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Nuclear magnetic resonance spectroscopy-based urinalysis for a young girl with extreme hypoglycaemia

To the Editor,

Hypoglycaemia is not an uncommon medical emergency in paediatric patients. Untreated hypoglycaemia can be rapidly fatal in young vulnerable subjects. Unlike hypoglycaemia in adults, inherited metabolic disorders (IMD) should be considered as one of the key differential diagnoses in paediatric patients and early diagnosis is important to guide appropriate management and prevent further metabolic insult. Here, we report a case of recurrent and severe hypoglycaemia in a 1-year-old girl who was diagnosed with fructose-1,6-bisphosphatase (FBP1) deficiency, a life-threatening and rare IMD condition which can be effectively managed by lifestyle and dietary modification. We will discuss the clinical use of NMR-based urinalysis in the rapid diagnosis of FBP1 deficiency and describe the use of the glycerol-kinase-based triglycerides assay as a quick approach to quantitate glycerol, a pathognomonic marker for this

condition. This study was approved by Institutional Review Board (Reference Number: UW 21–150).

The 1-year-old patient was referred to us for metabolic investigations, presenting with acute and ketotic hypoglycaemia. Before this admission, she had another episode of a similar nature at 11 months old, which was managed as a viral infection in another hospital. The patient had an unremarkable birth history and was born to non-consanguineous parents. A dried blood spot newborn screening test was performed in a private centre with unremarkable findings. She had normal development and growth all along. On admission, she presented with extreme hypoglycaemia (plasma glucose 0.5 mmol/L), ketosis [plasma beta-hydroxybutyrate (BOHB) 5.5 mmol/L, reference interval (RI) <0.6], lactic acidosis (plasma lactate 10.1 mmol/L, RI 0.7–2.1; pH 7.29; RI 7.35–7.45) and hyperuricaemia (0.7 mmol/L, RI 0.11–0.3). She also had an elevated lactate-to-pyruvate (L:P) ratio of 42 and an elevated BOHB-to-acetoacetate (AcAc) ratio of 8. On physical examination, the patient appeared irritable and lethargic with decreased tone over four limbs. She had warm peripheries and a satisfactory hydration status. Her pulse was 106 BPM, and she was tachypnoeic. There was no significant hepatomegaly. The patient remained arousable during physical examination despite a fluctuating consciousness. There was no known positive family history of hypoglycaemia, IMD or sudden infant death. Her twin brother was otherwise healthy with unremarkable development. The patient was given intravenous dextrose infusion, and her condition gradually improved with no other complications upon discharge.

A urine sample was saved during hypoglycaemic attack in this patient and was sent to our laboratory for further metabolic work-up. Urine organic acid (UOA) analysis with ethyl acetate extraction by gas chromatography-mass spectrometry (GC-MS; 5973N Gas Chromatography system; Agilent, USA) detected marked increases in urinary glycerol, lactate, AcAc and BOHB. Nuclear magnetic resonance (NMR)-based urinalysis (¹H-NMR; 600 MHz Avance; Bruker, USA) showed hyperexcretion of glycerol, measuring 47.5 mmol/mmol creatinine (97.5th percentile 0.05 mmol/mmol Cr), BOHB 49 mmol/mmol Cr (97.5th percentile 0.008 mmol/mmol Cr), AcAc 10 mmol/mmol Cr (97.5th percentile not available), lactate 71 mmol/mmol Cr (97.5th percentile <0.116 mmol/mmol Cr) and creatinine 1.2 mmol/L (Fig. 1).

Clinically, ketotic hypoglycaemia with glyceroluria can be seen in FBP1 or glycerol kinase (GK) deficiency. Since GK deficiency is an X-linked condition, our patient will be a manifested female if she is affected by GK deficiency. She also has an unaffected twin brother and therefore we consider FBP1 deficiency is more likely in our case. Genetic analysis was arranged and we identified a homozygous variant, NM_000507.4:c.959dupG; NP_000498.2:p.Ser321-IlefsTer13 in the *FBP1* gene and this is a known disease-causing variant for FBP1 deficiency. This patient had a good clinical response after lifestyle and dietary modifications.

