# Original Article

# Metabolic Tools for Identification of New Mutations of Enzymes Engaged in Purine Synthesis Leading to Neurological Impairment

*(de novo* purine synthesis / aminoimidazole ribotide / 5-formamidoimidazole-4-carboxamide ribotide / PAICS / PFAS)

M. KRIJT, O. SOUCKOVA, V. BARESOVA, V. SKOPOVA, M. ZIKANOVA

Research Unit for Rare Diseases, Department of Paediatrics and Adolescent Medicine, First Faculty of Medicine, Charles University and General University Hospital, Prague, Czech Republic

**Abstract. The cellular pool of purines is maintained by *de novo* purine synthesis (DNPS), recycling and degradation. Mutations in genes encoding DNPS enzymes cause their substrates to accumulate, which has detrimental effects on cellular division and organism development, potentially leading to neurological impairments. Unspecified neurological symptoms observed in many patients could not be elucidated even by modern techniques. It is presumable that some of these problems are induced by dysfunctions in DNPS enzymes. Therefore, we determined the concentrations of dephosphorylated DNPS intermediates by LC-MS/MS as markers of yet unpublished mutations in *PFAS* and *PAICS* genes connected with dysfunctions of carboxylase/phosphoribosylaminoimidazolesuccinocarboxamide synthase (PAICS) or phosphoribosylformylglycinamidine synthase (PFAS). We determined the criteria for normal values of metabolites and investigated 1,447 samples of urine and 365 dried blood spots of patients suffering from various forms of neurological impairment. We detected slightly elevated aminoimidazole riboside (AIr) concentrations in three urine samples and a highly elevated 5-formamidoimidazole-4-carboxamide riboside (FGAr) concentration in one urine sample. The accumulation of AIr or FGAr in body fluids can indicate PAICS or PFAS deficiency, respectively, which would be new disorders of DNPS caused by mutations in the appropriate genes. Measurement of DNPS intermediates in patients with neurological symptoms can uncover the cause of serious cellular and functional impairments that are otherwise inaccessible to detection. Further genetic and molecular analysis of these patients should establish the causal mutations for prenatal diagnosis, genetic consultation, and reinforce the DNPS pathway as a therapeutic target.**

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Corresponding author: Marie Zikanova, Department of Paediatrics and Adolescent Medicine, First Faculty of Medicine, Charles University and General University Hospital, Prague, Czech Republic, Ke Karlovu 2, 121 08 Prague 2, Czech Republic, E-mail: marie.zikanova@lf1.cuni.cz

Abbreviations: ADSL – adenylosuccinate lyase, AICAR/r – aminoimidazole carboxyamide ribotide/riboside, AIR/r – aminoimidazole ribotide/riboside, ATIC – 5-aminoimidazole-4-carboxamide ribonucleotide transformylase/inosine monophosphate cyclohydrolase, CAIR/r – carboxyaminoimidazole ribotide/riboside, CSF – cerebrospinal fluid, DBS – dry blood spot, DNPS – *de novo* purine synthesis, FAICAR/r – 5-formamidoimidazole-4-carboxamide ribotide/riboside, FGAM – formylglycineamidine ribotide, FGAR/r – formylglycineamide ribotide/riboside, GAR/r – glycineamide ribotide/riboside, GART – glycinamide ribonucleotide synthetase/aminoimidazole ribonucleotide synthetase/glycinamide ribonucleotide transformylase, HPLC – high-performance liquid chromatography, IMP – inosine monophosphate, LC-MS/MS – liquid chromatography tandem mass spectrometry, PAICS – phosphoribosylaminoimidazole carboxylase/ phosphoribosylaminoimidazolesuccinocarboxamide synthase, PFAS – phosphoribosylformylglycinamidine synthase, PPAT – amidophosphoribosyltransferase, SAdo – succinyladenosine, SAICAR/r – succinylaminoimidazolecarboxamide ribotide/riboside

