A pioneer study on human 3-nitropropionic acid intoxication: Contributions from metabolomics

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Abstract
The neurotoxin 3-nitropropionic acid (3-NPA) is an inhibitor of succinate dehydrogenase, an enzyme participating both in the citric acid cycle and the mitochondrial respiratory chain. In human intoxications, it produces symptoms such as vomiting and stomach ache in mild cases, and dystonia, coma, and sometimes death in severe cases. We report the results from a liquid chromatography-Orbitrap mass spectrometry metabolomics study mapping the metabolic impacts of 3-NPA intoxication in plasma, urine, and cerebrospinal fluid (CSF) samples of a Norwegian boy initially suspected to suffer from a mitochondrial disease. In addition to the identification of 3-NPA, our findings included a large number of annotated/identified altered metabolites (80, 160, and 62 in plasma, urine, and CSF samples, respectively) belonging to different compound classes, for example, amino acids, fatty acids, and purines and pyrimidines. Our findings indicated protective mechanisms to attenuate the toxic effects of 3-NPA (e.g., decreased oleamide), occurrence of increased oxidative stress in the patient (such as increased free fatty acids and hypoxanthine) and energy turbulence caused by the intoxication (e.g., increased succinate). To our knowledge, this is the first case of 3-NPA intoxication reported in Norway and the first published metabolomics study of human 3-NPA intoxication worldwide. The unexpected identification of 3-NPA illustrates the importance for health care providers to consider intake-related intoxications during diagnostic evaluations, treatment and follow-up examinations for neurotoxicity and a wide range of metabolic derangements.

Keywords
3-nitropropionic acid, 3-NPA, beta-nitropropionate, bovinocidin, global metabolomics, LC-MS, metabolomics, 3-nitropropionate, β-Nitropropionic acid

1 | INTRODUCTION

3-Nitropropionic acid (3-NPA) is a neurotoxin (Wang et al., 2017) naturally produced by some fungi that infest plants and vegetables.
enzyme in the citric acid cycle and the mitochondrial respiratory chain, where it constitutes complex II (Alston et al., 1977; Brouillet et al., 2005; Coles et al., 1979; Liot et al., 2009; Scallet et al., 2001). Typically, patients with mild cases of intoxication vomit, feel nauseous and exhausted, and have diarrhea, stomach ache and headache, and most recover after a few days (Ming, 1995). Severe cases have more serious symptoms, usually developing dystonia and coma (Ming, 1995). No known antidote for 3-NPA intoxication is available (Ming, 1995; Xin Chen et al., 2018). The toxin is widely used in phenotypic animal models of Huntington’s disease (HD), as it produces neurological derangements and mitochondrial damage and alterations in rodents similar to that of patients with HD (Ayala-Peña, 2013; Brouillet et al., 2005; Cano et al., 2021; Wiprich et al., 2020). 3-NPA has also been used in a model of mitochondrial dysfunction and oxidative stress (Lahiani-Cohen et al., 2019).

Human intoxications have mostly been reported from China following ingestion of moldy sugarcanes infected by Arthrinium spp., producing 3-NPA in toxic amounts (Liu et al., 1992; Ming, 1995; Xin Chen et al., 2018). From its first report (1972), the cases roughly reached 884 in 17 years, with 88 fatalities (Liu et al., 1992). Outside of Asia, 3-NPA has been detected in trees, plants and fruit juice in, for example, New Zealand (MacAskill et al., 2015), Australia (Ossedryver et al., 2013), Serbia (Janić Hajnal et al., 2020), Nigeria (Ayeni et al., 2020) and North America (Anderson et al., 2005; Darby et al., 2020; Liu et al., 2017), and has been reported to cause intoxication in animals. Contaminated stale coconut water with 3-NPA was identified as the cause of death in a Danish man (Birkelund et al., 2021). Individual variation in susceptibility to 3-NPA toxicity may be due to differences in age, genetics, environment, epigenetics, and overall health (Aldridge et al., 2003). For example, only one of two children who consumed the same sugarcanne in 1995 manifested typical symptoms and clinical signs (Ming, 1995). The fact that different parts of a plant can be unequally affected by fungi and thereby have varying amounts of 3-NPA, may have contributed to this difference.

