS Deficiency

Reduced GCS activity causes glycine accumulation and may also compromise folate-bound one-carbon metabolism. In *GLDC-deficient* mouse models, abnormal folate profiles, reduced levels of one-carbon-carrying folates, growth impairment, and developmental abnormalities have been reported. Formate supplementation partially restored folate balance, improved embryonic growth, and prevented neural tube defects, supporting a functional link between impaired GCS-derived one-carbon supply and abnormal development.[12]

Recent metabolic profiling further demonstrates that GLDC deficiency is associated with elevated cortical glycine, reduced folate one-carbon charging, secondary reduction in methionine, and decreased levels of alternative one-carbon donors such as L-serine and formate. Region-specific decreases in D-serine and alterations in sphingolipid species suggest effects on myelination and neuronal support.[13] Together, findings from animal and cellular models indicate that NKH pathophysiology extends beyond isolated glycine accumulation to involve interconnected disturbances in glycine, serine, and one-carbon metabolism, with potential consequences for astrocyte maturation, glial function, and neuronal homeostasis.[13]

Mechanisms of Neurological Injury

The neurological manifestations of NKH likely result from the combined effects of excessive glycine signaling, metabolic disturbances, and abnormal neurodevelopment rather than from a single pathogenic mechanism. Elevated glycine levels are proposed to bind to and overactivate NMDA receptors, disrupting glutamatergic neurotransmission and increasing Ca^{2+} influx into the postsynaptic neuron. Elevated intracellular Ca^{2+} activates phospholipases and nitric oxide synthase, leading to the production of reactive oxygen and nitrogen species. Glycine causes oxidative damage to lipids and disrupts the antioxidant system, ultimately resulting in oxidative stress. Glycine also impairs the citric acid cycle and the electron transport chain, and inhibits mitochondrial creatine kinase and Na^{+},K^{+}-ATPase activity.[10,18]

MOLECULAR GENETICS

Pathogenic variants in the genes encoding the GCS proteins disrupt glycine metabolism. *GLDC* mutations affect the P-protein and account for about 70-80% of classic NKH cases; missense variants are particularly common in *GLDC*, reflecting the gene's large size and functional complexity. Mutations in *AMT* affect the T-protein and account for approximately 20% of NKH cases.[19] The major genetic causes of classic NKH, their relative contributions, and the principal variant classes are summarized in Table 1. The predominance of *GLDC* variants is particularly relevant because this gene also exhibits substantial allelic and functional heterogeneity.

No clinically significant differences have been observed between individuals with biallelic pathogenic variants in the *GLDC* and *AMT* genes. GCS activity is a decisive predictor of the severity of the clinical course of NKH. Biallelic variants that completely abolish GCS activity (e.g., exon copy number changes, frameshift, non-sense, and splice-site variants) usually result in absence of residual activity; however, some exceptional GLDC variants (e.g., *c.2203-2A>G* and c.2999delG) are associated with severe NKH. In contrast, biallelic variants that preserve some GCS activity are expected to be associated with a milder phenotype and better prognosis; attenuated NKH is typically observed when at least one allele retains residual activity. Because the functional effects of missense variants are difficult to assess, predicting the genotype–phenotype relationship remains challenging. Taken together, the type of genetic variant and the extent of residual GCS activity largely determine NKH severity and clinical outcome, although exceptions exist.[20,21] Functional studies of GLDC variants have demonstrated a broad spectrum of residual activity, ranging from undetectable to greater than 10% of wild-type activity; even very low residual activity may suffice to produce an attenuated phenotype in some individuals.[20] As illustrated in Table 2, the relationship between genotype, residual GCS activity, and clinical phenotype is best understood as a continuum rather than a binary distinction between severe and attenuated disease. Nevertheless, genotype-based prediction of disease severity remains imperfect. In a cohort of 124 patients, the presence of one allele with residual activity was necessary but not sufficient for an attenuated outcome, and individuals with the same genotype had divergent clinical outcomes.[22]

Table 1. Mutation spectrum in non-ketotic hyperglycinemia.

Gene	Encoded protein	Approximate frequency	Mutation types
GLDC	P-protein (glycine decarboxylase)	~70–80%	Missense, non-sense, splice-site, frameshift, deletions, CNV
AMT	T-protein (amino methyltransferase)	~20%	Missense, non-sense, frameshift, splice variants
GCSH	H-protein	Rare (<1%)	Missense, small deletions
DLD	L-protein	Very rare	Missense variants

CNV, copy number variation.

These observations suggest that residual GCS activity provides important prognostic information but does not fully account for phenotypic variability. Accordingly, genotypes should be interpreted together with biochemical parameters, age at onset, neuroimaging findings, neurological phenotype, and longitudinal clinical course.

GCSH mutations affect both glycine metabolism and mitochondrial energy production. The H-protein functions within the GCS and is also required for protein lipoylation, a process essential for energy metabolism enzymes such as pyruvate dehydrogenase and 2-ketoglutarate dehydrogenase.[9] The clinical phenotype depends on which function is more strongly affected by the mutation.

CLINICAL MANIFESTATIONS

NKH is classified as classical when caused by GCS mutations, and as variant (formerly termed atypical) when due to defects in lipoic acid metabolism.

NKH presents with varied symptoms, ranging from severe neonatal encephalopathy to milder forms in later childhood. The disease is divided into severe (classic) NKH and attenuated NKH, depending on clinical severity and neurological outcomes.[20,23]

Severe (Classic) NKH

Neurological impairment, the hallmark of NKH, results from glycine accumulation in the central nervous system. Elevated glycine acts as a co-agonist at NMDA receptors, leading to excitotoxic neuronal injury and epileptic encephalopathy. In early infancy, hypotonia, increased lethargy, coma, apnea, myoclonic jerks, seizures, recurrent hiccups, and pinpoint pupils may be observed in the severe form and may require ventilator assistance during the initial 10 to 20 days.[3,24] Basic skills such as smiling, purring, and turning can be acquired; however, developmental stages such as sitting, reaching, grasping objects, and communicating cannot be learned. Spastic quadriparesis and trunk hypotonia are often observed. Seizures gradually increase during infancy and become treatment-resistant. In addition, cortical vision loss, microcephaly, and nutritional problems may accompany the clinical picture.[3,23,25] Children who survive the neonatal period often fail to achieve developmental milestones or lose previously acquired skills over time.

