

**Univerzita Karlova**  
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**UNIVERZITA KARLOVA**  
**1. lékařská fakulta**

Patologie a fyziologie *de novo* syntézy purinů

*Pathology and physiology of de novo purine synthesis*

**Mgr. Matyáš Krijt**

2021

**Doktorské studijní programy v biomedicině**  
*Univerzita Karlova a Akademie věd České republiky*

Obor: Biochemie a patobiochemie

Předseda oborové rady: prof. MUDr. Libor Víttek, Ph.D., MBA

Školící pracoviště: Klinika pediatrie a dědičných poruch metabolismu 1. lékařské fakulty  
Univerzity Karlovy a VFN

Školitel: Ing. Marie Zikánová, Ph.D.

Disertační práce bude nejméně pět pracovních dnů před konáním obhajoby zveřejněna k nahlížení veřejnosti v tištěné podobě na Oddělení pro vědeckou činnost a zahraniční styky Děkanátu 1. lékařské fakulty.

# Abstrakt

Puriny jsou organické sloučeniny s rozmanitými funkcemi, které se nacházejí ve všech živých organismech ve složitých molekulách, jako jsou nukleotidy, nukleosidy nebo jako purinové báze. Jejich přirozená rovnováha v organismu je udržována syntézou, recyklací a degradací. Přebytek purinů se vylučuje močí jako kyselina močová. Purinové nukleotidy mohou být recyklovány záchrannými cestami, které katalyzují reakci purinové báze s fosforibosylpyrofosfátem. Zcela nová ústřední molekula purinového metabolismu, inosinmonofosfát, může být syntetizována z prekurzorů při *de novo* purinové syntéze (DNPS), která je aktivována v případech zvýšené potřeby purinů jako je např. vývoj organismu. DNPS zahrnuje deset kroků katalyzovaných šesti enzymy, které formují multienzymový komplex purinosom umožňující tok substrátů skrz tuto dráhu.

Dosud byly popsány tři poruchy DNPS: deficit ADSL, AICA-ribosidurie a deficit PAICS. Všechny tři poruchy jsou způsobeny genetickými mutacemi vedoucími ke špatné funkci příslušného enzymu, které se dále projevují nedostatečnou aktivitou daného kroku DNPS. Biochemicky to znamená akumulaci substrátu poškozeného enzymu, biologicky narušení formování purinosomu a klinicky nespecifická neurologická postižení, což přispívá k problematické diagnostice poruch DNPS. Předpokládali jsme, že defekty ostatních DNPS enzymů zůstávají nepopsané z důvodu vzácnosti těchto pacientů a také malé míře rozšíření diagnostických metod způsobené komerční nedostupností většiny substrátů DNPS.

Naše hypotéza byla podpořena údaji z databáze gnomAD, které predikují existenci jedinců s mutacemi v pěti ze šesti možných genů kódující enzymy DNPS. Proto jsme přistoupili k biochemické a anorganické syntéze jednotlivých substrátů DNPS a jejich izotopicky značených analogů. Všechny připravené sloučeniny byly použity jako standardy pro vývoj diagnostických metod využívající techniku LC-MS/MS. Dále jsme vyvinuli modely lidských buněk simulující známé i teoreticky možné poruchy DNPS. Buněčné linie byly charakterizovány genetickým sekvenováním, proteinovými aktivními esejemi a stanovením hladin substrátů DNPS v buněčném médiu a lyzátech.

Naše výsledky umožnily iniciaci mezinárodní spolupráce vedoucí k popisu nové poruchy DNPS, deficitu PAICS, a dále podpořily screening vzorků moči a suchých krevních kapek (DBS) pacientů s nespecifickým neurologickým postižením bez určené diagnózy. Stanovili jsme fyziologické hodnoty substrátů DNPS detekovatelných v moči a DBS. Ve vzorcích DBS jsme nedekovali žádné významně odlehle hodnoty. Nicméně, ve vzorcích moči jsme identifikovali tři mírně a jednu extrémně zvýšenou hodnotu, což vedlo k dalšímu testování s cílem prokázat přítomnost poruchy v DNPS.

**Klíčová slova:** *De novo* syntéza purinů, Deficit Adenylosukcinátlyázy, AICA-ribosidurie, Deficit PAICS, Lidský buněčný model, Purinosom, Suchá krevní kapka (DBS), Screening, Tandemová hmotnostní spektrometrie, HPLC-MS/MS, Nespecifické neurologické symptomy.

# Abstract

Purines are organic compounds with miscellaneous functions that are found in all living organisms in complex molecules such as nucleotides, nucleosides or as purine bases. The natural balance of purine levels is maintained by their synthesis, recycling and degradation. Excess purines are excreted in the urine as uric acid. Purine nucleotides may be recycled by salvage pathways catalysing the reaction of purine base with phosphoribosyl pyrophosphate. A completely new central molecule of purine metabolism, inosine monophosphate, can be synthesized from precursors during the *de novo* purine synthesis (DNPS). DNPS involves ten steps catalysed by six enzymes that form a multienzymatic complex, the purinosome, enabling substrate channelling through the pathway. DNPS is activated under conditions involving a high purine demand such as organism development.

Currently, three DNPS-disrupting disorders have been described: ADSL deficiency, AICA-ribosiduria and PAICS deficiency. All three disorders are caused by genetic mutations leading to the impaired function of particular enzyme causing insufficient activity of respective DNPS step, manifested biochemically by accumulation of substrate of deficient enzyme, biologically by disruption of purinosome formation and clinically by unspecific neurological features, which contributes to difficulties in DNPS disorders diagnosis. We assumed, that defects in other DNPS enzymes remain unseen due to the rarity of DNPS patients and the lack of diagnostic methods caused by the commercial unavailability of most of the DNPS substrates.

Our hypothesis was supported by the data from gnomAD database revealing the possibility of mutations in five of six genes coding enzymes of the pathway. Therefore, we prepared biochemical and inorganic procedures for synthesis of DNPS substrates and their multiple isotopically labelled analogues. All prepared compounds were utilized as standards for the development of LC-MS/MS diagnostic methods. We also produced human cell models of known and putative DNPS disorders. Cell lines were characterized by genetic sequencing, protein activity assays and determination of DNPS substrates accumulation in cell medium and lysates.

Our results initiated an international collaboration leading into description of a new DNPS disorder the PAICS deficiency and encouraged us to screen the urine and dry blood spot (DBS) samples of patients with nonspecific neurological impairment lacking a diagnose. We determined physiological values of DNPS substrates detectable in urine and DBS. DBS samples did not reveal any significantly altered values. However, we identified three modestly and one extremely elevated value within urine samples resulting in further investigation with the aim to prove a presence of DNPS disorder.

**Keywords:** *De novo* purine synthesis, Adenylosuccinate lyase deficiency, AICA-ribosiduria, PAICS deficiency, Human cellular model, Purinosome, Dried blood spots, DBS, Screening, Tandem mass spectrometry, HPLC–MS/MS, Unspecific neurological symptoms.