## Introduction

Purines are essential molecules for nucleic acid synthesis and universal carriers of chemical energy in all dividing cells (Yin et al., 2018). The cellular metabolism of purines is managed by *de novo* purine synthesis (DNPS), salvage pathways and purine degradation. DNPS is one of the metabolic pathways crucial to cell division and to neural development. Mutations in genes encoding DNPS enzymes may lead to congenital neurological disorders. DNPS includes 10 reactions, in which six enzymes are involved, with three of them being multifunctional (Fig. 1). DNPS enzymes are organized into

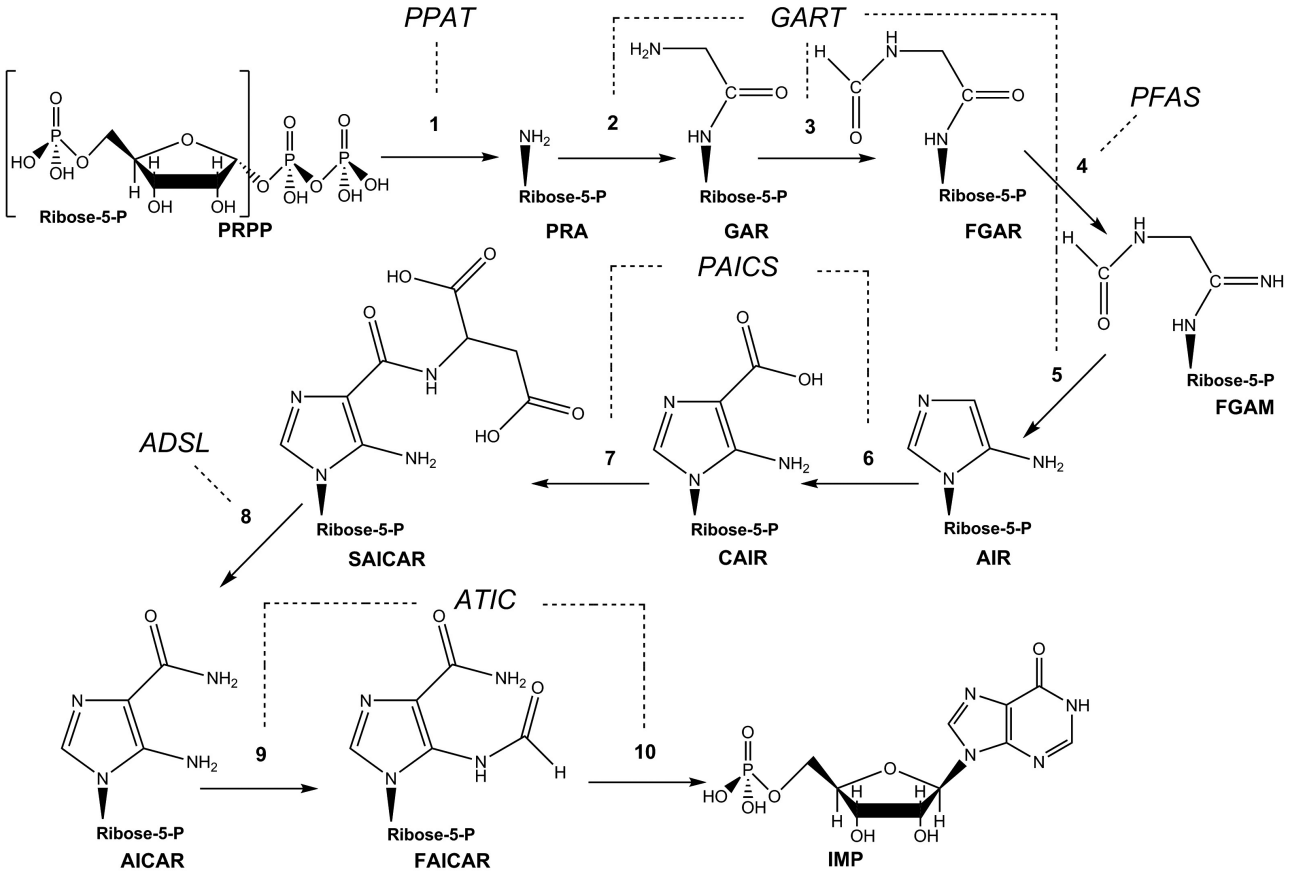


*Fig. 1.* Scheme of *de novo* purine synthesis (DNPS)
Phosphoribosylpyrophosphate (PRPP) substrate is metabolized by enzyme amidophosphoribosyl transferase (PPAT) to phosphoribosylamine (PRA) in the step 1. Steps 2, 3 and 5 are catalysed by trifunctional enzyme glycinamide ribonucleotide synthetase/aminoimidazole ribonucleotide synthetase/glycinamide ribonucleotide transformylase (GART). In step **2**, the whole molecule of glycine is connected to the amine group of PRA, resulting into glycineamide ribotide (GAR). The $N^{10}$-formyl tetrahydrofolate provides the formyl group to produce formylglycineamide ribotide (FGAR) during step **3**. In step **4**, the enzyme phosphoribosylformylglycinamidine synthase (PFAS) exchanges the oxo group of FGAR for the imino group provided by glutamine in order to create formylglycineamidine ribotide (FGAM). Step **5** leads to cyclization of FGAM into aminoimidazole ribotide (AIR). Steps **6** and **7** are catalysed by bifunctional enzyme phosphoribosylaminoimidazole carboxylase/ phosphoribosylaminoimidazolesuccinocarboxamide synthase (PAICS), which produces carboxyaminoimidazole ribotide (CAIR) and succinylaminoimidazolecarboxyamide ribotide (SAICAR), respectively. The bifunctional enzyme adenylosuccinate lyase (ADSL) catalysis occurs in step **8**, when the cleavage of fumarate from SAICAR occurs and forms aminoimidazolecarboxamide ribotide (AICAR). Steps **9** and **10** are catalysed by bifunctional enzyme 5-aminoimidazole-4-carboxamide ribonucleotide transformylase/inosine monophosphate cyclohydrolase (ATIC), which forms 5-formamidoimidazole-4-carboxamide ribotide (FAICAR) and inosine monophosphate (IMP), respectively.