Attenuated NKH

Patients with attenuated NKH demonstrate better cognitive and neuromotor development than those with classical NKH. However, during infancy, hypotonia, coma, and seizures may occur. Brain malformations seen in the classical type are not detected in this form. Seizures can usually be controlled with one or two anticonvulsants. According to the degree of intellectual development, those with a DQ of 50-80 are classified as "attenuated good"; although hyperactivity, behavioral problems, and seizures are not seen in this group, late-onset chorea may develop. Those in the DQ range of 20-50 (attenuated intermediate) show moderate development; hyperactivity, chorea, and speech retardation are frequently observed. Those with a DQ <20 are classified as having poor, attenuated NKH and typically present with autistic features, absence of speech, and spasticity.[20,22] The behavioral phenotype of attenuated NKH includes intellectual disability, impaired motor and communication skills, and pronounced adaptive behaviors. ADHD-like traits frequently occur in these cases.[26]

Variant NKH

Variant NKH has an overlapping phenotype with classical NKH and results from mutations affecting lipoic acid biosynthesis. It is classified within the broader group of lipoate synthesis disorders. Lipoate is not only the cofactor for GCS but also for pyruvate dehydrogenase, 2-ketoglutarate dehydrogenase, and branched-chain ketoacid dehydrogenase. These disorders are also known as multiple mitochondrial dysfunction syndromes. Disorders affecting active pyridoxal-5-phosphate result in decreased activity of many pyridoxal-P-dependent reactions,

Table 2. Genotype–phenotype correlation in non-ketotic hyperglycinemia.

Genetic finding	Enzyme activity	Clinical phenotype	Age of onset	Neurological outcome
Null mutations (non-sense, frameshift) in *GLDC*	Absent or minimal	Classic severe NKH	Neonatal	Severe developmental delay, refractory epilepsy
Compound heterozygous variants (one severe + one milder variant) in *GLDC*	Low residual activity	Intermediate NKH	Neonatal or infantile	Variable neurological impairment
Missense variants with residual activity in *GLDC*	Partial activity	Attenuated NKH	Infantile/childhood	Milder developmental delay, variable epilepsy
Pathogenic variants in *AMT*	Reduced activity	Classic or intermediate NKH	Neonatal or infantile	Variable severity
Variants in *GCSH*	Variable	Rare phenotypes	Variable	Limited data

NKH, non-ketotic hyperglycinemia.

including the GCS; an example is pyridox(am)in 5′-phosphate oxidase deficiency (caused by PNPO mutations).[23]

Biochemically, variant NKH is characterized by markedly elevated plasma glycine, mildly to moderately increased CSF glycine, and a lower or borderline CSF-to-plasma glycine ratio than in classical NKH. GCS activity is reduced, although mutations in *GLDC, AMT*, or *GCSH* are typically absent. Instead, impaired protein lipoylation may be observed.[23]

The diagnosis should be considered in patients with moderate CSF glycine elevation, absence of *GLDC/AMT/GCSH* mutations, and additional findings such as elevated lactate or alanine, which suggest broader mitochondrial involvement. In such cases, sequencing of genes involved in lipoate synthesis and Fe–S cluster biogenesis is recommended (*NFU1, BOLA3, IBA57, LIAS* and *GLRX5*).[27]

Clinically, variant NKH manifests a heterogeneous phenotype depending on the underlying gene defect. Presentations may range from childhood-onset spastic paraplegia with relatively preserved cognition to severe early-onset neurodegeneration. Features such as leukodystrophy, cardiomyopathy, optic atrophy, and elevated lactate are more suggestive of variant NKH than classical NKH.[28]

DIAGNOSTIC APPROACH

Diagnosing NKH is a multistep process that integrates clinical suspicion, biochemical markers, neuroimaging, and genetic or enzymatic confirmation.[20] In contemporary clinical practice, diagnostic evaluation relies primarily on biochemical assessment of glycine metabolism, particularly the simultaneous measurement of plasma and CSF glycine concentrations and the calculation of the CSF-to-plasma glycine ratio, followed by molecular genetic confirmation. Enzyme activity studies and the 13C-glycine breath test provide functional information on GCS activity and have historically contributed to diagnostic evaluation, but they are not the primary basis of contemporary diagnosis.

Biochemical Analysis

Elevated plasma glycine is typically the initial finding; however, this alone is insufficient for diagnosis. Plasma glycine may be falsely elevated in conditions such as valproic acid treatment, administration of glycine-buffered intravenous immunoglobulin, neonatal herpes infection, and branched-chain organic acidemias. Urinary glycine excretion may also increase. However, isolated hyperglycinuria may be observed in renal transport disorders and should not be interpreted as evidence of NKH. Concomitant elevation of CSF glycine is required, and plasma and CSF glycine concentrations should be measured simultaneously by quantitative amino acid analysis.[20]

CSF-to-plasma glycine ratio: This ratio is 0.02 or lower in healthy individuals. Although the diagnostic threshold for classic NKH is generally considered to be greater than 0.08, it may be lower (0.04-0.08) in variant NKH.[29] However, the ratio is meaningful only when CSF glycine is elevated. Blood contamination, as reflected by a high CSF protein concentration, may falsely elevate results. Valproate inhibits glycine degradation and increases plasma and CSF glycine concentrations, as well as their ratio. Rare cases of attenuated NKH may have normal CSF glycine concentrations.

In NKH, the urinary organic acid profile is expected to be normal; this finding distinguishes NKH from organic acidurias associated with ketotic hyperglycinemia.[29]

GCS Activity Measurement

GCS activity assays represent an earlier functional approach to diagnosing NKH. Measurement of GCS activity in a liver wedge biopsy is the most precise method, but it is reserved for selected cases because it is invasive and unavailable in many centers. Because the enzyme is highly sensitive, rapid sample processing and deep freezing are essential. This approach may be used for prenatal diagnosis, particularly when the familial mutation is unknown. However, because the false-negative rate is approximately 1%, it is not routinely recommended.[20,30]

Although enzyme analysis in cultured lymphoblasts has been investigated, the results are unreliable because enzyme activity values overlap between controls and patients with NKH.[31]

Although enzyme activity assays provide functional information on GCS activity, their invasive and technical requirements, together with the limitations of certain assay methods, have restricted their role in routine diagnostic evaluation.