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## List of abbreviations

ADSL	<i>Adenylosuccinate lyase</i>
AICAR/r	Aminoimidazolecarboxamide ribotide/riboside
AICARFT	<i>Aminoimidazole carboxamide ribonucleotide formyltransferase</i>
AIR/r	Aminoimidazole ribotide/riboside
AIRC	<i>Phosphoribosyl aminoimidazole carboxylase</i>
AIRS	<i>Phosphoribosyl aminoimidazole synthetase</i>
ATIC	<i>Aminoimidazole carboxamide ribonucleotide formyltransferase/IMP cyclohydrolase</i>
Cas	CRISPR associated
CAIR/r	Carboxyaminoimidazole ribotide/riboside
CIP	Calf intestine phosphatase
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CSF	Cerebrospinal fluid
DAD	Diode array detector
DNPS	<i>De novo</i> purine synthesis
FAICAR/r	Formamidoimidazolecarboxamide ribotide/riboside
GAR/r	Glycinamide ribotide/riboside
GARFT	<i>Phosphoribosyl glycinamide formyltransferase</i>
GARS	<i>Phosphoribosyl glycinamide synthetase</i>
GART	<i>Phosphoribosyl glycinamide formyltransferase, phosphoribosyl glycinamide synthetase, phosphoribosyl aminoimidazole synthetase</i>
FGAMR/r	Formylglycine amidine ribotide/riboside
FGAR/r	Formylglycinamide ribotide/riboside
HeLa	Human epithelial carcinoma cell line
HPLC	High pressure liquid chromatography
HRMS	High resolution mass spectrometry
IMPCH	<i>Inosine monophosphate cyclohydrolase</i>
MBP	Maltose binding protein
MS/MS	Tandem mass spectrometry
PAICS	<i>Phosphoribosyl aminoimidazole carboxylase and Phosphoribosyl aminoimidazole succinocarboxamide synthase</i>
PD	Purine depleted
PFAS	<i>Phosphoribosyl formylglycinamide synthase</i>
PPAT	<i>Phosphoribosyl pyrophosphate amidotransferase</i>
PR	Purine rich
PRA	Phosphoribosylamine
PRPP	Phosphoribosyl pyrophosphate
PRPP	Phosphoribosyl pyrophosphate
<i>PurD</i>	<i>Phosphoribosyl glycinamide formyltransferase</i>
<i>PurL</i>	<i>Phosphoribosylformylglycinamide synthase</i>
<i>PurM</i>	<i>Aminoimidazole ribonucleotide synthetase</i>
<i>PurN</i>	<i>Phosphoribosyl glycinamide synthetase</i>
SAdo	Succinyladenosine
SAICAR/r	Succinylaminoimidazolecarboxamide ribotide/riboside
SAICARS	<i>Succinyl aminoimidazole carboxamide ribonucleotide synthetase</i>

SAMP	Succinyladenosine monophosphate
SNP	Single nucleotide polymorphism
TLC	Thin layer chromatography
wt	Wild type

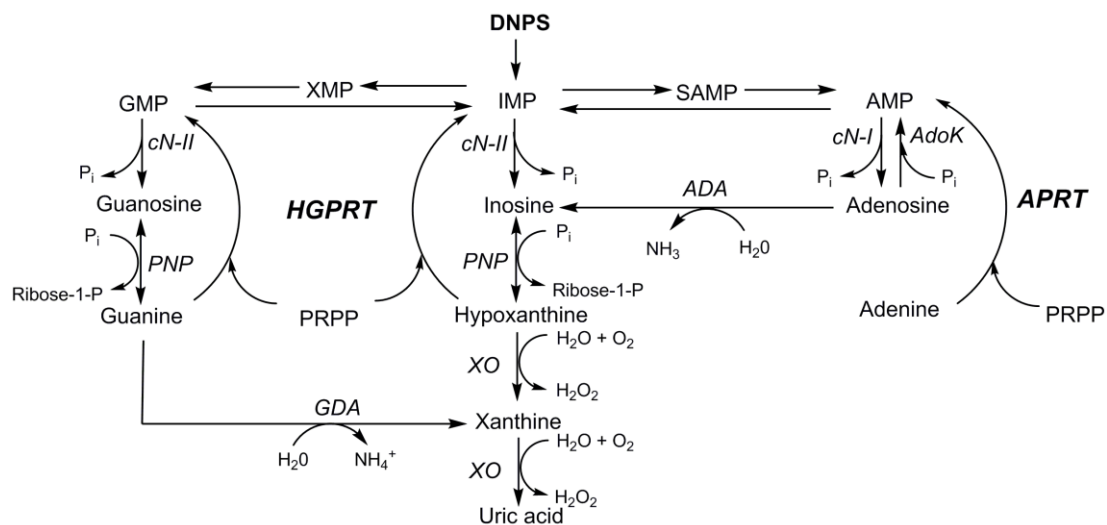


# 1 Introduction

Purines are organic compounds with miscellaneous functions found in all living organisms. They have been shown to provide energy for many metabolic processes, constitute the building blocks of deoxyribonucleic acid, ribonucleic acid and many cofactors, i.e. nicotinamide adenine dinucleotide, flavin adenine dinucleotide or acetyl coenzyme A. In addition, purines participate in cell signalling or function as neuromodulators and co-transmitters. The purine molecule consists of two heterocycles – the imidazole ring and the pyrimidine ring, which appears in an organism with various modifications.

## 1.1 Metabolism of purines

The natural balance of purine levels is maintained by their synthesis, recycling (salvage pathways) and degradation pathways (Fig. 1). Under normal conditions are nucleotides supplied by salvage pathways. Excess amount of nucleotides is catabolized to ribose, phosphate residue and purine base, which is in humans converted to uric acid by the degradation pathway and excreted from organism in the urine. On the other hand, during conditions when there is a high purine demand is activated the *de novo* purine synthesis (DNPS).