The research to date has tended to focus on biochemical changes and alterations in metabolite levels in exposed organisms using targeted approaches (Binienda & Kim, 1997; Olsen et al., 1999; Tadros et al., 2005). These hypothesis-testing procedures may provide analysis of a small number of specific metabolites, but fail to provide a more complete presentation of all the metabolic derangements (Kell & Oliver, 2004). This is particularly relevant in clinical settings when differences between two study groups (e.g., case and control) are only partly known or the cause of disease, and thereby the affected metabolic pathway(s) and metabolites, are unclear. Thus, untargeted approaches (so-called metabolomics, metabonomics, metabolic profiling, etc.) can better provide vital information to health care providers for diagnosis, prognosis, and the choice and efficacy of therapy (Ashrafian et al., 2020; Gowda et al., 2008). For regulatory toxicology and risk assessment, the utility of metabolomics has been discussed as a novel and efficient tool (Olesi et al., 2021).

Metabolomics is the study of low-molecular weight (typically <1500 Da) compounds present in a biological sample (Viant et al., 2017; Wishart, 2013). Liquid chromatography-mass spectrometry (LC-MS) is one of the most widely used analysis methods for metabolomics (Patti et al., 2012). Compared with nuclear magnetic resonance (NMR) spectroscopy, LC-MS provides more sensitive analyses (Allwood & Goodacre, 2010) as low-concentration compounds are more easily detected, especially when using high resolution, high sensitivity MS instruments (e.g., Orbitraps (Ghaste et al., 2016)).

So far, metabolic profiling has only been applied to three non-human models exposed to this neurotoxin (Chang et al., 2011; Novoselov et al., 2015; Tsang et al., 2009). Therefore, for the first time, we report the results from an LC-Orbitrap MS metabolomics study of a 14-year-old Norwegian boy severely intoxicated with 3-NPA.

2 | MATERIALS AND METHODS

2.1 | Chemicals

Water used was of type 1 (>18 MΩ cm), obtained from MilliQ ultrapure water purification system (Merck Millipore, Darmstadt, Germany). Methanol was purchased from Rathburn Chemicals (Walkerburn, Scotland). Formic acid (98%) and 3-NPA were from Merck Millipore.

2.2 | Patient, clinical details and ethics statement

The patient was a 14-year-old Caucasian boy with a previous history of unilateral iridocyclitis, eczema and bilateral Achilles tendinitis, and several years of mild abdominal pain and diarrhea. His abdominal symptoms had worsened for 4–5 months ahead of an acute illness episode with 24 h of progressive abdominal pain, vomiting and confusion. He was admitted to the emergency department with reduced consciousness and dystonic movements, but with stable respiration and circulation. Blood tests showed elevated lactate (14 mmol/L, reference range 0.5–2.2 mmol/L) and ammonia (97 μmol/L, reference range 10–50 μmol/L) and compensated metabolic acidosis with increased anion gap of 29.6 mmol/L. Cerebral and medullary MRI revealed symmetric acute infarctions in the putamen without other findings. The changes were suspicious of methanol intoxication, but no methanol was detected in his blood, and an organic acidemia or mitochondrialopathy was thus considered. Treatment with high caloric glucose infusion, biotin, thiamine, riboflavin, carnitine, antibiotics, and antiviral treatment was initiated. The lactic acidosis reversed and ammonia normalized within a few hours.

The subsequent days, his level of consciousness and dystonia were fluctuating, and periodically he could follow instructions with latency. On Day 4, he deteriorated to a comatose state. Cerebral MRI at this stage showed progression of T2 weighted high signal intensity and lactate peaks in the dorsal striatum, as well as lesions in the brainstem, consistent with Leigh syndrome. Mitochondriopathy remained a relevant diagnosis, but thiamine transporter two
deficiency and intoxication with metronidazole were also suggested. Cerebral MRI on Day 6 showed no additional progression. Clinically, he gradually recovered from Day 10. He developed axonal polyneuropathy, possibly a critical illness neuropathy. Four weeks after hospital admittance, he could start rehabilitation at a specialist hospital for physical medicine and rehabilitation. After four more weeks, he was discharged to his home. After several months of fatigability and minor residual symptoms from his neuropathy, the patient has since been stable (for more than 2 years); that is, no clinical neurological sequelae and no similar episodes of acute illness.