a multienzyme complex called purinosome (An et al., 2008; Baresova et al., 2012). To date, two genetic defects in DNPS have been described: adenylosuccinate lyase (ADSL) deficiency (OMIM 103050) (Van den Berghe and Jaeken, 1986) and AICA-ribosiduria (OMIM 608688) (Marie et al., 2004). Both of these deficiencies result in serious damage of cellular homeostasis and functions manifested as neurological symptoms present at birth, causing accumulation of substrate(s) for the defected enzyme in cellular models or body fluids such as urine, plasma and/or cerebrospinal fluid (CSF) (Van den Bergh et al., 1991; Baresova et al., 2016).

The ADSL enzyme (ADSL, EC 4.3.2.2) catalyses the eighth reaction of DNPS (Fig. 1) and the second reaction of the purine nucleotide cycle. Mutations in this enzyme lead to ADSL deficiency (Kmoch et al., 2000). The cellular impairment clinically manifests in various disabilities classified as three basic types: (1) a neonatal form consisting of prenatal hyperkinesia, pulmonary hypoplasia and prenatal abortion of growth, followed by fatal neonatal encephalopathy and early death (Mouchegh et al., 2007); (2) a severe infantile form associated with severe psychomotor retardation and frequently with early death (Jaeken et al., 1988); (3) a mild/moderate form associated with less severe psychomotor retardation, hypotony and autism (Jaeken et al., 1988). The biochemical diagnostics of this condition is based on the detection of two dephosphorylated substrates of ADSL

– succinyladenosine (SAdo) and succinylaminoimidazolecarboxamide riboside (SAICAr). These substrates can be measured in the urine, plasma and/or CSF (Van den Bergh et al., 1991; Krijt et al., 2013). The genetic diagnosis of this condition is made through the detection of mutations in the *ADSL* gene (Jurecka et al., 2015).