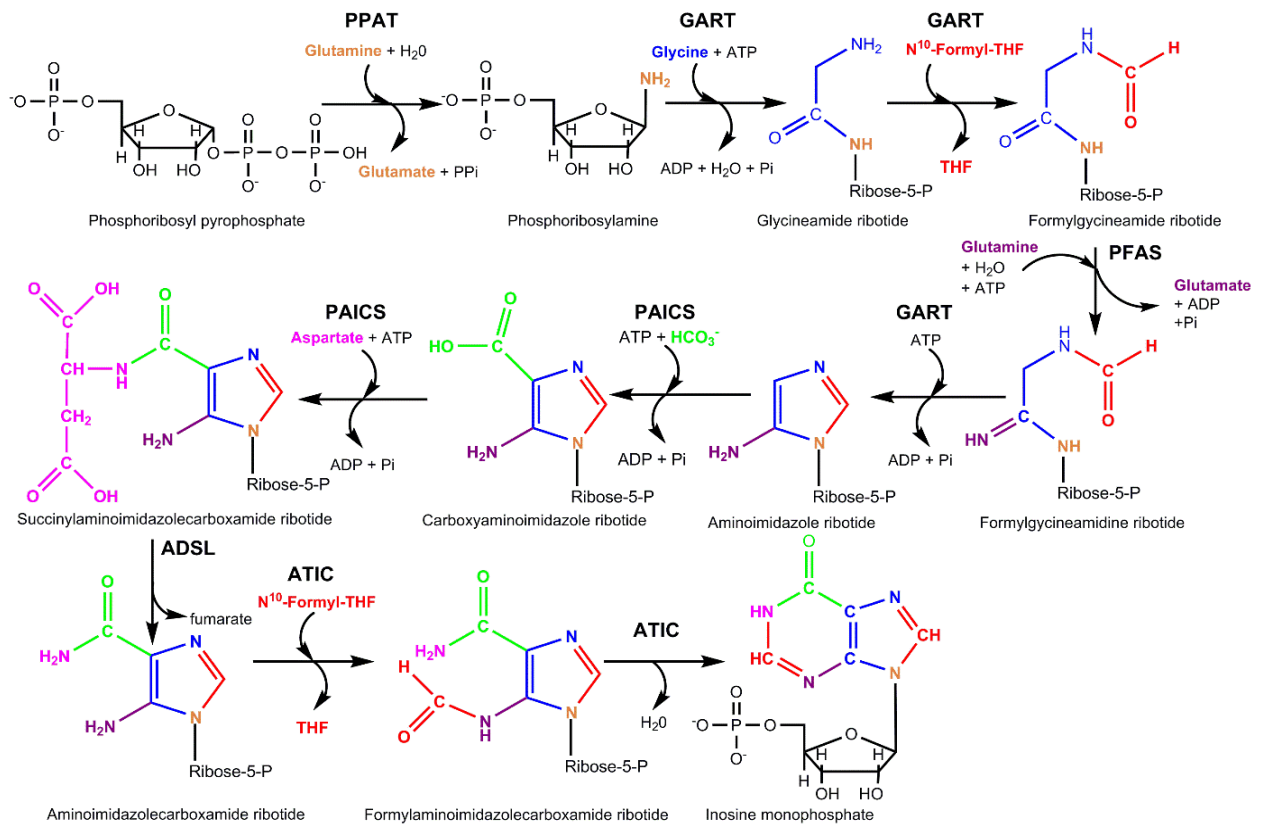
**Figure 1. Salvage and degradation pathways of purine metabolites.** Guanine, hypoxanthine and adenine may be converted into nucleotides by salvage pathways or catabolized by degradation pathway into uric acid which is the end product of human purine metabolism.

cN-I or cN-II = cytosolic 5'-nucleotidase-I or II. AdoK = adenosine kinase. PNP = purine nucleoside phosphorylase. APRT = adenosine phosphoribosyl pyrophosphate transferase. HGPRT = hypoxanthine-guanine phosphoribosyl pyrophosphate transferase. GDA = guanine deaminase. XO = xanthine oxidase. ADA = adenosine deaminase.

### 1.1.1 The *de novo* purine synthesis

The DNPS supplies organism with newly synthesized molecules of purines. The first substrate of DNPS is phosphoribosyl pyrophosphate (PRPP), which is subsequently modified in ten reactions catalysed by six enzymes (Tab. 1) in order to build up one new molecule of IMP. The conversion of PRPP into IMP

is extremely energetically demanding with a consumption of five molecules of ATP. Some of the DNPS pathway reactions are catalysed substitutions and some of them result in cleavages of individual atoms, functional groups, amino acids molecules or larger organic or inorganic segments (Fig. 2).



**Figure 2. The *de novo* purine synthesis.** The synthesis of IMP occurs in ten reactions catalysed by six enzymes.

**Table 1. Overview of DNPS enzymes, their abbreviations and a number of the catalysed step in a reaction sequence.**

Step	Enzyme name	Abbreviation
1	<i>Phosphoribosyl pyrophosphate amidotransferase</i>	PPAT
2	Trifunctional enzyme: <i>Phosphoribosyl glycinamide synthetase</i>	GART:
3	<i>Phosphoribosyl glycinamide formyltransferase</i>	GARS
5	<i>Phosphoribosyl aminoimidazole synthetase</i>	GART
4	<i>Phosphoribosyl formylglycinamidase synthase</i>	PFAS
6	Bifunctional enzyme: <i>Phosphoribosyl aminoimidazole carboxylase</i>	PAICS:
7	<i>Succinyl aminoimidazole carboxamide ribonucleotide synthetase</i>	AIRC
8	<i>Adenylosuccinate lyase</i>	SAICARS
8	<i>Adenylosuccinate lyase</i>	ADSL
9	Bifunctional enzyme: <i>Aminoimidazole carboxamide ribonucleotide formyltransferase</i>	ATIC:
10	<i>Inosine monophosphate cyclohydrolase</i>	AICARFT
		IMPCH

### 1.1.1.1 The purinosome

The facts that the DNPS pathway consists of ten steps and is driven only by six enzymes, from which four are multifunctional and that the DNPS substrate PRA is unstable put forward a hypothesis that DNPS enzymes form a multienzymatic complex that enables substrate channelling through

the pathway (Rudolph J. and Stubbe J.,1995). Whether DNPS enzymes form a multienzymatic complex was studied in HeLa cells transiently transfected with constructs coding human DNPS proteins fused with fluorescent proteins (An S. *et al.*,2008). This study revealed co-localization of DNPS proteins in cells grown in purine depleted (PD) medium and diffuse distribution in purine rich (PR) medium. The possibility to dynamically regulate signal overlap and diffusion of all individual DNPS proteins led to a statement about formation of a multienzymatic complex, the purinosome (An S. *et al.*,2008). Existence of the purinosome was further supported by detection of purinosome formation of immunolabeled DNPS endogenous proteins in various cell types (Baresova V. *et al.*,2012).

## 1.2 DNPS disorders

Mutations in genes coding enzymes of DNPS lead to genetically determined disorders of purine metabolism. Until recently, two DNPS disorders were described – a deficiency of enzyme ADSL and bifunctional enzyme ATIC, named ADSL deficiency (OMIM 103050) and AICA-ribosiduria (OMIM 608688), respectively. Both disorders manifest with unspecific neurological symptoms and accumulation of substrates of affected enzymes. Data obtained from gnomAD database (Karczewski K.J. *et al.*,2020) show that there are no evolutionary constraints against loss of function or missense mutations in DNPS genes except that of PPAT. Data provided herein represent the range of genetic variability and the probability of gene loss of function intolerance.

### 1.2.1 Adenylosuccinatelyase deficiency

The ADSL patients are divided into four groups: neonatal fatal form, severe childhood form (Type I), mild to moderate form (Type II) and very mild form (Type III) based on the severity of symptoms and ratio of SAdo/SAICAr in body fluids (van den Bergh F. *et al.*,1993; Mouchegh K. *et al.*,2007; Macchiaiolo M. *et al.*,2020).