As one of the tentative diagnoses was a mitochondrial disease, his samples were analyzed at our routine diagnostics laboratory and our laboratory for inborn errors of metabolism. Except from compound heterozygosity for two mutations in POLG, both associated with valproate induced liver toxicity ([NM_002693.2] c.[3428A > G](;)[3708G > T]), no genetic defects were detected from neither exome analysis of a Mendelian gene panel (“mendeliome”) including genes relevant for known mitochondrial diseases, nor full sequencing of mitochondrial DNA. The decision to perform metabolomics was made on the basis of the results from the diagnostic analyses: deviations in the glycolysis pathway and citric acid cycle were found, giving strong indications of affection of complex II in the mitochondrial respiratory chain, in combination with the detection of an unknown compound in the GC–MS trace for organic acids in urine. Because an intoxication of some kind was also one of the initial tentative diagnoses, a full forensic toxicology screening of samples from the patient was also performed, but with no findings. Figure 1 gives an overview of the identification process performed in this study.

The Regional Ethics Committee assessed the project in line with Norwegian laws and legislations, and concluded in decision 108825/2020 that the project did not need their approval. The Data Protection Officer at Oslo University Hospital (GDPR art. 37) assessed the project (20/20905) and confirmed that the processing of personal data was in line with GDPR art. 6 (1) (a) and art. 9 (2) (a), since written informed consent from the patient, now aged 17 years, was obtained. The use of a urine sample from a healthy volunteer (written, informed consent) was approved by the Regional Committee for Medical and Health Research Ethics (case number: 173346).

2.3 | Samples, preparations and LC–MS analysis

Heparin plasma, urine and cerebrospinal fluid (CSF) samples were collected from the patient for analysis. Table 1 provides an overview of the specimens with sampling time points. Collected samples were stored at −20°C prior to preparation and analysis. A urine sample...
from a healthy volunteer (control) was taken right before analysis. For establishment of retention time, accurate mass and fragmentation pattern, an aqueous 3-NPA standard solution was analyzed using the same methods (described below) as the patient and control samples.

For peak area comparison, two plasma samples taken the same day (“acute phase” Day 2) were compared with two plasma samples taken 9 and 12 months later (“follow-up phase”), respectively. Regarding urine samples, one from the acute phase (Day 2) was compared with a sample taken 12 months later (follow-up phase). For CSF, a sample taken during the acute phase (Day 2) was compared with a sample taken two days (45.5 h) later (Day 4) (subacute phase, during the second round of deterioration), since no CSF sample from the acute phase (Day 2) was compared with a sample taken 12 months later (follow-up phase). The length of storage before analysis and the number of freeze–thaw cycles for each sample varied in this study. In short, samples were stored about 7, 10.5, or 19.5 months before metabolomics mapping and the number of freeze–thaw cycles was two for most samples.

All patient samples were thawed, vortex-mixed (Reax top vortex mixer, Heidolph [Schwabach, Germany]) and centrifuged (Megafuge 1.0R, Heraeus [Hanau, Germany]) for 10 min (3 600 RCF). Urine (patient and control) and CSF samples were filtered using a syringe filter (Millex-GV, Merck) before injection into the LC–MS. Plasma samples were mixed with cold methanol (4°C) (1:3 sample: methanol v:v), vortex-mixed, and centrifuged (Fresco 21 Microcentrifuge, Thermo Scientific [Waltham, MA, USA]) for 10 min (21 100 RCF) at 4°C, before injection of the supernatant into the LC–MS.

LC instrumentation used was Dionex Ultimate 3000 UHPLC system pump, column department, and autosampler from Thermo Scientific. The MS used was a Q Exactive Orbitrap (Thermo Scientific) with electrospray ionization. Full MS analysis was performed according to the procedure described in Skogvold et al. (2021) (including liquid chromatography and electrospray ionization parameters and settings). MS/MS analysis was performed with the same chromatographic and electrospray parameters and settings as described in Skogvold et al. (2021), and with the MS settings shown in Data S1 as additional experimental information.