AICA-ribosiduria is a recently identified autosomal recessive inherited disorder of DNPS (Marie et al., 2004). The cause of this condition is deficiency of bifunctional enzyme ATIC-5-aminoimidazole-4-carboxamide ribonucleotide transformylase (EC 2.1.2.3)/inosine monophosphate cyclohydrolase (EC 3.5.4.10), which catalyses the last two reactions of DNPS (Fig. 1). Similarly to patients with ADSL deficiency, the dephosphorylated substrate of the defective ATIC enzyme, aminoimidazolecarboxamide riboside (AICAr), accumulates in body fluids. SAICAr and SAdo accumulate as well, but at a lower level than in ADSL deficiency. The main pathogenic mechanism of ADSL and ATIC deficiencies has been attributed to the cytotoxicity of the accumulated dephosphorylated substrates (Stone et al., 1998; Marie et al., 2004; Jurecka et al., 2015).

To date, no other genetically determined defects of the DNPS pathway have been identified. However, the existence of such defects is highly probable. It is hypothesized that these defects will similarly manifest as nonspecific neurological symptoms accompanied by accumulation of DNPS intermediates in body fluids. Based on this hypothesis, we previously prepared and characterized DNPS deficiencies in a HeLa cell model system (Baresova et al., 2016; Madrova et al., 2018). Accumulation of dephosphorylated substrate(s) of the defective enzyme occurred in four DNPS knock-out cell lines, with the exception of cells deficient in trifunctional enzyme GART – glycinamide ribonucleotide synthetase (EC 6.3.4.13)/aminoimidazole ribonucleotide synthetase (EC 6.3.3.1)/glycinamide ribonucleotide transformylase (EC 2.1.2.2).

The main problem in developing analytical methods for detection of DNPS defects is the lack of commercial availability of DNPS intermediates. In our previous work, we developed procedures for preparation of phosphorylated and dephosphorylated DNPS substrates, which we characterized and detected by LC-MS/MS (Madrova et al., 2018).

In the presented work, we established physiological levels of DNPS metabolites 5-formamidoimidazole-4-carboxamide riboside (FGAr), aminoimidazole riboside (AIr) and carboxyaminoimidazole riboside (CAIr) in 40 control urine samples and in 50 control dried blood spots (DBS), and detected these metabolites in 1,447 urine samples and in 365 DBS of patients with various undiagnosed neurological defects. FGAr can serve as a marker for mutations in *PFAS*, which encodes phosphoribosylformylglycinamidine synthase (PFAS, EC 6.3.5.3) – for the fourth reaction of DNPS (Fig. 1). AIr and CAIr are markers for mutations of *PAICS*, which encodes the phosphoribosylaminoimidazole carboxylase (EC 4.1.1.21)/phosphoribosylaminoimidazolesuccinocarboxamide synthase (EC 6.3.2.6) (PAICS) bifunctional enzyme – for the sixth and seventh reactions of DNPS (Fig. 1). We hypothesized that the cytotoxicity of these markers would lead to neurological impairment similar to ADSL and AICA-ribosiduria deficiencies. Recently, a study of PFAS and PAICS expression in the rat brain and hippocampal neurons suggests the possibility of direct influence on neuronal functions caused by the cytotoxicity of accumulated substrates due to DNPS enzymatic deficiencies (Williamson et al., 2017).

## Material and Methods

### *Chemicals*

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated in the text.

### *Preparation of DNPS intermediates*

DNPS substrates FGAr, AIr, CAIr and SAdo-$^{13}C_4$ were synthesized utilizing human and bacterial recombinant enzymes as described previously (Zikanova et al., 2005; Baresova et al., 2016; Madrova et al., 2018).

Briefly, enzymes catalysing each individual step of DNPS were prepared and used for synthesis of DNPS intermediates. Ribotidic forms of DNPS substrates obtained in enzymatic reactions were dephosphorylated in potassium phosphate buffer, pH 8.0 with calf intestine phosphatase (CIP) (New England Biolabs, Ipswich, MA) to ribosidic forms.

### *Preparation of samples*

Control and neurologically impaired samples were anonymized prior to analysis.