The neonatal fatal form is defined with SAdo/SAICAr ratio lower than 1 and these patients suffer from microcephaly, respiratory failure, lack of spontaneous movement and seizures not responding to anticonvulsive treatment (Mouchegh K. *et al.*,2007; Zikanova M. *et al.*,2010). The severe childhood form (Type I) is defined with SAdo/SAICAr ratio between 1 – 2 and the clinical manifestation includes microcephaly, seizures, psychomotor retardation, lack of eye-to-eye contact and early death (Jurecka A. *et al.*,2015). The mild to moderate form (Type II) is defined with SAdo/SAICAr ratio larger than 2. The ASDLD Type II patients have moderate psychomotor retardation and transient contact disturbances (Jaeken J. *et al.*,1992), ataxia, which could cause gait disturbance (Jurecka A. *et al.*,2015) and seizures may occur later at the age between 2 and 4 (Castro M. *et al.*,2002; Jurecka A. *et al.*,2008) or even up to the age 9 (Gitiaux C. *et al.*,2009). The very mild form (Type III) was described in two individuals with SAdo/SAICAr ratio larger than 3.5 (Macchiaiolo M. *et al.*,2020).

ADSLD is not causing a decrease in levels of nucleotides in various tested tissues in patients (Jaeken J. and van den Berghe G.,1984). Therefore, the pathogenic effect of ADSLD was attributed to accumulated SAICAr and SAdo (Stone T.W. *et al.*,1998). High levels of SAdo may have even a protective effect and compensate for the toxic effect of SAICAr (Jaeken J. *et al.*,1988; van den Bergh F. *et al.*,1993).

The prevalence and incidence of ADSL deficiency are unknown. More than 80 cases have been reported to date, mostly from Europe and the Mediterranean region followed by cases from Brazil and US. The disorder may be underdiagnosed as it is probably panethnic (Knoch S. *et al.*,2000).

### 1.2.2 AICA-ribosiduria

Only one case of AICA-ribosiduria accompanied with severe neurological symptoms was identified since 2004 (Marie S. *et al.*,2004). However, three new cases suffering from neurodevelopmental impairment were discovered in 2020 (Ramond F. *et al.*,2020).

The first case described is a 4 years old girl. The sister of a healthy brother has shown alarming neurological conditions including psychomotor retardation, epilepsy, dysmorphic features and congenital blindness. The newly described cases were preceded with intrauterine growth retardation followed by neonatal hypotonia, scoliosis and dysmorphic features. Thereafter, delayed cognitive skills, ophthalmological disorders, basic or no spoken language were observed (Ramond F. *et al.*,2020). Similarly to ADSL deficiency, AICA-ribosiduria cytotoxicity is presumably caused by AICAr accumulation (Baresova V. *et al.*,2012).

### 1.2.3 Treatment

Currently, there is no effective treatment for ADSLD and AICA-ribosiduria available. Therapeutic approaches are primarily supportive. Treatment with anticonvulsive drugs (e.g. valproic acid, phenobarbital, carbamazepine, topiramate, levetiracetam, phenitoin, clobazam) depends on the type of seizures.

## 1.3 DIAGNOSTIC METHODS of DNPS disorders

### 1.3.1 Biochemical methods

Biochemical methods for DNPS diagnostics are based on detection of elevated substrates in body fluids of affected individuals. The first case of ADSLD was identified with contribution of thin layer chromatography (TLC) and high-pressure liquid chromatography (HPLC) coupled with UV detection (Jaeken J. and van den Berghe G.,1984). Another method applied to screening of ADSLD was the Bratton-Marshall (BM) test. The BM test was modified and established for screening of DNPS substrates with a free aromatic amino group (AIR, CAIR, SAICAR, AICAR).

However, more specific, high throughput, but also more expensive methods, such as capillary electrophoresis (Hornik P. *et al.*,2007) or HPLC coupled with UV diode array detection (DAD) (Lee B.L. and Ong C.N.,2000), have been lately introduced. The most common DAD enables to detect compounds in the region of 190 – 1100 nm. The substrates of DNPS pathway containing imidazole ring, the first of which appear in the form of AIR or Alr, respectively, absorb UV light between 245 and 275 nm. Inconveniences with compounds that do not absorb the UV light could be circumvented by use of high pressure liquid chromatography coupled with tandem mass spectrometry (HPLC-MS/MS) or high resolution MS (HPLC-HRMS) as a detection technique.

### 1.3.2 Genetic methods

Individuals with elevated concentrations of DNPS substrates were investigated for presence of mutations in DNPS genes. Samples of peripheral blood or fibroblasts are used for the determination of mutations in genes coding individual enzymes of DNPS and related regions. Up to date, around 80 patients were diagnosed and subsequently sequenced for mutations in the ADSL gene (<http://www1.lf1.cuni.cz/udmp/adsl>, November 2019). The majority of identified mutations were missense mutations occurring as compound heterozygotes (Jurecka A. *et al.*,2015).

### 1.3.3 Function studies

The determination of enzyme activity in various cell type provides another possibility to back up the diagnosis of DNPS disorders and contribute to a detailed explanation of pathogenesis. Functional and biochemical studies of mutations may be performed by experiments utilizing recombinant enzymes (Race V. *et al.*,2000; Kmoch S. *et al.*,2000; Zikanova M. *et al.*,2010).

## 1.4 Cell model systems to study DNPS disorders

### 1.4.1 Chinese hamster ovary cell model

In the beginning, Chinese hamster ovary cells were selected to develop mammalian model (Kao F.T. and Puck T.T.,1968) with aberrant metabolism in each individual step of DNPS (Patterson D. *et al.*,1974;.1975, 1976; Tu A. S. and Patterson D.,1977).

### 1.4.2 Genetically modified HeLa model cell lines

The Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) allied with CRISPR associated (Cas) proteins are endowed with an endonuclease activity (Garneau J.E. *et al.*,2010). The CRISPR/Cas9 gene silencing was used in preparation of HeLa cell lines deficient in enzyme activity of each individual step of DNPS (Baresova V. *et al.*,2016).

## 2 AIMS AND HYPOTHESIS

We assume that DNPS disorders are underdiagnosed and partially neglected due to the absence of commercially available substrates of this pathway and demanding diagnostic methods. Therefore, we had to prepare unavailable substrates and their dephosphorylated forms in order to develop diagnostic methods for known and putative DNPS disorders. Thus, we enabled screening of patients lacking diagnosis and suffering from unspecific neurological symptoms.

### 2.1 Preparation of DNPS substrates

Only PRPP, AICAR, IMP and SAMP may be commercially obtained. Therefore, we had to develop procedures for the preparation of remaining substrates and their dephosphorylated forms in order to gain standards. Obtained or prepared substrates of DNPS may be used for development of qualitative and quantitative methods intended for use in detection of DNPS substrates of known and putative DNPS disorders.

### 2.2 Development of detection methods

A suitable analytical tool for development of detection methods for all DNPS substrates is HPLC-MS/MS. HPLC-MS/MS is a semiquantitative method that requires standards for the purposes of tuning and evaluation. This method is utilizing a separation of sample content by liquid chromatography and detection in selected reaction monitoring regime executed by triple quadrupole mass spectrometer.