### RESULTS

#### 3.1 Annotated, identified and altered metabolites

3-NPA was annotated in the patient urine sample from the acute phase using an untargeted analysis (metabolomics). This finding was verified by analysis of a 3-NPA reference standard, using the same metabolomics method, and supported by the acquired spectrum of the unknown peak in the GC–MS trace for organic acids. The level of metabolites detected in samples collected during three phases (Table 1) was compared to identify the metabolic changes. Tables S1–S3 illustrate for each material the annotated or identified metabolites with a ratio ≤0.50 or ≥2.0 between samples from two different clinical phases. The numbers of such comparison were as follows: 80 (plasma), 160 (urine), and 62 (CSF). In plasma, most of these metabolites belonged to the groups of amino acids and related compounds, fatty acids, and glycolysis and citric acid cycle. In urine and CSF, the majority belonged to the group of amino acids and related compounds, fatty acids, and glycolysis and citric acid cycle, and purines and pyrimidines and related compounds. Some compounds were altered in available phases for more than one sample material (Table 2). Among them, the six metabolites including 3-hydroxybutyrate, hypoxanthine, lactate, succinic anhydride, succinate and methylmalonate presented high ratio of acute as well as subacute to follow-up phase (i.e., 2.0–9.9 or ≥10) for all materials. However, the first three metabolites showed decreasing pattern (from ≥10 to 2.0–9.9) when considering subacute to follow-up phase. In contrast, theobromine had ≥10 times lower
TABLE 2  Metabolites with altered amounts between acute phase and subacute phase (a/S in plasma and CSF), acute phase and follow-up phase (a/F in plasma and urine) as well as subacute phase and follow-up phase (S/F in plasma) sam

| Metabolite                      | Plasma                      | Urine                   | CSF         |
|--------------------------------|-----------------------------|-------------------------|-------------|
|                                | A/F | A/S | S/F | A/F | A/S | |
| 2-Hydroxyvaleric acid          | ↑   |     |     | ↑   |     | |
| 3-Hydroxybutyrate              | ↑↑  | ↑   |     | ↑   |     | ↑↑ |
| 4-Acetamidobutyric acid        |     |     |     |     |     | ↓ |
| 6-Hydroxyxcaproic acid         | ↑   |     |     | ↑↑  |     | ↑ |
| Acetylcarnitine                | ↑   |     |     | ↑   |     | |
| Acetoacetate                   |     |     |     | ↑↑  |     | ↑ |
| Alanine                        |     |     |     | ↑↑  |     | ↓ |
| Alpha-glycerylphosphorylcholine|     |     |     | ↑↑  |     | |
| Aminolevulinic acid            |     |     |     | ↑    |     | |
| Asparagine                     |     |     |     | ↑↑  |     | |
| Citrate                        |     |     |     | ↑    |     | |
| Cis-4-hydroxy-proline          |     |     |     | ↑   |     | ↑ |
| Dihydrothymine                 |     |     |     | ↑↑  |     | ↓ |
| Fumarate                       | ↑   |     |     | ↑   |     | |
| Glucose                        |     |     |     | ↑   |     | |
| Glucose-6-phosphate            |     |     |     | ↑   |     | |
| Glutamate                      | ↑   |     |     | ↑   |     | |
| Glutamine                      | ↓   |     |     | ↑   |     | |
| Glutaric acid                  |     |     |     | ↑   |     | |
| Hydroxyphenyllactic acid       | ↑   |     |     | ↑   |     | |
| Hypoxanthine                   | ↑↑  | ↑   |     | ↑   |     | ↑ |
| Indole-3-lactic acid           | ↑   |     |     | ↑   |     | |
| Kynurenine                     | ↑   |     |     | ↑   |     | |
| Lactate                        | ↑   |     |     | ↑   |     | |
| Leucine and isoleucine\(a\)    |     |     |     | ↑↑  |     | ↑ |
| Malate                         | ↑   |     |     | ↑   |     | |
| Methionine                     |     |     |     | ↑   |     | |
| N-acetylaspartate              |     |     |     | ↑   |     | |
| N-acetylleucine                |     |     |     | ↑   |     | |
| N-acetylvaline                 |     |     |     | ↑   |     | |
| N-phenylacetylglutamine        |     |     |     | ↑   |     | |
| Proline                        | ↑   |     |     | ↑   |     | |
| Propionate                     | ↑↑  | ↑   |     | ↑   |     | |
| Propionylcarnitine             | ↑   |     |     | ↑   |     | |
| Pyruvate                       |     |     |     | ↑   |     | |
| Succinate and methylmalonate\(b\) | ↑↑  | ↑   |     | ↑   |     | ↑ |
| Succinic anhydride             | ↑↑  | ↑   |     | ↑   |     | ↑ |
| Taurine                        | ↑   |     |     | ↑   |     | |
| Theobromine                    | ↑↑  |     |     | ↑   |     | |
| Xanthine                       | ↑   |     |     | ↑   |     | |