The gluconeogenic pathway and the glycolytic pathway proceed in opposite directions, involving mostly bidirectional enzymes intercalated with key irreversible enzymes unique to each pathway. In particular, FBP1, pyruvate carboxylase, phosphoenolpyruvate carboxykinase and glucose 6-

Fig. 1 (Upper) NMR-based urinalysis detects hyperexcretion of glycerol (red box, measuring 47.5 mmol/mmol creatinine). An enlarged illustration for the 3.50–3.80 ppm region indicates the proton NMR fingerprint of glycerol. (Lower) Proton NMR spectra from patients with exudate pleural effusion (PE) and transudate PE are included for comparison with the urine NMR spectrum of the patient. Signals are normalised using the proton signal from the internal standard, i.e., TMSP. Marked increases in lipoprotein particles (red arrows) are noted in exudates but not in transudates. No proton lipoprotein signals were detected in the patient's urine, which excludes the possibility of chyluria in this case.

phosphatase are such examples of the latter, and they mainly contribute to gluconeogenesis. Since FBP1 is the rate-limiting step in gluconeogenesis, deficiency in FBP1 would significantly impair this process, which can precipitate hypoglycaemia. Hypoglycaemic attacks are often triggered by febrile illness, poor feeding, catabolic stress, or potentially after consumption of a large amount of fructose. Nevertheless, this life-threatening crisis is preventable by lifestyle and dietary modification.¹

Moreover, FBP1 deficiency will impair the normal metabolic flux in gluconeogenesis with the accumulation of gluconeogenesis substrates, including lactate, pyruvate, alanine, ketones and glycerol (Fig. 2). These markers are elevated in patients affected by FBP1 deficiency.² Such impaired gluconeogenic flux would lead to NADH accumulation with an increase of NADH-to-NAD⁺ ratio. Although direct measurement of NADH and NAD⁺ is not routinely available, this redox can be monitored using surrogate biomarkers, blood L:P and/or BOHB:AcAc ratios which reflected the redox status in cytosol and mitochondrion respectively.³ Elevated L:P and BOHB:AcAc ratios were also seen in this case.

Interestingly, metformin has been shown to inhibit FBP1 enzyme on hepatic gluconeogenesis, in addition to its known suppressive effect on the mitochondrial respiratory complex.⁴ This may be one of the pathomechanisms in metformin-associated lactic acidosis (MALA) in this regard. Nevertheless, more investigations are needed to carefully study whether FBP1 deficiency patients or carriers are more vulnerable to MALA.

Like many other IMD, the diagnosis of FBP1 deficiency relies on the positive identification of pathognomonic markers, such as glycerol and/or glycerol-3-phosphate (G3P). GC-MS is commonly used for the investigation of IMDs in

organic aciduria. However, both sample preparation and analysis will take a considerable time (days). In our experience, a 600 MHz NMR-based urinalysis will take <15 minutes. Sample preparation is simple, involving centrifugation and mixing of centrifuged urine supernatant with buffer solution. Another advantage of NMR is the ability to identify and quantitate hundreds of metabolites in a single analysis. An enlarged NMR spectrum of glycerol is included in Fig. 1 as an example in which its characteristic proton chemical shifts (expressed numerically in parts per million, ppm) and proton splitting pattern are shown. The proton chemical shift and the proton splitting pattern are the NMR fingerprint of a molecule. The concentration of the studied molecule can be quantitated according to its proton signal intensity relative to that of a known amount of internal standard (IS); for example, trimethylsilylpropanoic acid (TMSP). To date, the annotations and quantifications can be done automatically in a short time for a panel of metabolites without the need of high computational power.⁵