### 2.3 Characterization of cell lines as model of individual DNPS enzymatic defects

Prepared standards and analytical methods were applied to verification of previously prepared model organisms simulating known and putative DNPS disorders. Such model organisms are represented by HeLa cell lines with defects in individual enzyme of DNPS (Baresova V. *et al.*, 2016). The hypothesis was to confirm that such model organism would accumulate substrate for defected enzyme.

### 2.4 Screening for biomarkers of known and putative DNPS disorders in urine and dry blood spots

DNPS disorders manifest with unspecific neurological symptoms ensuing clinical diagnosis extremely difficult. An outcome of lack of commercially available standards of DNPS substrates is that not enough effort is put into extension of routinely employed biochemical methods of their detection. Thus, we have established and validated accessible methods for screening DNPS disorders in urine and dry blood spots.

## 3 Materials and methods

### 3.1 Preparation of DNPS substrates

Expression vectors coding recombinant DNPS enzymes

Preparation of cDNA

cDNA cloning

Transformation of competent E. Coli DH5 $\alpha$ F'IQ strain

Plasmid isolation from E. Coli DH5 $\alpha$ F'IQ strain (Mini-prep)

Expression and purification of recombinant proteins

Preparation of bacterial culture

Preparation of bacterial cell lysate

Affinity chromatography

SDS-PAGE gel electrophoresis

Preparation of non-labelled DNPS substrates

Preparation of isotopically labelled substrates

### 3.2 Analytical methods for detection of DNPS substrates

HPLC-UV

HPLC-MS/MS

### 3.3 BIOLOGICAL SAMPLE PREPARATION

Urine

Cell medium and lysates

Plasma

DBS

## 4 RESULTS

### 4.1 Expression and purification of human and bacterial recombinant enzymes

A special strain DH5 $\alpha$ F'IQ of *E. coli* was transfected and used in production of DNPS enzymes. These bacterial enzymes were prepared: MBP-GARS (PurN), MBP-GARFT (PurD), 6H-PFAS (PurL), MBP-AIRS (PurM) as well as human MBP-GART, MBP-PFAS, MBP-PAICS and MBP-ADSL.

### 4.2 Preparation of DNPS substrates

Most of the DNPS substrates (GAR/r, FGAR/r, FGAMR/r, AIR/r, CAIR/r, SAICAR/r, SAMP and SAdo) were produced by the forward or reverse enzymatic reactions catalysed with human or bacterial recombinant enzymes. FAICAR was prepared by the inorganic formylation of commercially supplied AICAR.

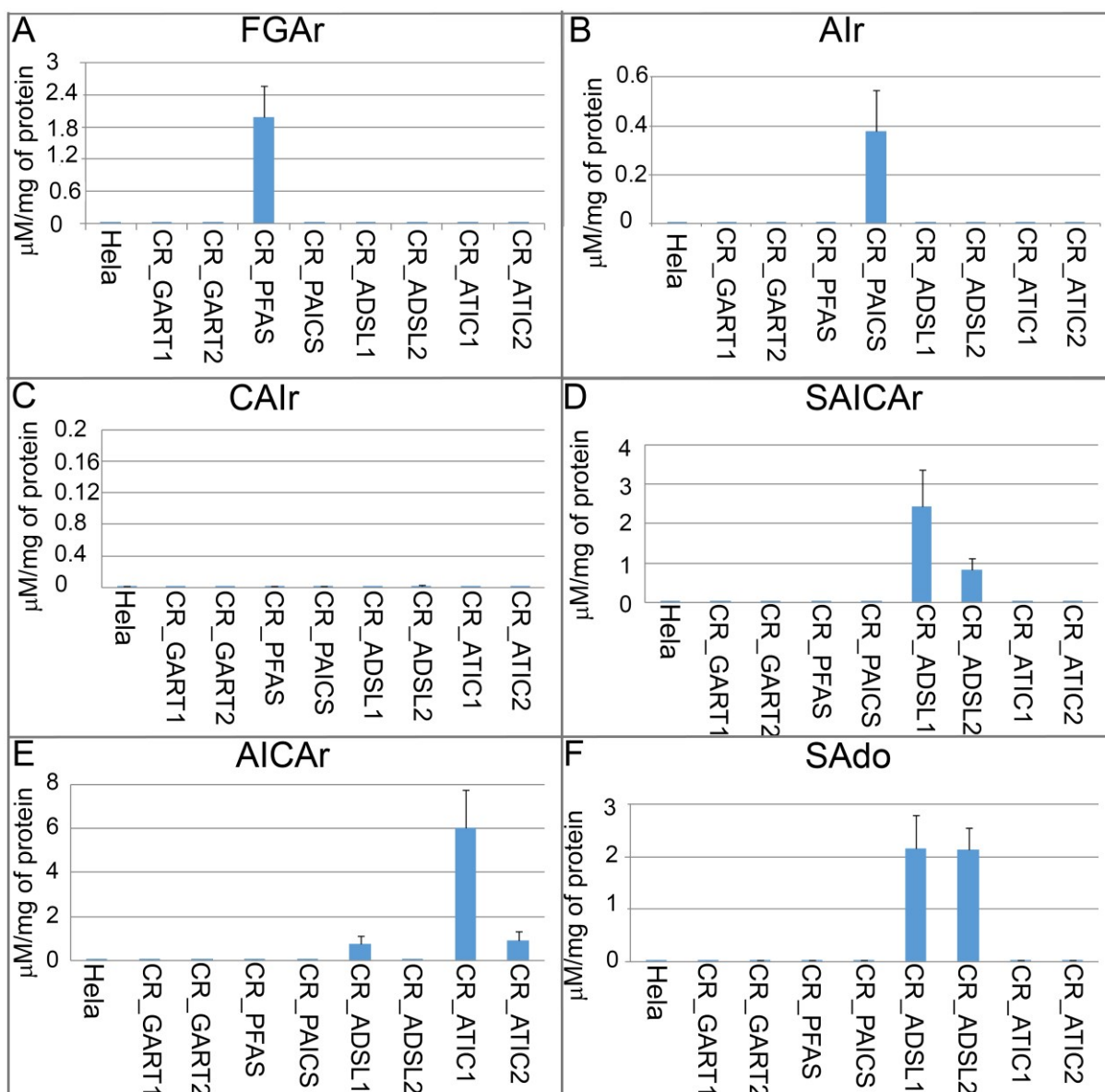
### 4.3 Development of detection methods

Prepared or purchased DNPS substrates were utilized in the development of detection methods based on HPLC-MS/MS or HPLC-HRMS techniques. HPLC-MS/MS was carried out on Agilent 1290 Infinity LC System (Agilent Technologies, Palo Alto, CA, USA) coupled with an API 4000 triple quadrupole mass spectrometer operated with Analyst software version 1.4 (Applied Biosystems, Foster City, CA, USA). HPLC-HRMS analysis was achieved in cooperation with a group of professor Adam from Palacky University Olomouc and carried out on Ultimate 3000 RS LC System (ThermoFisher Scientific, MA, USA) coupled with Orbitrap Elite (ThermoFisher Scientific, MA, USA) hybrid mass spectrometer operated with MassFrontier 7.0.5.09 SP3 software (HighChem, SK). Developed methods were utilized for analysis of cell model organisms (lysates and medium), bodily fluids (urine, plasma and CSF) and DBS.