Note: Symbol explanation: ↓: ratio 2.0–9.9, ↑↑: ≥10, ↓↓: 0.10–0.50, ↑↑: ≤0.10.

Abbreviation: CSF, cerebrospinal fluid.

\(a\)Not chromatographically separated: leucine and isoleucine,

\(b\)Not chromatographically separated: succinate and methylmalonate.
concentration in plasma and urine samples from the acute and subacute phases relative to the follow-up phase.

As shown in Figure 2, several compounds in the glycolysis pathway and citric acid cycle were altered in amounts in the acute phase compared with the subacute phase in CSF samples and with the follow-up phase in both plasma and urine samples. Figure 3 depicts how the amount of 3-NPA rapidly decreased (86% decrease in peak area) from the first to the second plasma sample obtained as little as 10 h later during the acute phase (see Table 1 for sample ID). In the third sample, 35 h later (and in the following three samples), 3-NPA was no longer detectable. This indicates an intoxication in which the toxin is rapidly degraded and eliminated while the metabolic and toxic effects and damages linger on for a long time before biochemical and clinical recovery and normalization can occur. A similar pattern of rapidly decreasing amounts as that of 3-NPA was observed for, for example, propionate and lactate in plasma samples (Figure 3).

### 3.2 Source of 3-NPA

A thorough investigation was done to find the source of 3-NPA intoxication, including interviews with the patient and family and observations in the home and surrounding neighborhoods. There were no positive results with respect to identification of the origin of the toxin. The family lives in a relatively modern house and did not report any problems with moisture, and so forth. The neighborhood is typically suburban, with relatively modern detached houses. The family lives a typical active Norwegian life. Neither the environment nor the activities of the family gave any clues to an exposure to 3-NPA. The patient had not traveled outside Norway but had stayed at home. The patient’s diet was found to be normal and similar to that of his family with no exotic ingredients or food imported from China or other Eastern regions. The family mostly bought their food in the local food store, with one exception: they frequently bought a type of flour in Denmark, but there was none left in the house or shop to analyze. The patient took a nutritional supplement during the last months before he became ill. This supplement was analyzed with our metabolomics platform and there was no 3-NPA detected.

### 4 DISCUSSION

This study mapped the metabolic impacts of 3-NPA intoxication in plasma, urine and CSF samples of a single patient. Prior to our analyses, a full forensic toxicology screening of collected samples during the acute phase revealed no indication of any exogenous toxin. This
illustrates how untargeted approaches enable unexpected findings when targeted ones with a pre-defined list of analytes fail to detect relevant metabolites that are not included in the panel.

4.1 Comparative metabolomics studies of 3-NPA intoxication

To our knowledge, no other publications on metabolomics of 3-NPA toxicity in humans exist to compare our results with, and available reports on biochemical alterations are very few and limited. Reported analysis of blood, urine, and CSF specimens generally fell within the normal range (Liu et al., 1992; Ming, 1995; Xin Chen et al., 2018). One exception (Birkelund et al., 2021) highlighted metabolic acidosis along with elevated lactate, 3-hydroxybutyrate (as we, too, observed in plasma) and glucose in blood. An alternative source for comparison is patients with complex II deficiency, but our literature search resulted in no metabolomics research similar to ours.