13C-Glycine Breath Test

The 13C-glycine breath test is another functional method historically used to assess GCS activity. Normally, hepatic GCS metabolizes glycine, generating CO_2. When glycine is labeled with [1-13C], its breakdown produces $13CO_2$, which is exhaled and can be measured to evaluate GCS activity *in vivo*.

$NH_2\text{-}CH_2 \rightarrow 13COO \rightarrow 13CO_2 + NH_3$

The patient receives 10 mg/kg (maximum 100 mg) of [1-13C] glycine orally. A baseline breath sample is collected before administration, followed by breath samples collected at intervals between 15 and 300 minutes. An infrared analyzer measures the change in $13CO_2$ ($\Delta 13CO_2$). Results are expressed as cumulative percentage recovery, accounting for the administered dose and the patient's height and weight. Because 13C is non-radioactive, it can be used safely in children.

In healthy individuals, the mean 13C recovery within 5 hours is approximately 24%, whereas it is substantially lower in patients with NKH (approximately 8%). Thus, the test can readily distinguish patients with NKH from healthy individuals. Although the test is non-invasive, safe, and easy to perform, results may vary in patients with organic acidemias (e.g., methylmalonic and propionic acidemias), and the patient's metabolic status may affect the findings.[32,33] Although the 13C-glycine breath test provides functional information on GCS activity, it is not part of the primary biochemical-molecular diagnostic pathway used in contemporary clinical practice.

Neuroimaging and Functional Tests

Neuroimaging and electrophysiological studies provide complementary information, particularly to support the clinical diagnosis and assess disease severity and prognosis.

Magnetic resonance imaging (MRI): MRI findings vary with age. When symptoms begin within the first 3 months, diffusion restriction is usually detected in the posterior arm of the internal capsule, anterior brainstem, posterior tegmental tracts, and cerebellum. After three months, the restriction in the infratentorial regions decreases; between 3 and 14 months, spread toward the motor cortex and supratentorial white matter can be observed . Additionally, the corpus callosum may be thin and short, but does not completely disappear. In some infants, retrocerebellar cystic enlargement may occur in association with hydrocephalus. Atrophy, by contrast, occurs with advancing age in severe cases of NKH, while it is usually not observed in mild cases.[23,28,29]

Studies have found a significant relationship between the appearance of the corpus callosum and the clinical phenotype; the former is considered one of the first objective indicators of severe NKH (sensitivity 60-70%). This relationship has greater predictive value during infancy. After one month, no patient with severe NKH had a normal-appearing corpus callosum. The diffusion-limitation pattern is valuable for diagnosing NKH, but its severity is insufficient to distinguish the phenotype. NKH is characterized mainly by a brain growth disorder; spongiosis (microvacuoles) does not have prognostic significance. Early MRI findings are important for diagnosis, particularly for differentiating it from transient NKH.[29]

MR spectroscopy (MRS) offers a non-invasive method for measuring glycine levels in the brain. Detecting a significant glycine peak at 3.6 ppm can aid in diagnosis. At a short echo time (TE = 35 ms), the glycine signal at 3.55 ppm overlaps with myo-inositol. At an intermediate echo time (TE=135 ms), the glycine signal at 3.6 ppm separates and is clearly visible. While a significant glycine peak is seen in severe NKH, the glycine peak is lower in attenuated NKH and is sometimes even difficult to distinguish.[20] Magnetic resonance spectroscopy reveals the NAA/myo-inositol-glycine ratio as a prognostic indicator for adverse outcomes.[30]

EEG: The "burst suppression" pattern is common in the neonatal period, but this can also be observed in atypical cases. This pattern consists of periodic high-amplitude activity at 1-3 Hz on a background of low activity and shows no spatial or temporal differentiation. The prognostic value of EEG is limited because changes such as hypsarrhythmia and burst-suppression patterns, which are common in severe forms of NKH, can also occur in attenuated NKH.[31,32]

Molecular Genetic Testing and New Diagnostic Approaches

It is an autosomal recessive disease. The molecular basis of classical NKH is largely attributed to two main genes: *GLDC* (9p24.1) and *AMT* (3p21.31). GCSH (16q24) encodes the H-protein, mutations in this gene are extremely rare (1<% of cases) and are usually associated with "variant NKH" or combined bioenergetic defects. Furthermore, mutations in the *SLC6A9* gene, which encodes the glycine transporter, result in GLYT1 encephalopathy, leading to a similar clinical picture despite normal GCS activity.[15,19,33]

Genetic heterogeneity is high in NKH; most mutations are private. Missense mutations are the most common type. In addition, non-sense (early stop), splicing (clipping zone), and small insertions/deletions (indels) were observed. Copy Number Variants: Approximately 20-21% of mutations in the *GLDC* gene are large deletions or duplications that are undetectable by traditional sequencing methods. Therefore, methods such as MLPA or microarray should be used alongside sequencing for a complete molecular analysis. Next-generation sequencing: today, multiple-gene panels or whole-exome sequencing have become the primary tools for diagnosing NKH and similar inherited metabolic diseases.[19,20,33]

Advances in functional genomics have improved the interpretation of genetic variants and the assessment of genotype-phenotype relationships in NKH. Recent reports have demonstrated that synonymous GLDC variants, which may initially be classified as variants of uncertain or low clinical significance, can cause aberrant splicing and loss of normal gene function when evaluated using functional minigene assays. These findings highlight the importance of incorporating functional evidence into variant classification, particularly for non-canonical or apparently synonymous variants.[34,35] *In silico* prediction alone may be insufficient for variant interpretation in NKH; functional assays, particularly splicing assays and residual enzyme activity studies, can substantially refine pathogenicity classification.