### *Urine samples*

Urine creatinine measurements were performed in a standard manner. All samples were adjusted to a creatinine concentration of 1 mmol/l and frozen. Prior to the analysis, the defrosted samples were spun down for 60 s in a mini centrifuge in order to remove any residual proteins. The supernatant, with a volume of minimum 30 µl to maximum 200 µl, was then pipetted into the insert and embedded into an HPLC vial for the purpose of LC-MS/MS analyses.

### *Dried blood spots*

From each DBS sample, three 3-mm diameter disks were punched from a Guthrie card and placed in 100 µl of extraction buffer containing acetonitrile : methanol : water (1 : 1 : 1 ratio) with 100 nmol/l SAdo-$^{13}C_4$ internal standard. Samples were incubated for 15 min in an ultrasonic bath. An 80 µl aliquot of the extract was transferred to a clean tube and centrifuged for 5 min at 8,000 *g*. The supernatant was evaporated to dryness under a stream of nitrogen, dissolved in 30 µl of LC-MS water and embedded into an HPLC vial for the purpose of LC-MS/MS analyses.

*HPLC-MS/MS analysis*

The Agilent 1290 Infinity LC System (Agilent Technologies, Palo Alto, CA) coupled with an API 4000 triple quadrupole mass spectrometer operated with Analyst software version 1.4 (Applied Biosystems, Foster City, CA) was applied for identification and quantification of all DNPS substrates. The separation was performed with Prontosil 120 – 3 C18 – AQ column (200*4 mm, 3 μm) (Bischoff Chromatography, Leonberg, Germany) at 30 °C. The gradient elution consisted of 0.1% formic acid solution in water (mobile phase A) and 0.1% formic acid solution in acetonitrile (mobile phase B). The flow rate of 400 μl/min was performed by changing % B as follows: 0.0–12.0 min: 0 % to 20 %, 12.0–13.0 min: 20 % to 60 %, 13.0–15.5 min: 60 %, 15.5–16.0 min: 60 to 0 %. The column was then regenerated with 100 % A for 9 min, with an increased flow rate 700 μl/min between 16.2 and 24.2 min. The injection sample volume was 5 μl. The parameters of MS detection were set as previously (Baresova et al., 2016).

## Results

The aim of our work was to study the flux of DNPS intermediates under physiological conditions and its alteration under pathological conditions designated by neurological symptoms. The levels of DNPS intermediates FGAr, AIr and CAIr were determined by LC-MS/MS in the urine and DBS in patients with undiagnosed neurological impairment.

Linearity and limits of detection (LOD) and quantification (LOQ) were defined using signal-to-noise ratios of 3 : 1 and 10 : 1, respectively. We confirmed the linearity of the method by generating linear calibration curves with high correlation coefficients ($r^2 > 0.9911$) in the indicated concentration range (Table 1).

Reference values of metabolites in the urine and DBS were determined by analysis of 40 control urine samples and 50 control DBS samples. The physiological values of FGAr, AIr and CAIr detected in urine samples vary from tens to hundreds of nmol/l concentrations (Table 2). In DBS samples, only FGAr was detected; AIr and CAIr were below LOD.

Screening of DNPS metabolites was performed in 1,447 anonymized samples of urine and 365 samples of DBS of patients suffering from neurological disability (Fig. 2). We detected an elevated FGAr level (386 times vs controls) in one patient (Fig. 2A); the elevated FGAr levels may be a marker of defective PFAS enzyme. In three urine samples, we detected slightly elevated AIr levels, a marker for defective PAICS enzyme (Fig. 2C). In DBS samples, we did not detect any markedly elevated values of the analysed DNPS metabolites.