### 4.4 DNPS substrates in model organisms of individual DNPS disorders

The hypothesis that the DNPS disorders caused by defective enzymes manifest biochemically by accumulation of their substrates were tested on model cell lines simulating known and putative DNPS disorders prepared previously in our lab by CRISPR/Cas9 method (Fig. 3) (Baresova V. *et al.*, 2016).





**Figure 3. HeLa cell lines mutated with CRISPR/Cas9 technique in individual genes coding DNPS enzymes.** The target mutation of individual DNPS genes resulted in cell lines with characteristic elevation of dephosphorylated substrates of defective enzyme in cell growth medium. HPLC-MS/MS analysis detected elevated FGAr (A) in CR\_PFAS cells, elevated Alr (B), but not CAIr (C) was observed in CR\_PAICS, high concentrations of SAICAr (D) and SADo (F) were detected in CR\_ADSSL and AICAr (E) was accumulated in CR\_ATIC cells.

#### 4.4.1 Transfection with wild type protein construct

CRISPR/Cas9 model cell lines were transfected with vectors coding fluorescently labelled wild type (wt) protein related to defective enzyme to study purinosome restoration and effect on levels of accumulated substrates. CR\_PFAS, CR\_PAICS, CR\_ADSSL and CR\_ATIC cells grown 24 h in PD medium were analysed by HPLC-MS/MS for presence of accumulated substrates of defective enzymes before and after transfection with construct encoding appropriate wt protein (Table 2) (Baresova V. *et al.*,2018).

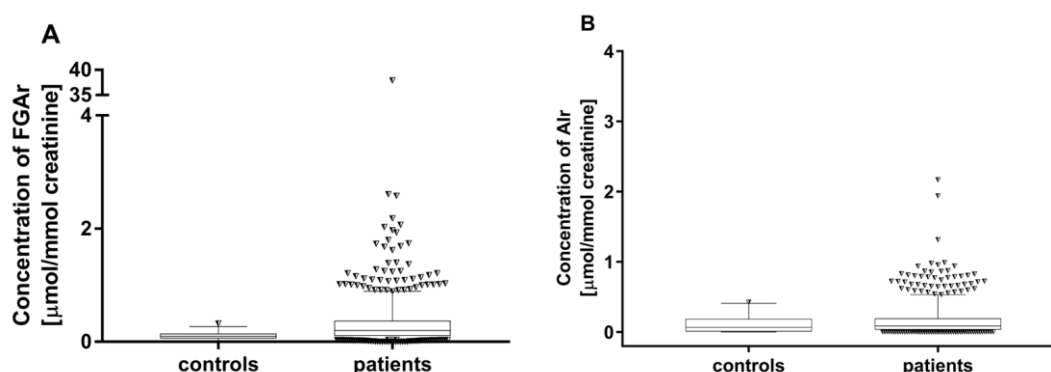
**Table 2. Effect of transfection with wt protein associated with the previously CRISPR/Cas9 mutated protein.** CR\_PFAS, CR\_PAICS, CR\_ADSL and CR\_ATIC cell lines were transfected with corresponding wt protein. The decrease in concentration of substrate of defective protein substituted by transfected protein was detected by HPLC-MS/MS.

metabolite	HeLa	CR_PFAS		CR_PAICS		CR_ADSL		CR_ATIC	
	$\mu\text{mol/L}$	non TR $\mu\text{mol/L}$	TR $\mu\text{mol/L}$	non TR $\mu\text{mol/L}$	TR $\mu\text{mol/L}$	non TR $\mu\text{mol/L}$	TR $\mu\text{mol/L}$	non TR $\mu\text{mol/L}$	TR $\mu\text{mol/L}$
FGAr	nd	12.1 $\pm$ 2.1	0.32 $\pm$ 0.22	0.28 $\pm$ 0.06	nd	nd	nd	nd	nd
FGAR	nd	2.1 $\pm$ 0.9	0.07 $\pm$ 0.06	0.13 $\pm$ 0.04	nd	nd	nd	nd	nd
Alr	nd	nd	nd	1.0 $\pm$ 0.1	0.004 $\pm$ 0.001	nd	nd	nd	nd
AIR	nd	nd	nd	2.93 $\pm$ 0.16	0.33 $\pm$ 0.01	nd	nd	nd	nd
SAICAr	nd	nd	nd	nd	nd	0.40 $\pm$ 0.04	0.072 $\pm$ 0.002	0.05 $\pm$ 0.01	$\approx$ LOQ
SAICAR	nd	nd	nd	nd	nd	0.21 $\pm$ 0.03	nd	nd	nd
SAdo	$\approx$ LOQ	$\approx$ LOQ	$\approx$ LOQ	$\approx$ LOQ	$\approx$ LOQ	10.5 $\pm$ 1.1	1.9 $\pm$ 0.2	$\approx$ LOQ	$\approx$ LOQ
SAMP	$\approx$ LOQ	$\approx$ LOQ	$\approx$ LOQ	nd	nd	22.7 $\pm$ 2.5	nd	nd	nd
AICAr	nd	nd	nd	nd	nd	nd	nd	0.09 $\pm$ 0.03	nd
AICAR	nd	nd	nd	nd	nd	nd	nd	1.2 $\pm$ 0.4	nd

nd – not detected,  $\approx$  LOQ – close to limit of quantification

## 4.5 Screening for biomarkers of known and putative DNPS disorders in urine and dry blood spots

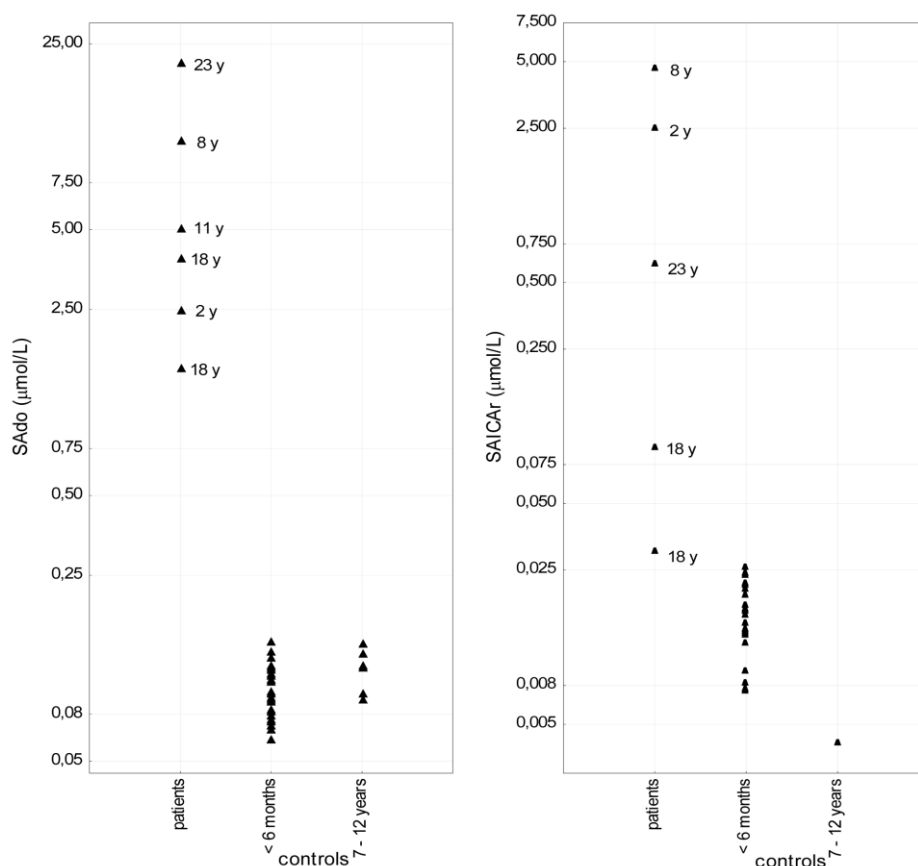
Our studies of model cell lines confirmed the hypothesis that to date undescribed defects of DNPS will almost certainly manifest by accumulation of substrates of defective enzyme in body fluids. Therefore, we started the screening of patient cohort with unspecific neurological impairment lacking a diagnosis to study the prevalence of putative DNPS disorders. To achieve this, anonymised samples of urine and DBS were analysed. No increased values were detected in 365 DBS samples. One extremely elevated concentration of FGAr was detected in one sample and slightly elevated levels of Alr were detected in 3 samples (Fig. 4).