Molecular diagnostics provide important information for predicting disease severity. In severe cases of NKH, mutations in both alleles that eliminate enzyme activity (e.g., non-sense, frameshift, or large deletions) are detected. The attenuated form, on the other hand, is characterized by mutations that maintain residual enzyme activity in at least one allele (p. Ala389Val, p. Ala802Val in *GLDC*; p. Ile106Thr in *AMT*). These patients often show more favorable neurodevelopmental outcomes.[19] Kuseyri Hübschmann et al.[36] reported that genotype-based predictions indicate that two mild variants lead to attenuated phenotypes, whereas two severe variants result in severe disease. The presence of one mutation with residual enzyme activity is necessary but not sufficient for an attenuated outcome. However, divergent outcomes for the same genotype indicate that other genetic or non-genetic factors contribute to disease severity.[22] Genotype-phenotype discordance limits molecular prediction, as identical genotypes produce divergent outcomes, and 206 of 255 *GLDC* missense mutations remain uncharacterized.[22,37]

Molecular testing fails to identify defects in the *GLDC* or *AMT* genes in approximately 5% of cases. These conditions should be classified as variant NKHs resulting from mutations in genes involved in the synthesis or transport of lipoic acid, a GCS cofactor (including *LIAS, BOLA3, GLRX5, NFU1, IBA57, LIPT1*, *and LIPT2*). Such cases commonly present with additional clinical features, such as pyruvate dehydrogenase deficiency and lactic acidosis, alongside elevated glycine levels.[19,23] In addition, plasma alanine, serum lactate, and pyruvate may increase. An increase in 2-ketoglutarate can be detected in urinary organic acids. Those with *NFU1* mutations also have elevated levels of 2-aminoadipic acid and 2-ketoadipic acid. In addition, pyruvate dehydrogenase (PDH) activity has consistently been reduced. Deficiencies of respiratory chain complexes I and II are seen in iron-sulfur cluster synthesis disorders (*NFU1, ISCA2, IBA57, BOLA3*). Amounts of Lipoyl-H protein, the E2 subunit of PDH and 2-ketoglutarate dehydrogenase, can be shown to be decreased by Western blotting.

Although the disease is autosomal recessive, *de novo* mutations have been reported in about 1% of cases. This significantly reduces recurrence risk when the parents are not carriers; therefore, molecular confirmation of parental carrier status is recommended. Prenatal diagnosis through chorionic villus sampling and pre-implantation genetic testing are possible in families with known mutations.[20]

Diagnostic standards remain CSF/plasma glycine and GCS genetics, but several newer tools are refining earlier detection and severity prediction. Functional assays of missense variants (enzyme activity, protein stability, structural modeling) help classify variants as severe vs. hypomorphic and predict phenotype, suggesting which patients may have an attenuated course.[22,38] A 2025 microRNA sequencing study identified 10 candidate miRNAs differentially expressed in NKH patients versus heterozygous carriers and controls, the first such profiling in *AMT/GLDC*-related disease, proposed as future blood-based early biomarkers and a framework for novel diagnostic strategies.[39]

Some integrative severity prediction models combine age at onset, CSF and plasma glycine thresholds, and genotype and pathogenicity scores to stratify NKH as severe versus attenuated, achieving high AUCs (0.87-0.97).[36,40] The following were suggested as diagnostic criteria for severe NKH: onset of symptoms within the first week, CSF glycine >230 µM, brain malformation on MRI, two non-missense mutations, and glycine index >3. In attenuated NKH, symptom onset after 4 months, CSF-to-plasma glycine ratio ≤0.08, no use of antiepileptic drugs, and at least one functional allele are determinants. For early diagnosis, these markers provide 78% accuracy for the severe form and 49% for the milder form.[22,31] Although high CSF glycine correlates with the severe form, low CSF glycine does not rule it out.

Beyond the traditional CSF/plasma glycine ratio, CSF serine and threonine levels provide additional diagnostic evidence; the CSF serine/threonine ratio discriminates classic NKH from transient NKH, in which the glycine ratio is elevated but the serine/threonine ratio remains normal. Untreated NKH patients show a specific alteration in D-serine metabolism, with a greater reduction in D-serine than in L-serine and a decreased D/L-serine ratio, reflecting a perturbation of the serine shuttle and one-carbon metabolism.[41]

New diagnostic methods that combine genetics, functional assays, and omics and imaging tools are improving the detection of NKH, and further research is needed to achieve precise and widely accessible diagnostics.

DIFFERENTIAL DIAGNOSIS

The differential diagnosis of NKH can be challenging, particularly in neonates who present with hypotonia, seizures, apnea, feeding difficulties, or altered consciousness, as these manifestations overlap with those of several metabolic and genetic encephalopathies. The differential diagnosis includes urea cycle disorders, organic acidemias, maple syrup urine disease, disorders of amino acid metabolism, mitochondrial disorders, and other causes of neonatal epileptic encephalopathy. Distinguishing NKH from these conditions requires integrating clinical presentation with targeted biochemical investigations rather than relying on a single abnormal laboratory parameter. Table 3 summarizes the principal clinical and biochemical features that distinguish NKH from these major mimics.

For practical diagnostic purposes, the distinction between NKH and other neonatal metabolic encephalopathies should not

Table 3. Disorders showed elevated plasma glycine.