## Discussion

Purines play irreplaceable roles in all living organisms, utilized as universal energy source and storage, coenzymes, neurotransmitters, basic building blocks of nucleic acids, as well as for signal transduction and enzyme activity alterations. DNPS supplies the organism with newly synthesized molecules of purines during times of higher purine consumption, such as cell division and development. This pathway consists of 10 reactions driven by six enzymes. Mutations in genes encoding enzymes of DNPS lead to genetically determined disorders with a primary effect on neurological functions and physiological growth. Detection and investigation of DNPS disorders is difficult due to the nonspecific nature of the neurological findings caused by cytotoxic accumulation of substrate(s) of deficient enzymes and by the absence of commercially available DNPS substrates for development of analytical methods. In the

*Table 1. Limits of detection (LOD) and quantification (LOQ) of DNPS metabolites in the urine and DBS*

| | URINE | | DBS | |
|---|---|---|---|---|
| | **LOD [μmol/l]** | **LOQ [μmol/l]** | **LOD [μmol/l]** | **LOQ [μmol/l]** |
| **FGAr** | 0.001 | 0.004 | 0.05 | 0.16 |
| **AIr** | 0.023 | 0.076 | 0.03 | 0.11 |
| **CAIr** | 0.003 | 0.009 | 0.03 | 0.09 |

*Table 2. Physiological ranges of DNPS intermediate concentration detected in the urine and DBS*

| | URINE [μmol/mmol creat.] | | | DBS [μmol/l] | | |
|---|---|---|---|---|---|---|
| | **min** | **max** | **median** | **min** | **max** | **median** |
| **FGAr** | 0.00 | 0.32 | 0.098 | 0.24 | 0.55 | 0.35 |
| **AIr** | 0.00 | 0.43 | 0.068 | n.d. | n.d. | n.d. |
| **CAIr** | 0.00 | 0.17 | 0.052 | n.d. | n.d. | n.d. |