Regarding non-human models, only three papers (Chang et al., 2011; Novoselov et al., 2015; Tsang et al., 2009) described changes in the metabolome upon exposure to 3-NPA. In treated larvae, Novoselov et al., (2015) identified 3-NPA amides of glycine, serine, threonine and alanine as important detoxification pathways. These four amino acid amides were absent in our samples. In metabolic profiling of exposed rats, Chang et al. (2011) found 16 and 3 alterations in brain and plasma samples, respectively. Increased succinate and decreased oleamide in the brains of the study group versus controls corroborated our observation in CSF samples of the acute phase vs. the subacute phase. Inversely, the increased amount of glycerol, methionine and citrate in our CSF opposed what they reported for murine brain. Another rat study focusing on the brain of treated animals was conducted by Tsang et al. (Tsang et al., 2009). The authors found 14 altered metabolites in neural tissues concomitant with modified methylene lipids and methyl lipids. In line with their findings, we also recorded increased amounts of succinate and glycerol in the CSF acute phase sample compared with the subacute phase sample. For lactate, Tsang et al. (Tsang et al., 2009) pointed out the increased amounts in the frontal cortex, dorsal striatum and frontal cortex aqueous extracts, while decreased amounts in the cerebellum. Cerebral MRI performed in the subacute phase included spectroscopy (MRS), and showed lactate peaks in the putamina and the dentate nuclei, and reduced levels of N-acetylaspartate in the putamina. We measured an increase of lactate in the CSF acute phase sample vs. the subacute phase sample. Concerning N-acetylaspartate and N-acetylaspartylglutamate, Tsang et al. (2009) observed decreased amounts, while we observed increased amounts in CSF.

The few discrepancies between our results and the three above-mentioned studies might have been caused by differences in sample material, analytical methods (e.g., GC-TOF MS and NMR vs. LC-Orbitrap MS) or biochemical pathways among species. Additionally, our second time point of CSF sampling, during the subacute phase, is not representative of the controls of the three animal studies, since our patient was still gravely ill at the time of the second CSF sampling. Thus, some biochemical alterations were not yet normalized at this time-point and others might even be more profound a few days after the intoxication took place, as some pathways and metabolites could be affected by changes induced by the damages from intoxication and the following downstream effects. As such, the biochemical alterations in our subacute phase CSF sample portrayed the pathophysiological state at a later stage than the acute inhibition and damage of complex II/the glycolysis pathway and citric acid cycle.
4.2 | Protective responses to 3-NPA intoxication

The level of 3-NPA rapidly decreased from plasma sample 1 to 2, indicating a quick excretion and/or metabolism/detoxification of the toxin. During and after this process, some protective mechanisms are naturally coupled to attenuate the toxic effects of 3-NPA. For example, 3-hydroxybutyrate was demonstrated to be neuroprotective in a 3-NPA HD mouse model, likely due to its bioenergetic effects (Lim et al., 2011). In the present study, 3-hydroxybutyrate was increased in all acute phase samples compared with the subacute phase (CSF) and the follow up-phase (plasma and urine). We also observed decreased CO-carnitine in the acute phase CSF sample versus the subacute phase sample. This might imply increased carnitine consumption due to compensatory mechanisms to maintain normal mitochondrial metabolism. The higher carnitine level in the subacute phase sample may represent a partial biochemical normalization, but is probably also explained by carnitine supplementation in-between the two sample time-points. In a plasma sample taken day two of the acute phase, prior to the carnitine treatment, the level of CO-carnitine was reported to be in the lower area of the reference range (13 μmol/L, reference range 10–49 μmol/L). In rat models, pre-treatment with carnitine provided a protective effect through enhanced mitochondrial metabolism of long chain free fatty acids, thus preventing 3-NPA induced inhibition of mitochondrial function and the resulting brain temperature decrease (Binienda, 2003), reduction of mortality and neuronal degeneration (Binienda et al., 2004), as well as protection against oxidative stress (Binienda & Ali, 2001) induced by 3-NPA. Oleamide, another protective metabolite, was also decreased in CSF of our patient, similar to the metabolome of treated rats by Chang et al. (2011). Oleamide is a cannabinoid receptor 1 agonist (Kruk-Slomka et al., 2016) and such agonists can have a neuroprotective role through stimulation of the receptor, as examined by Maya-López et al. (2017). In their experiment, pre-treatment of rats with a synthetic cannabinoid receptor agonist prior to 3-NPA treatment protected against dysfunctional mitochondria and oxidative damage (Maya-López et al., 2017).