Condition	Distinguishing features vs. NKH
Organic acidemias (propionic, methylmalonic, isovaleric acidemia)	Metabolic acidosis, ketosis, abnormal urine organic acids; NKH shows no ketoacidosis and normal organic acids despite high glycine
"Variant NKH" due to lipoate defects (*LIAS, BOLA3, NFU1, GLRX5,* some *GCSH*)	Hyperglycinemia plus combined deficiency of pyruvate/α-ketoglutarate dehydrogenase, lactic acidosis, multisystem disease, leukodystrophy; not an isolated GCS defect
Glycine transporter defect (*SLC6A9*)	Hyperglycinemia with abnormal CSF/plasma ratio but primary transport defect; may have overlapping neonatal encephalopathy, requires genetic confirmation

NKH, non-ketotic hyperglycinemia; CSF, cerebrospinal fluid; GCS, glycine cleavage system.

rely solely on plasma glycine concentration. The combined assessment of plasma and CSF glycine, particularly the CSF-to-plasma glycine ratio>0.08 (often ≥0.15 in severe NKH), provides an important discriminator, while concomitant evaluation of ammonia, lactate, acid-base status, ketones, plasma amino acids, and urine organic acids helps distinguish NKH from urea cycle disorders, organic acidemias, and other aminoacidopathies.[41,42]

NKH also mimics some clinical situations;

Hypoxic-ischemic encephalopathy (HIE) presents with similar features, including hypotonia, coma, and seizures; unlike NKH, it is not associated with isolated glycine elevation and shows distinct MRI patterns. At intermediate echo times, MRS may be misinterpreted as showing glycine, whereas long echo times help distinguish glycine from myo-inositol and reduce the risk of misdiagnosing NKH in patients with HIE.[43-49]

Infectious encephalitis/meningitis–CSF pleocytosis, elevated protein, systemic infection markers; no characteristic CSF glycine pattern.

Other inborn errors of metabolism (urea-cycle defects, maple syrup urine disease, and mitochondrial disorders) usually present with hyperammonemia, characteristic organic acid abnormalities, or lactate elevation—findings that are absent in isolated NKH. Hyperglycinuria caused by renal glycine transport defects, including familial iminoglycinuria and benign hyperglycinuria, should also be distinguished.[50]

Transient NKH-early hyperglycinemia that normalizes as glycine-cleavage activity improves; milder course. Requires follow-up amino acids and genetics to avoid over-diagnosis of classic NKH.[44]

Clinical "red flags" that should keep NKH high on the list are: neonatal onset within days, profound hypotonia, apnea/ventilation need, hiccups, refractory seizures, and very high CSF glycine with normal organic acids.[28,34] Overall, establishing NKH and excluding mimics depends on combined clinical phenotype, CSF/plasma glycine profile, absence of ketoacidosis/alternative metabolic signatures, neuroimaging, and confirmatory genetic testing for GCS or related genes.[36,34]

TREATMENT STRATEGIES

Treatment remains challenging and is primarily supportive, aiming to reduce glycine levels and mitigate neurotoxicity. Current therapeutic strategies include pharmacological reduction of glycine levels (e.g., sodium benzoate), NMDA receptor antagonists (e.g., dextromethorphan), dietary interventions such as the ketogenic diet for seizure control, and emerging experimental approaches such as gene therapy (Table 4).[28,45,46] Early intervention may improve outcomes in milder cases of classic NKH, but no curative therapy exists.

Glycine-Lowering Therapy:

Sodium benzoate reduces plasma glycine levels by conjugating them to hippuric acid; the target range is 120-300 µmol/L. Measurements should be performed on blood samples taken 1-2 hours after benzoate administration.

Benzoate + CoA → Benzoyl-CoA +Glycine → CoA+ Hippurate

• Dose for attenuated NKH: 200-550 mg/kg/day. For children and adults, the initial dose is recommended to be 5.5 g/m^2 based on body surface area.

• Dose for severe NKH: 550-750 mg/kg/day. In adults, the maximum dose should be limited to 16.5 g/m^2/day.

• Division of dosing: It should be administered at least three doses/day. Six doses per day should generally be preferred for newborns.[20,28,47]

Treatment starts at a low dose and plasma glycine levels are monitored regularly. If the target level is not reached, the dose is increased by 50 mg/kg/day each time, and the plasma glycine level is measured again after 24-48 hours. Follow-up frequency by age group is as follows:

• In infants: Every two weeks,

• In young children: Once a month,

• In older children: Every three months.

Table 4. Treatment options for NKH.

Modality	Primary goal	Evidence/limitations
Sodium benzoate	Lower plasma/CSF glycine	Standard care; improves arousal/seizures in some, no cure; effect greater in attenuated NKH
NMDA antagonists (dextromethorphan, ketamine)	Block glycine-NMDA excitotoxicity	Mixed evidence; no clear added long-term benefit; mechanistic concerns and side-effects
Ketogenic diet	Seizure and glycine reduction	Lowers plasma glycine more consistently than high-dose benzoate; variable clinical impact
Conventional ASMs (e.g., levetiracetam)	Seizure control	Helpful in some refractory cases; no standardized regimen
VNS, other non-drug epilepsy therapies	Refractory epilepsy	Case-level evidence of seizure reduction
Palliative, multidisciplinary care	Quality of life, survival	Crucial in severe NKH; can extend survival and support families
AAV-GLDC gene therapy (preclinical)	Causal correction	Normalizes metabolic profile in mouse model; human trials not yet available

NKH, non-ketotic hyperglycinemia; NMDA, modulate N-methyl-D-aspartate; AAV-GLDC, adeno-associated virus - glycine decarboxylase; ASMs, anti-seizure medications; VNS, vagus nerve stimulation; CSF, cerebrospinal fluid.

Benzoate therapy is effective in the liver and kidneys but does not normalize glycine levels in the brain or CSF; therefore, monitoring CSF glycine levels is unnecessary.[47]

High doses of benzoate (500-750 mg/kg/day) can lead to gastritis, carnitine loss, and toxicity. Overdose may cause hypocalcemia, low plasma glycine, nausea, vomiting, lethargy, coma, seizures, acidosis, and electrolyte disturbances; the threshold for toxicity is plasma benzoate >2.5 mmol/L. Treatment includes discontinuing benzoate, supplementing glycine, and/or performing hemodialysis.[20] The taste of benzoate can cause incompatibilities. In some countries, a 75% granule form is available.