n.d. – not detected

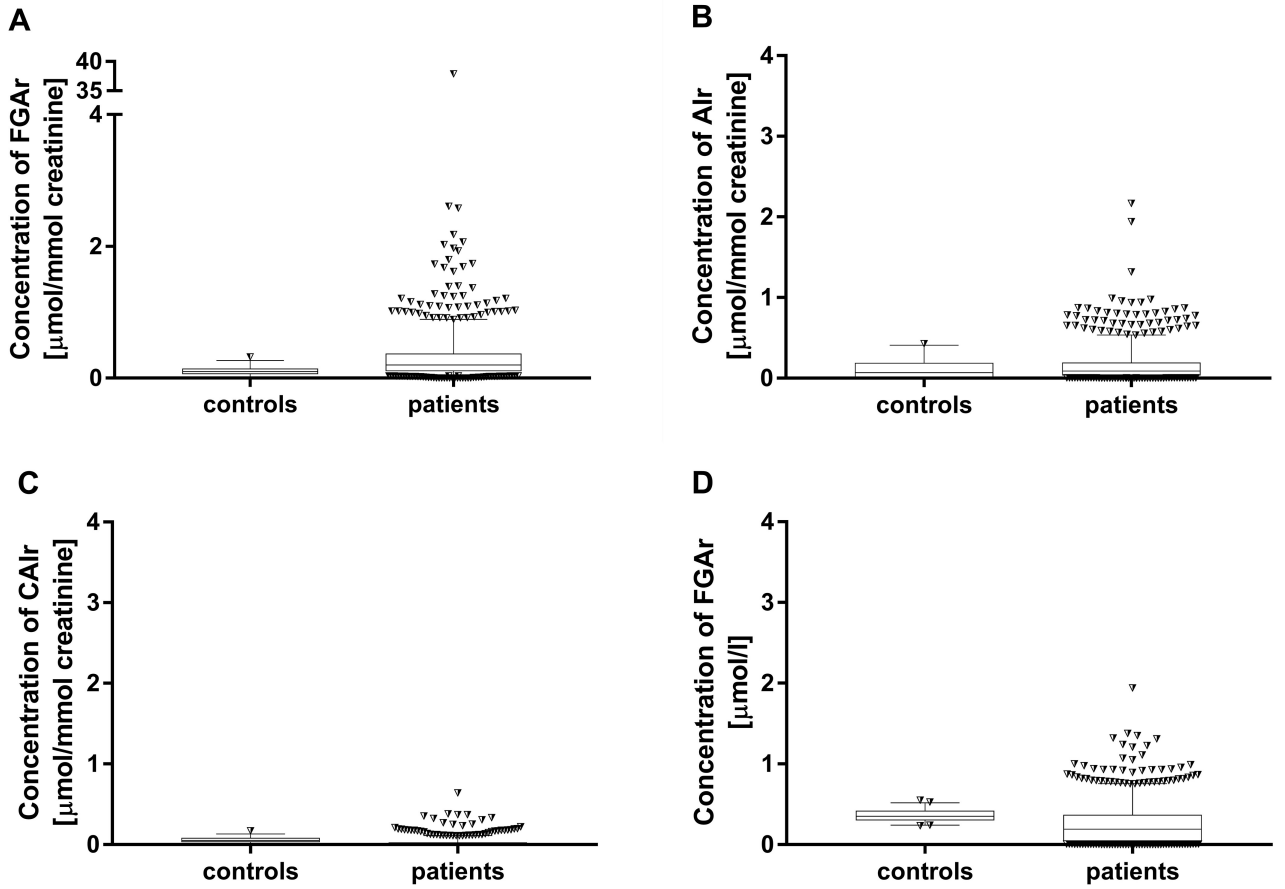


Fig. 2. *De novo* purine synthesis (DNPS) metabolites determined in urine samples and dried blood spots (DBS) Randomized urine samples of 40 controls and 1,447 patients with neurological impairment were screened for the presence of A/ formylglycineamide riboside (FGAr), B/ aminoimidazole riboside (AIr) and C/ carboxyaminoimidazole riboside (CAIr) metabolites by the HPLC-MS/MS method. With the same approach we also screened DBS samples of 50 controls and 365 patient samples; however, only D/ formylglycineamide riboside (FGAr) was detected. The box extends from the 25$^{th}$ to 75$^{th}$ percentiles with plotted median by the horizontal line, and whiskers mark the 5$^{th}$ and 95$^{th}$ percentiles.

case of severe phenotypes, mostly resulting in early death, the disease often remains undiagnosed (Mouchegh et al., 2007). This lack of diagnosis results in inadequate genetic counselling and inability to predict the outcomes of future pregnancies.

Furthermore, specialized laboratory investigation for metabolic disorders may not show satisfactory results due to the limited number of diagnostic methods that can detect DNPS disorders (Hartmann et al., 2006; Chrastina et al., 2007). Most of the specialized metabolic laboratories test only for ADSL deficiency, and rarely also AICA-ribosiduria. To date, no other genetically determined defects of DNPS enzymes have been identified. However, the range of the genetic variation provided in the gnomAD database indicates that, except for *PPAT,* there are no evolutionary constraints against loss of function or missense mutations in these genes in the population (Baresova et al., 2016). It can be anticipated that these mutations will be manifested by severe neurological impairment caused by the cytotoxicity of accumulated DNPS intermediates in body fluids, as shown in HeLa cell models (Baresova et al., 2016; Madrova et al., 2018).

In this work, we determined DNPS intermediates in the urine and DBS samples from patients with undiagnosed neurological impairment. We detected a higher urinary concentration of AIr in three samples and accumulation of FGAr in one sample. The accumulation of AIr or FGAr in body fluids can indicate mutations in the *PAICS* or *PFAS* gene and lead to the discovery of currently undiagnosed genetic disorders. Subsequently, AIr and FGAr nominate among applicable diagnostic markers.

Based on our results, we conclude that detection of DNPS intermediates, especially AIr and FGAr in patients with neurological symptoms, can show serious cellular and functional impairments resulting in novel metabolic defects of DNPS that have previously been inaccessible to detection. Genetic and molecular analysis of these patients should follow to establish the causal mutation(s) for future prenatal diagnosis and genetic consultation. Proving the existence of putative DNPS

disorders provides a new insight in the cellular metabolism. Broader cognitive knowledge brings possibilities to investigate the physiological and pathological conditions of the DNPS pathway and the effects on general and neurological development. Better understanding of the DNPS metabolism may also reveal the mechanisms of the pathway modulation, which could support not only the treatment of DNPS disorders, but also manage uncontrolled cell division (Chakravarthi et al., 2018; Meng et al., 2018), reinforcing DNPS as a potential therapeutic target.

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