Compared with MS instruments, NMR instruments are not universally available and would require a higher cost upon initial establishment. Nevertheless, NMR is an emerging technology in laboratory medicine. Its clinical potential has been demonstrated in newborn screening, IMD, infection, pleural effusions and lipoprotein profiling.^{6–10}

An alternative approach for urine glycerol measurement is proposed using the lipoprotein lipase-glycerol kinase (GK) method for triglycerides measurement. Glycerol is an intermediate formed after triglycerides are hydrolysed. The glycerol formed in this process, together with any endogenous or exogenous glycerol in the sample, would be converted into G3P by GK followed by a further reaction to produce a coloured product via Trinder reaction. Without a glycerol blanking, measurement of urine triglycerides would

Fig. 2 Key metabolic flux in patients with fructose-1,6-bisphosphatase (FBP1) deficiency during hypoglycaemia or catabolic stress. Defects in FBP1 impair gluconeogenesis which can lead to hypoglycaemia (1) and with accumulations of gluconeogenic substrates, for example, glycerol (2), pyruvate (3) and lactate (4). Ketosis (5) is secondary to lipolysis from a breakdown of triglycerides (only the key metabolites are shown; consecutive arrows imply multiple steps).

reflect the glycerol and G3P concentrations. To verify this approach, we measured the amount of glycerol using urine samples spiked with glycerol of 0–20 mmol/L by a triglycerides assay in our laboratory (Roche Cobas 6000). We confirmed an excellent linear relationship between spiked glycerol (x) and measured glycerol (y) with $R^2 = 0.9996$ where $y = 1.228x + 0.027$. Using this method, the concentration of urine glycerol measured 44.2 mmol/mmol Cr (or 53 mmol/L) which is slightly lower than that measured by NMR spectroscopy, i.e., 47.5 mmol/mmol Cr.

This GK-based approach could provide a quick estimation of urine glycerol. However, the effect of G3P (one of the intermediates in the GK-based reaction) has not been taken into consideration. Indeed, elevated G3P has been described in a patient with FBP1 deficiency where the patient has a urinary glycerol of 23.8 mmol/mmol Cr and a urinary G3P of 0.38 mmol/mmol Cr.¹¹ It appears that the absolute level of G3P, although elevated, was still much lower (~50-fold) than that of glycerol. Retrospectively, we had re-analysed the urine sample using a non-extraction protocol for UOA, and an elevated G3P was seen in the UOA profile. Nevertheless, the peak intensity is much lower than that of glycerol (data not shown).

This approach assumes the patient has a negligible amount of urinary triglycerides, and it is usually the case in normal subjects unless the patient is affected by chyluria.¹² Through NMR analysis, the absence of lipoprotein particles had excluded obvious chyluria. For comparison, typical exudate and transudate pleural effusion NMR spectra were included in Fig. 1 which showed excess lipoprotein particles in exudates, but not in transudates or the patient's urine.

In conclusion, we envisage NMR-based urinalysis can streamline the diagnosis of IMD and is an emerging diagnostic tool in advanced laboratory medicine. The current case

report is one of the many examples in IMD in which an early diagnosis could prevent further metabolic complications. We propose the use of GK-based triglyceride assay as a quick approach for suspected FBP1 deficiency where an urgent organic acid analysis is not available.

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Estimating blood nitrate concentration from interference on radiometer ABL800 chloride electrode

To the Editor,

Nitrate species reportedly interfere with blood chloride measurement by ion selective electrodes (ISEs), which are commonly used to measure chloride in clinical laboratories. This phenomenon has been described with multiple instruments, including ABL700 and ABL800 (Radiometer Medical, Denmark),^{1,2} and Cobas Integra (Roche Diagnostics, USA).³ There is evidence that nitrate anions do not interfere with chloride measurements on Ci8200 Architect (Abbott Diagnostics, USA). The relationship between plasma nitrate concentration and changes in chloride readings is positive and linear. Plasma nitrate concentrations can become significant after ingestion of sodium nitrate or from the use of topical creams containing cerium nitrate.¹