The glycine index evaluates whether plasma glycine concentration is maintained within the target range (120-300 µmol/L). The glycine index helps determine a person's specific benzoate requirement. Especially in patients on a ketogenic diet, the benzoate dose should be reduced as the glycine pool shrinks; the glycine index guides this adjustment.[47]

$$Glycine\ index = \frac{benzoate\ dose\ (mg/kg/day)}{plasma\ glycine\ (\mu mol/l)}$$

A low glycine index indicates that the benzoate dose does not adequately reduce the glycine level; in this case, the dose may need to be increased. A high glycine index, on the other hand, indicates that the benzoate dose reduces glycine below the target range, so dose reduction is recommended because this reduction may pose a risk of toxicity. Target values: plasma glycine ≤300 µmol/L and benzoate level should be at the non-toxic limit (≤2.5 mmol/L).[28,48]

The ketogenic diet (KD) has been explored in NKH primarily as an adjunctive therapy for refractory epilepsy, a major clinical feature, particularly in classical neonatal forms. Given the limited efficacy of standard treatments such as sodium benzoate and NMDA receptor antagonists, KD has emerged as a potential supportive strategy.

The therapeutic rationale for KD in NKH is based on its antiepileptic and neuroprotective effects rather than addressing the disease itself. Ketone bodies may decrease neuronal excitability, modulate glutamatergic neurotransmission (important because glycine acts as an NMDA co-agonist), and enhance mitochondrial energy metabolism.[49] In cases of glucose deprivation, glycine is converted to serine, which is then converted to pyruvate by serine dehydratase to be used as the starting substrate for gluconeogenesis. The ketogenic diet may enhance endogenous glycine utilization through metabolic pathways involved in energy production and gluconeogenesis, potentially influencing glycine homeostasis.[48] Importantly, emerging evidence suggests that the effects of KD in NKH may extend beyond seizure control to include modulation of glycine metabolism. In a multicenter study involving six infants with NKH, the patients initially treated with high-dose sodium benzoate were subsequently switched to KD with low-dose benzoate. KD was associated with an approximately 28% reduction in plasma glycine concentrations compared with high-dose benzoate therapy. In two patients evaluated by serial magnetic resonance spectroscopy, brain glycine concentrations decreased by approximately 30% in the basal ganglia and by 50% in the white matter, although concentrations remained above the normal range. Concentrations of CSF glycine, serine, and threonine also decreased during KD, consistent with increased utilization of these substrates during gluconeogenesis. However, the clinical response was heterogeneous, with seizure reduction observed in about half of the patients. These findings suggest that KD may influence glycine homeostasis, although

biochemical improvement does not necessarily translate into uniform neurological benefit.[48]

More recent clinical evidence from Türkiye further supports a potential role for KD in NKH. In a retrospective comparative study of 14 patients with classical NKH, eight received KD and six received a glycine-restricted diet (GRD). KD was associated with significantly greater reductions in seizure frequency and seizure scores, as well as a reduced requirement for antiseizure medications, compared with GRD. However, neither dietary intervention significantly improved plasma glycine concentrations, motor function, or swallowing ability. These findings are particularly important because they suggest that the clinical antiseizure effects of KD may occur independently of a measurable reduction in systemic glycine concentrations.[50]

Additional reports support KD's potential antiseizure benefit in severe NKH. The ketogenic formula has been associated with complete seizure control and EEG improvement within approximately one month, whereas other cases have reported reduced focal seizures, lower CSF glycine levels, and improved quality of life when KD is combined with conventional therapy.[44,51-55]

Taken together, the available evidence supports KD as a potential adjunctive treatment for seizure control in selected patients with NKH, particularly those with drug-resistant epilepsy.

NMDA Receptor-Targeted Therapy:

In NKH, glycine degradation is impaired, leading to accumulation of glycine in the central nervous system. Glycine overactivates the NMDA receptor, causing neuronal damage through increased calcium entry and excitotoxicity. This causes severe epileptic encephalopathy, especially in classical neonatal NKH.

Dextromethorphan and ketamine are commonly used non-competitive NMDA receptor antagonists. Clinical studies report reduced seizure frequency, partial EEG improvement, and frequent coadministration with sodium benzoate to block glycine co-agonism.[23,28] Early findings demonstrated improved alertness, improved sucking, and partial control of seizures in neonates. A 17-year-old patient treated with high-dose benzoate and dextromethorphan experienced extended survival but persistent severe disability and refractory seizures; a 2023 case report showed it reduced myoclonic seizures resistant to multiple drugs.[56,57] Recent reassessment argues that reduced brain D-serine in NKH may actually under-activate NMDA receptors, and that clear added benefit of dextromethorphan/ketamine beyond glycine reduction has not been demonstrated; routine use should be critically reconsidered because of potential adverse effects.[16]

Dextromethorphan is typically administered at doses ranging from 3 to 15 mg/kg/day, with higher doses required in younger patients (approximately 10 mg/kg/day in neonates, 5 mg/kg/day in children, and 3 mg/kg/day in adolescents and adults). The total daily dose is usually divided into three to four administrations or given as a sustained-release formulation twice daily. Interindividual variability in response is significant and partly explained by pharmacogenomic differences involving CYP2D6, CYP3A4, and UGT1A1 enzymes. Concomitant use of medications such as cimetidine may inhibit metabolism and increase the risk of toxicity. Routine therapeutic drug monitoring is not recommended because a defined therapeutic range has not been established. Adverse effects are generally dose-related and include sedation and movement disorders at higher doses. Additionally, suppression of the cough reflex may increase the risk of aspiration and pneumonia in this group. Long-term high-dose use may lead to tolerance, and abrupt discontinuation can result in withdrawal symptoms; therefore, gradual tapering over several months is recommended when discontinuation is required.[58,59]

Ketamine is available as R-, S-, and racemic formulations and has low oral bioavailability (8-24%) due to extensive first-pass metabolism via CYP3A and CYP2B6, producing norketamine, a less active metabolite. It exerts its primary effect via non-competitive antagonism of NMDA receptors, which reduces GABAergic interneuron activity; it also interacts with opioid receptors and inhibits monoamine transporters, thereby increasing adrenergic activity. Oral dosing is approximately 15 mg/kg/day in neonates and approximately 9 mg/kg/day in infants (range, 1-32 mg/kg/day). At high doses, ketamine induces dissociative anesthesia and may be used in refractory status epilepticus, whereas lower doses provide analgesic and antidepressant effects, though they may also cause cognitive impairment and hallucinations. Its metabolite, hydroxynorketamine, acts as an AMPA receptor agonist and may contribute to antidepressant effects. However, long-term safety data are limited, and chronic use has been associated with urinary tract toxicity, including cystitis and bladder dysfunction.[16]

Evidence supporting dextromethorphan and ketamine for NKH remains limited and derives primarily from case reports, small case series, and clinical observations. Although some patients may experience improved seizure control or neurological status, controlled clinical trials have not established its efficacy.