Oxidative stress plays an essential role in the pathogenesis of many diseases, and it occurs when there is an imbalance between the generation of reactive oxygen (and nitrogen) species (ROS) and the antioxidant defense systems (Halliwell & Gutteridge, 2015). The administration of 3-NPA gave rise to formation of ROS (Olsen et al., 1999), contributing to 3-NPA’s neurotoxicity. Increased amounts of total free fatty acids was seen in a rat model investigating different brain regions after 3-NPA injection (Binienda & Kim, 1997). Elevated free fatty acids in plasma were shown to be associated with increased amounts of free radicals in plasma (Paolillo et al., 1996). The current study found free fatty acids such as oleic acid, linoleic acid, and arachidonic acid increased in acute phase plasma samples, which could indicate increased oxidative stress in our patient. Moreover, taurine was increased in plasma and decreased in urine when comparing acute phase versus follow-up phase samples. The antioxidant effect of taurine was reported in pre-treated rats before 3-NPA exposure (Tadros et al., 2005). The occurrence of oxidative stress was further supported in our patient by increased amounts of hypoxanthine in all acute phase sample materials versus samples from the subacute (CSF) and follow-up (plasma and urine) phases. Elevated hypoxanthine is an indicator of hypoxia (Saugstad, 1988), and 3-NPA has been shown to cause histotoxic hypoxia (Hamilton & Gould, 1987). Hypoxia is known to result in oxidative stress by generating ROS (Abramov et al., 2007; Hernansanz-Agustín et al., 2014; Pialoux & Mounier, 2012).

4.3 | Energy turbulence caused by 3-NPA intoxication

Complex II inhibition, where 3-NPA directly targets, is biochemically characterized by grossly elevated succinate, often in combination with accumulation of other citric acid cycle metabolites (Bourgeois et al., 1992; Zieliński et al., 2016). Hence, a large amount of this dicarboxylic acid was detected in all our sample materials taken during the acute phase. In addition to the presence of or elevated succinate in urine and brain (Brockmann et al., 2002; Rustin et al., 1997), respectively, high levels of lactate in plasma (Birch-Machin et al., 1996) have been reported in patients with complex II deficiency. In compliance with this, we found increased amounts of lactate in plasma when comparing samples taken during the acute phase with the follow-up phase. Interestingly, there were also decreased amounts of acetate in urine comparing the acute phase with the follow-up phase. In a study administering 3-NPA to neurons and astrocytes under perinatal conditions (Tovar-Franco & Criollo-Ray, 2009), lactate and acetate were shown to enable neurons and astrocytes to sustain energy metabolism despite the inhibition of complex II. Therefore, the decreased amounts of acetate in our patient could indicate its consumption in an effort to maintain energy metabolism. It is also possible that some of the excess amount of lactate was used for this purpose.

There was a lower level of pyruvate in plasma samples from our patient in the acute phase compared with the follow-up phase. However, pyruvate was higher in the acute phase urine sample. Several scenarios of compensation mechanisms for complex II inhibition was previously described by Zieliński et al. (2016) using a computer model of a human cardiomyocyte. One case involved the pentose phosphate pathway in which the formation of pyruvate was minimized. Additionally, they found that increased availability of glutamate, arginine, proline, valine, aspartate, and glutamine had a large (positive) effect on ATP production during complex II inhibition. One explanation was accelerated amino acid degradation for NADH production. Despite differences in sample material and analytical methods, our results showed higher levels of some amino acids in plasma (glutamate and proline) or urine (glutamate, valine, and glutamine), and lower levels in plasma (glutamine and arginine) or urine (proline and aspartate), when the acute phase was compared with the follow-up phase. In a human body experiencing extreme stress like our severely ill patient, several factors (e.g., catabolism and ketosis) take part. It is difficult to assess the widespread effects on different metabolic pathways affected and thereby the timeline of changes in the metabolites found to be altered in this study.
Malate has been shown to have a positive effect on ATP production in a model of a human heart mitochondrion investigating succinate dehydrogenase deficiency (among other disorders) (Smith & Robinson, 2011). Based on our results, malate was increased in plasma and urine. It could be that we detected larger amounts of malate because of the very large amounts of succinate, which, at the time of sampling during the acute phase, had begun to transform to fumarate and malate as the 3-NPA inhibition of succinate dehydrogenase was easing. Most likely, the patient had residual and recovering complex II activity, and fumarate and malate would therefore be increased due to the large amount of their substrate and precursor succinate.