**Figure 4. Urine analysis from patients with neurological impairment.** Urine samples analysed with HPLC-MS/MS provided physiological range of FGAr, Alr and CAIr in 40 control samples and the screening of 1447 randomized patients' samples showed one extremely increased level of FGAr in one case (A) and three moderately upregulated concentrations of Alr (B). The box extends from the 25<sup>th</sup> to 75<sup>th</sup> percentiles with plotted median by the horizontal line, and the whiskers mark the 5<sup>th</sup> and 95<sup>th</sup> percentiles.

## 4.6 Retrospective analysis of DBS from ADSL-deficient patients

In the first set of experiments, Guthrie cards collected from 6 neonatal patients with diagnosed ADSLD stored for 2 – 23 years at RT were used and compared with values of 31 controls stored at RT up to 6 months. Isotopically labelled SAICAr- $^{13}\text{C}_4$  and SAdo- $^{13}\text{C}_4$  were utilized as internal standards in the precise detection and quantification of studied metabolites (Fig. 5) (Zikanova M. *et al.*,2015).



**Figure 5. Detection of SAICAr and SAdo in DBS of ADSL-deficient patients by HPLC-MS/MS.** Samples of ADSL-deficient patients prepared from DBS stored for 2 and up to 23 years revealed elevated concentrations of SAICAr and SAdo when compared to control samples.

## 4.7 Description of new DNPS disorder

Our colleagues from the Universite de Paris in France used the Affymetrix 250K Nspl SNP array method for genotyping of genomic DNA. The data from single nucleotide polymorphism (SNP) probes did not reveal any copy-number variants pointing out autosomal recessive inheritance, however, three homozygous regions (Chromosome 6 (chr6: 26393539–28555894), Chromosome 8 (chr8: 70174745–73185849) and Chromosome 4 (chr4: 54484130–62069735) were identified in agreement with expected founder effect (Pelet A. *et al.*,2019). Further analysis included Sanger sequencing suitable for sequencing exomes of father, mother and the two affected children with emphasis on variants with allele frequencies less than 0.1 % (Lek M. *et al.*,2016). Thus, a single homozygous

variant chr4: 57307970 A/G located in exon 2 of gene *paics* was detected. This SNP variant causes substitution of lysine to arginine (p.Lys53Arg) (Pelet A. *et al.*,2019).

#### 4.7.1 Protein analysis and functional studies

The enzyme activity of patient measured in patients' skin fibroblasts was decreased to 10 % when compared to controls despite the mRNA levels and WB did not show any significant differences (Pelet A. *et al.*,2019). The mutated enzyme (p.Lys53Arg) was then transfected into *E. coli* and the activity of purified enzyme was determined to be 25 % activity of the control. Also, the disruption of purinosome was observed in patients' skin fibroblast cultured in PD medium (Pelet A. *et al.*,2019). The formation of purinosome was restored after transfection with wt enzyme and not after transfection with the mutated enzyme (Pelet A. *et al.*,2019).

#### 4.7.2 Metabolic studies

Known DNPS disorders manifest biochemically by the accumulation of substrates of defective enzyme in body fluids, which were unfortunately not available in case of PAICS deficient patients. Therefore, there were utilized samples prepared from lysates and growth medium derived from patients' skin fibroblasts. Patient' skin fibroblasts were cultured in PR and PD medium and then analysed by HPLC-MS/MS for presence of accumulated AIR and Alr, respectively. We expected similar results as in our previous study when CRISPR edited HeLa cells (CR\_PAICS) showed elevated levels of Alr in a growth medium (Baresova V. *et al.*,2016). However, neither AIR nor Alr were detected in lysates or growth media of patients' skin fibroblasts. The limits of detection and quantification set as ratios 3:1 and 10:1 signal to noise were 12 and 62 nmol/L in growth medium, respectively 3.6 and 18.6 nmol/L in water (Pelet A. *et al.*,2019).

## 5 DISCUSSION

At the beginning of our study, there were reported just two genetically determined disorders associated with DNPS, the AICA-ribosiduria with only one described patient (Marie S. *et al.*,2004) since 2020 when were diagnosed additional three cases (Ramond F. *et al.*,2020) and ADSL deficiency with less than 100 diagnosed patients worldwide (Jurecka A. *et al.*,2015; Banerjee A. *et al.*,2020).

Both disorders manifest biochemically by accumulation of substrate of deficient enzyme, biologically by disruption of purinosome and clinically by unspecific neurological features especially intractable seizures, psychomotor delay, symptoms analogous to autism and epilepsy. DNPS disorders occur very rarely, often lead to early death and even cause abortions, which all contribute to undiagnosed cases (Mouchegh K. *et al.*,2007). Furthermore, diagnostic methods of DNPS disorders are demanding and not routinely performed. This all contributes to neglect of the DNPS disorders. The major inconvenience during development of new diagnostic methods for determination of known and putative DNPS disorders is the commercial unavailability of DNPS substrates, which would be utilized as standards. Only PRPP, AICAR, SAMP and IMP could be purchased. Therefore, procedures for preparation of other DNPS substrates had to be developed in our laboratory (Zikanova M. *et al.*,2005; Baresova V. *et al.*,2016; Madrova L. *et al.*,2018).

The methods exploiting HPLC-DAD for analysis of Alr, CAIr, SAICAr, AICAr, Inosine and SAdo and their respective ribotides were validated in our group (Zikanova M. *et al.*,2005; Baresova V. *et al.*,2016). All these DNPS substrates absorb UV light but the more sophisticated mass spectrometric detection instead of UV detection was required to detect PRA, GAR, FGAr and FGAMr and their ribotides. For this purpose, HPLC-MS/MS and -HRMS methods for detection of GAR, FGAr, FGAMr, Alr, CAIr, SAICAr, AICAr, FAICAr, SAdo and their ribotides were established and used for the detailed studies of DNPS (Baresova V. *et al.*,2016; Madrova L. *et al.*,2018).