Partial clinical improvement with strychnine (GlyR antagonist) has been reported in a few historical cases. Although it improves tone and breathing, long-term use has been abandoned due to serious side effects.

Perampanel (an AMPA receptor antagonist) was administered as an intervention in a patient who did not benefit from sodium benzoate and dextromethorphan treatment. Gradually increasing the dose to 0.65 mg/kg/day resulted in a 50% reduction in epileptic spasms.[55]

A dose-dependent decrease in Gly levels induced by sodium cinnamate was detected in the animal model (GLDC def) and in the research phase in humans.

Epilepsy Management:

Seizures are frequently drug-resistant, especially in severe NKH.[28]

Benzodiazepines (clonazepam, clobazam) - most effective in myoclonic seizures in early infancy.

Levetiracetam, topiramate, and phenobarbital - are frequently used in older children.

Felbamate (NMDA blocker) - may be considered for resistant seizures, but its use is limited by serious side effects.

ACTH - is ineffective in West syndrome; it can even cause worsening, including coma.

Valproate and vigabatrin are contraindicated. Valproate inhibits the GCS and has precipitated worsening; vigabatrin has been reported to aggravate West syndrome in NKH.60

Vagal nerve stimulator - useful in some severe cases.

Supportive and Palliative Care:

Because current treatments do not halt neurodegeneration, long-term management is supportive: Respiratory, feeding (including gastrostomy), orthopedic and gastrointestinal care; monitoring for cardiopulmonary complications. Early, integrated pediatric palliative care can prolong survival and improve quality of life, even in severe NKH with profound disability. All recent series emphasize multidisciplinary follow-up.[3,61]

Emerging and Experimental Approaches:

Excitotoxicity, oxidative stress, and impaired brain bioenergetics are increasingly recognized as key mechanisms, suggesting future adjuvant therapies aimed at providing antioxidant and mitochondrial support, although these remain experimental.[18]

One major area of interest is gene-based therapy. A novel recombinant adeno-associated viral vector 9 expressing *GLDC* (rAAV9-GLDC) conferred 100% protection against disease progression and fatality in humanized NKH mice over a ten-month follow-up. A single intraperitoneal dose boosted astrogenesis without triggering an inflammatory response, which indicates a durable neuroprotective mechanism.[62] In a separate GLDC-deficient mouse model, neonatal administration of AAV9-GLDC restored GLDC mRNA and protein expression, leading to a significant reduction in plasma and brain glycine levels.[12] This vector also normalized the folate profile, restoring the glycine-derived one-carbon supply for folate metabolism. Unlike current treatments, gene therapy that restores GCS function simultaneously lowers glycine levels and supplies glycine-derived one-carbon groups to folate one-carbon metabolism. Preclinical studies in animal models have shown partial biochemical correction and improved survival, although translation to humans remains at an early stage.[12]

Methyl group donors have not been proven effective in humans, although they are effective in mice. Folinic acid is among the treatments that have not demonstrated benefit in humans. In addition, cofactor therapy has not been tried for rare mutations: GLDC mutations—pyridoxal phosphate; AMT mutations—folinic acid.

D-serine substitution has been proposed as a metabolite-based approach to address the under- activation hypothesis, but no clinical studies have been conducted.[26]

The evidence base for all NKH therapies is limited to case reports and small retrospective series and is therefore insufficient to support formal treatment recommendations.[54] The ketogenic diet shows the most reproducible benefit for seizure control and glycine reduction, though it does not normalize brain glycine or consistently improve neurodevelopment.[48] NMDA receptor antagonists have an increasingly contested mechanistic rationale, variable clinical responses, and emerging safety concerns.[26] Prospective controlled studies are needed across all interventions.

PROGNOSIS

The prognosis for NKH depends strongly on disease severity (severe versus attenuated), age of onset, brain imaging findings, EEG findings, and genotype. As summarized in Table 5, neonatal onset, severe neurological involvement, markedly elevated CSF-to-plasma glycine ratios, profound reduction in GCS activity, severe/null variants, burst-suppression EEG patterns, and extensive MRI abnormalities are generally associated with poorer outcomes, whereas residual enzyme activity, later onset, lower CSF-to-plasma glycine ratios, milder genetic variants, and less severe neurological abnormalities are more commonly associated with attenuated disease and a relatively better prognosis. However, these factors should not be interpreted as independent or absolute predictors because substantial clinical variability exists among patients. The prognosis is poor in the severe classical neonatal form and highly variable in attenuated cases.[12,63]

Table 5. Prognostic factors related to NKH.

Factor	Good prognosis	Poor prognosis
Clinic onset	Infant/childhood onset (attenuated NKH)	Neonatal onset (classical NKH)
Neurological findings	Mild hypotonia, controllable seizures	Refractory seizures, coma, severe encephalopathy
Developmental course	Partial motor and cognitive gains	Serious global developmental delay
CSF to plasma glycine ratio	Lower rate (close to <0.08)	High rate (0.08>)
GCS activity	Residual enzyme activity	Profound enzyme deficiency
Genetics	Mild/partial loss of function mutations	Null or severe mutations (*GLDC*, *AMT*)
EEG	Mild slowing, controllable epileptiform activity	Burst-suppression, severe epileptic encephalopathy
MRI findings	Minimal or non-specific changes	Hypoplasia of the corpus callosum, widespread brain damage
Response to treatment	Improvement in seizure control	Treatment-resistant epilepsy
Systemic status	Stable clinical course	Need for respiratory support, frequent intensive care

NKH, non-ketotic hyperglycinemia; CSF, cerebrospinal fluid; GCS, glycine cleavage system; EEG, electroencephalogram; MRI, magnetic resonance imaging.