4.4 | Biphasic onset, data accuracy and limitations

Our patient went through two periods of clinical deterioration and severe illness. The initial acute phase occurred on Days 1 and 2 of the episode, after which the patient had slight clinical improvement. Then, on Day 4, the patient surprisingly presented new signs of clinical deterioration requiring a new CSF sampling (hence, the “subacute phase CSF sample”) and an MRI investigation that revealed progression of the cerebral damage. Comparably, a study investigating mitochondria in 3-NPA treated cortical neurons (Liot et al., 2009) reported a similar unexpected biphasic rise in reactive oxygen species, leading to cell death. The mechanism for this possible biphasic clinical and biochemical course is unknown. To our understanding, previous reports of the numerous moldy sugarcane 3-NPA intoxications in China have not mentioned such a biphasic clinical course.

Repeatability using the same LC-MS method as in this study was mapped for a wide range of metabolites in our recent study (Skogvold et al., 2021), with retention time and peak area relative standard deviations of 0.1%–0.4% and 2%–10%, respectively. As such, we are confident that the highlighted differences in amounts of the metabolites reported in this work are not due to technical/instrumental variation, but represent real, biological differences at the time of sampling.

Compared with other 3-NPA studies, the list of altered metabolites in all three sample materials of ours was more comprehensive (Chang et al., 2011; Novoselov et al., 2015; Tsang et al., 2009). Having an individual patient as the sole unit of observation for investigating the changes in the metabolome during different clinical phases, limits the statistical strength. However, the fact that the patient was his own control can also be considered a strength since it neutralizes inter-individual differences that would otherwise be found even in the best of matched controls and that would have introduced random differences and statistical noise.

Further research should map the global metabolic alterations in both complex II deficiency and HD patients compared with controls to investigate the compliance with findings reported in this study. That will also increase the knowledge of the biochemical impacts of and possible treatment strategies for the two diseases.

5 | CONCLUSION

This is the first case of 3-NPA intoxication reported in Norway, and the first report of a full metabolomics mapping of human 3-NPA intoxication worldwide. Following the unexpected identification of 3-NPA in the urine of an acutely, seriously ill patient suspect of intoxication or mitochondrial disease, we performed metabolomics LC-Orbitrap MS studies of plasma, urine, and CSF samples to compare levels of metabolites during the acute phase and in the subacute phase with a second clinical deterioration (CSF) and in the long-term follow-up phase (plasma and urine). Our results revealed many changes not previously published. This is one of only a handful of cases of 3-NPA intoxications reported worldwide outside of China. The unexpected identification of 3-NPA illustrates the importance for health care providers to consider intake-related intoxications during diagnostic evaluations, treatment and follow-up examinations for neurotoxicity and a wide range of metabolic derangements. As 3-NPA has been found to cause intoxications in both Norway and Denmark, the possibility of including 3-NPA in forensic toxicology screenings in Europe should be considered.

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AUTHOR CONTRIBUTIONS

Conceptualization was performed by H. B. S., M. Y., H. R., and K. B. P. E. Methodology was performed by H. B. S., E. M. S., A. Ø., K. B. P. E. Formal analysis was performed by H. B. S., E. M. S., A. Ø., and K. B. P. E. Investigation was performed by H. B. S., E. M. S., A. Ø., E. K., D. H., and K. B. P. E. Resources were gathered by K. B. P. E. Writing of the original draft preparation was performed by H. B. S. and M. Y. Writing review was performed by E. M. S., A. Ø., E. K., D. H., H. R., and K. B. P. E. Visualization was performed by H. B. S., M. Y., E. M. S. Supervision was performed by M. Y., H. R., K. B. P. E. Project administration was carried out by H. R. and K. B. P. E.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.
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