Historically, nitrite (NO_2^-) and nitrate (NO_3^-) poisonings have been rare occurrences, with few published reports. However, in recent years a number of online sources have promoted sodium nitrite ingestion as a means of committing suicide, including Exit International. As a result, the number of presentations to emergency departments of nitrite poisonings (and nitrate poisonings) are expected to increase. One case report of nitrate ingestion describes a patient who presented with gastrointestinal symptoms and remained haemodynamically stable during his admission.⁴ Approximately 5% of ingested nitrate is converted to nitrite by flora of the alimentary canal.⁴ It is the absorbed nitrite species which are believed to be responsible for the life-threatening methaemoglobinaemia in cases of nitrate poisonings.⁴

Nitrate measurement is not routinely performed in clinical laboratories, however an estimation of a patient's blood nitrate could be made from discordance in chloride results between instruments that are susceptible to nitrate

interference (e.g., ABL800, Radiometer Medical) and those that are not (e.g., Alinity, Abbott Diagnostics). The disparity in susceptibility to nitrate interference can be explained by fundamental differences in electrode design. The Abbott instruments, including the Alinity, use a silver nitrate redox system to measure chloride, whereas the Radiometer instruments, including the ABL800, use an ion-selective polymer membrane.

Solid state silver will react with chloride anions to produce a measurable voltage, but will not react with nitrate because of its greater solubility. On the other hand, polymer membranes contain ion exchange molecules, often quaternary ammonium, which reside in channels of resin and could ferry nitrate as well as chloride from one side of the membrane to the other. Nitrate species have a high affinity for quaternary ammonium compounds due to their highly lipophilic nature. The aim of this study was to use a linear regression model to develop an equation that could be used in the acute care setting to estimate a patient's plasma nitrate concentration based on differences in chloride readings between Abbott and Radiometer instruments.

Nitrate-spiked patient samples were run by both Alinity and ABL800 methods. Three dilution series were produced, each comprising pooled plasma from a different random patient. Each dilution series consisted of five nitrate-spiked samples as well as a negative control. The volume of nitrate added to each sample was $\leq 1\%$. In detail, six 1 mL samples were aliquoted from the pooled plasma of each patient. A sodium nitrate master mix (6.4 mol/L) was produced by dissolving 5.44 g of sodium nitrate into a final volume of 10 mL of deionised water. The master mix was sequentially diluted 1:2 five times, and 10 μL of each dilution (except the original master mix) was added to a different pooled plasma aliquot. The final concentrations of nitrate in each dilution series were 2, 4, 8, 16 and 32 mmol/L.

The median concentration of nitrate (8 mmol/L) in each dilution series was selected by calculating a theoretical plasma nitrate concentration from an ingested dose of 10 g of sodium nitrate with an oral bioavailability of 100%⁵ and a volume of distribution of 0.3 L/kg body weight.⁶ Samples were run in triplicate on each instrument, and the mean result for each concentration of nitrate was calculated from the total results of all three pooled patient samples ($n=9$). For each concentration of nitrate, the mean Alinity chloride result was then subtracted from the mean ABL800 chloride result. Linear regression analysis was used to model the relationship between nitrate concentration and ABL800 chloride interference. Analysis of variance (ANOVA) was used to assess the chloride concentration by Alinity between samples of differing nitrate concentrations.

There were no significant differences in chloride concentration between samples of different nitrate concentrations (ANOVA, $p=0.990$) by the Alinity method. The chloride results exhibited a positive, dose-dependent relationship with nitrate concentration (Fig. 1) with the ABL800 method. The 32 mmol/L nitrate samples all produced chloride results greater than the upper limit of detection (350 mmol/L) and were thus omitted from further analysis. There was an increased variation in chloride readings between samples, with the highest included nitrate concentration (16 mmol/L), coefficient of variation (CV) of 14%, compared to CVs under 6% for lower concentrations of nitrate. For this reason, linear