Classical (neonatal severe) NKH is associated with a poor prognosis, characterized by early-onset encephalopathy, refractory seizures, and profound developmental impairment. Many patients require long-term ventilatory and nutritional support, and mortality is highest in the neonatal period or early infancy. Survivors typically exhibit severe intellectual disability, spasticity, and intractable epilepsy.[22,64]

In contrast, attenuated NKH demonstrates a more heterogeneous clinical course. Patients may achieve limited developmental milestones, including partial motor and cognitive skills; seizure control is often more achievable. However, long-term outcomes remain variable, and many individuals still experience persistent neurodevelopmental deficits.[3]

In a natural-history study of 65 NKH patients, one-third of patients died; a significant proportion of deaths occurred during the neonatal period. Most of the survivors had severe developmental delays, epilepsy, and spasticity; however, 10 of the 25 children (all boys) who were alive and reached the age of ≥3 were able to walk and communicate with words/signs.[63] In the Chinese cohort, 17 of the 20 patients were of the neonatal type; of the 18 patients, 9 died (8 during the neonatal period). All 9 surviving patients had varying degrees of neurodevelopmental impairment.[3] In the Turkish series, overall mortality was 30% among 10 classic NKH patients and 50% among those in the neonatal group; severe sequelae were reported in most survivors.[25]

Neuroimaging and electrophysiological findings also carry prognostic value. Severe EEG abnormalities, such as burst-suppression patterns, and structural brain anomalies on MRI are typically associated with worse outcomes. Overall, despite advances in supportive care, NKH remains a condition with significant morbidity, and current treatments are largely unable to alter the natural course of severe disease.[36] In a cohort of 124 patients, CSF glycine levels and the CSF-to-plasma glycine ratio were inversely correlated with the developmental quotient. A CSF glycine level >230 µM and a CSF/plasma ratio ≥0.15 were identified as strong predictors of severe disease, whereas ratios ≤0.08-0.09 were associated with attenuated phenotypes.[22] These findings were further supported by a smaller study of 25 patients, in which disease onset at ≥3 months and a CSF/plasma ratio ≤0.09 were confirmed as sensitive indicators of attenuated NKH.[36] At the molecular level, an attenuated phenotype requires at least one allele with residual enzyme activity, whereas biallelic mild mutations are associated with the most favorable developmental outcomes.[22] Additionally, clinical outcome scores and mutation severity scores derived from large-scale GLDC variant analyses demonstrate high specificity in distinguishing severe from attenuated forms.[40] Although most patients with severe NKH die in early childhood, some survive to age 7 with severe neurodevelopmental disorders, and reports indicate that long-term palliative care can prolong life.[61] In attenuated NKH, individuals can survive into adulthood. Individuals who have reached their 50s, who were diagnosed in childhood, and who have lifelong mild-to-moderate disabilities are reported.[65]

Taken together, the prognostic factors summarized in Table 5 may assist in early clinical stratification and counseling, particularly when age at onset, neurological severity, biochemical findings, neuroimaging, EEG, and genotype are considered. Nevertheless, they should be used cautiously because reliably predicting individual long-term outcomes remains challenging.

CONCLUSION

Despite advances in symptomatic management, NKH remains a devastating disorder with limited disease-modifying treatment options, highlighting significant unmet clinical needs. Emerging therapeutic strategies for NKH, including gene replacement targeting GLDC and *AMT,* read-through inducers for premature

stop codons, chemical chaperones for unstable missense variants, antisense oligonucleotides for selected splicing defects, and genotype-guided approaches, offer promising mutation-specific therapeutic options to restore or improve GCS activity. However, major limitations such as effective central nervous system delivery, durable expression, safety, and the need for robust functional validation remain unresolved, making genetic testing increasingly important for prognosis and individualized therapeutic planning.[19,38,66]

Targeting downstream neurotoxicity represents another critical area of development. Beyond glycine-mediated NMDA receptor overactivation, growing evidence suggests that excitotoxicity, oxidative stress, and bioenergetic dysfunction contribute to NKH neuropathology. These mechanisms support future therapeutic strategies, including antioxidants, mitochondria-targeted interventions, and modulators of non-NMDA receptor pathways.[18]

Demonstration of restructuring of serine-glycine-one-carbon metabolism and of a shift in glial differentiation in the iPSC-derived NKH astrocyte model provides a powerful platform for human cell-based drug screening.[14]

Severity-estimation models developed using multiparameter approaches (age at onset, CSF/plasma glycine ratio, CSF glycine threshold, genotype) accurately distinguish severe from attenuated cases.[22] This can help determine more rationally to whom we will give intensive treatment and to whom we will provide palliative care.[28,36] The Weighted Multiparametric Mutation Score, which combines *in silico* predictions of stability, conservation, and interaction, distinguished severe from attenuated disease with high statistical significance.[37] Brain MRI adds prognostic value: as corpus callosum growth is more pronounced in severe cases, with 71% of severe patients having brain malformations versus 7.5% of attenuated patients.[22,29] Small sample sizes constrain all models, and no external validation exists for any NKH severity model; the multiparametric approach is described as promising but unvalidated in independent cohorts. Clinical utility evaluation is equally absent, as no study has assessed whether these models improve clinical decision-making or patient outcomes. Without external validation and utility data, these models remain promising developmental tools rather than validated clinical instruments ready for implementation.

A major unresolved gap is the lack of human clinical trials evaluating gene-based and one-carbon metabolic therapies, despite encouraging preclinical findings in mouse models. NMDA receptor-targeted treatments also lack prospective controlled evidence, and their efficacy is increasingly questioned in light of biochemical data indicating D-serine deficiency.[41] Current prognostic models are constrained by small cohorts of rare-disease patients and require validation in larger retrospective datasets. Similarly, iPSC-derived models remain at an early stage and have not yet been used for therapeutic screening. Overall, disease rarity, insufficient biomarkers, and lack of controlled trials continue to limit therapeutic progress. Future studies should therefore focus on earlier diagnosis, biomarker discovery, and multicenter collaborative trials that can translate experimental approaches into clinically meaningful outcomes.

FOOTNOTES

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