The data from international human exome databases (Karczewski K.J. *et al.*,2020) indicate existing individuals with defective DNPS enzymes. To experimentally verify claims from genome databases and effects of known and putative DNPS disorders, HeLa cells genetically edited with CRISPR/Cas9 technology were utilized. HeLa cells were modified in order to get individual cell lines deficient for certain enzyme of DNPS (CR\_GART, CR\_PFAS, CR\_PAICS, CR\_ADSL and CR\_ATIC). All CR cell lines with exception of CR\_GART showed accumulation of the dephosphorylated substrate of defective enzyme in cell medium (Baresova V. *et al.*,2016; Madrova L. *et al.*,2018).

The accumulation of DNPS substrate(s) in human cell models of known and putative DNPS disorders encouraged us to screen of urine and DBS samples of patients suffering from unspecific neurological impairment lacking the diagnoses. The cohort of 1447 randomized urine samples and 365 DBS samples were tested for elevated levels of DNPS substrates. We detected a higher urinary concentration of Alr

in three samples and accumulation of FGAr in one sample. The accumulation of Alr or FGAr in body fluids can indicate PAICS or PFAS deficiency (Krijt M. *et al.*,2019). Similar approach was employed during screening of samples prepared from DBS. However, no elevated values of DNPS substrates were detected (Krijt M. *et al.*,2019). On the other hand, we used DBS (stored at RT from 2 to 23 years) for retrospective diagnosis of ADSLD (Zikanova M. *et al.*,2015). Therefore, DBS, which are routinely collected, represent a suitable material for diagnosis of DNPS disorders. Another advantage of DBS is a long-term storage even at RT and it may be easily transported to specialized analytical laboratories by regular mail.

The whole procedure of DNPS disorders diagnosis was introduced, studied and verified in our laboratory. Based on our expertise and thanks to GeneMatcher network (Sobreira N. *et al.*,2016), an international cooperation was established investigating a family with identified mutations in *PAICS* gene. The combination of Next generation sequencing and Sanger sequencing revealed mutation on chromosome 4 in exon 2 of *PAICS* gene resulting in substitution of the lysine 53 to the arginine (p.Lys53Arg) causing an early neonatal death and two siblings suffering from multiple malformations. The homozygous missense variant leads to production of enzyme PAICS with lower stability and transformed catalytic site (Pelet A. *et al.*,2019). The identified sequence of mutated *PAICS* was utilized for production of the recombinant enzyme. Further activity studies revealed significantly lowered activity when compared to controls. Body fluids of these patients were not available thus the HPLC-MS/MS analysis was performed on samples prepared from patients' fibroblast cultivated in PD medium. Samples prepared from lysates and cell medium showed neither accumulation of PAICS substrate AIR nor the dephosphorylated form, Alr. Nevertheless, the skin fibroblasts cultivated in PD medium were not able to form the purinosome, which supported all previous results. On the bases of the comprehensive study a new DNPS disorder was described as the PAICS deficiency (Pelet A. *et al.*,2019).

Our results support the future prospect of spreading the detection methods over diagnostic laboratories with a potential of decreasing the number of undiagnosed patients suffering with DNPS disorders (Kohler M. *et al.*,1999; van Werkhoven M.A. *et al.*,2013; Castro M. *et al.*,2014). Furthermore, the detailed investigation of DNPS and purinosome assembly aims to contribute to a discovery of effective treatment of DNPS disorders with the potential to target some types of cancer.

## 6 CONCLUSIONS

### 6.1 Preparation of DNPS substrates

We have developed procedures for preparation and purification of DNPS substrates GAR, FGAR, FGAMR, AIR, CAIR, SAICAR and FAICAR, their dephosphorylated analogues and isotopically labelled variants GAR- and GAR-<sup>13</sup>C<sub>2</sub> or -<sup>13</sup>C<sub>2</sub>, <sup>15</sup>N, FGAR- and FGAR-<sup>13</sup>C<sub>2</sub> or -<sup>13</sup>C<sub>2</sub>, <sup>15</sup>N, FGAMR- and FGAMR-<sup>13</sup>C<sub>2</sub> or -<sup>13</sup>C<sub>2</sub>, <sup>15</sup>N, or -<sup>13</sup>C<sub>2</sub>, <sup>15</sup>N<sub>2</sub>, AIR- and AIR-<sup>13</sup>C<sub>2</sub> or -<sup>13</sup>C<sub>2</sub>, <sup>15</sup>N, or -<sup>13</sup>C<sub>2</sub>, <sup>15</sup>N<sub>2</sub>, CAIR- and CAIR-<sup>13</sup>C<sub>2</sub> or -<sup>13</sup>C<sub>3</sub>, <sup>15</sup>N, or -<sup>13</sup>C<sub>3</sub>, <sup>15</sup>N<sub>2</sub>, SAICAR- and SAICAR-<sup>13</sup>C<sub>3</sub>, <sup>15</sup>N<sub>2</sub>, -<sup>13</sup>C<sub>3</sub>, <sup>15</sup>N or -<sup>13</sup>C<sub>4</sub>, AICAR- and AICAR-<sup>13</sup>C<sub>3</sub>, <sup>15</sup>N<sub>2</sub> and SAMP- and SAdo-<sup>13</sup>C<sub>4</sub> in vitro by enzymatic reactions.

### 6.2 Development of detection methods

Prepared DNPS substrates have served as standards for development of detection methods based on HPLC-MS/MS (Baresova V. *et al.*,2016) or HPLC-HRMS (Madrova L. *et al.*,2018). Additionally, the HRMS has enabled to capture fragmentation spectra providing valuable information for mass spectrometry databases lacking such experimental data (Madrova L. *et al.*,2018).

### 6.3 Characterization of cell lines as models of individual DNPS enzymatic defects

The hypothesis, that putative disorders of DNPS will manifest by accumulation of substrate of defective enzyme, has been verified in the studies with genetically modified HeLa cell lines with defects in individual enzymes of DNPS (CR\_GART, CR\_PFAS, CR\_PAICS, CR\_ADSL and CR\_ATIC) (Baresova V. *et al.*,2016; Madrova L. *et al.*,2018) and encouraged screening of urine and dry blood spot (DBS) samples.

### 6.4 Screening for biomarkers of known and putative DNPS disorders in urine and dry blood spots

We analysed 1447 urine and 365 DBS samples of patients with nonspecific neurological impairment lacking a diagnose and we have discovered modestly elevated concentration of AIR in three samples and extremely increased level of FGAR in one sample (Krijt M. *et al.*,2019).

### 6.5 PAICS patient

Genetic data indicating the presence of PAICS deficiency were carefully studied. After recognition of homozygous SNP, verified investigative methods including molecular genetic methods, protein functional and kinetic studies, purinosome visualisation and DNPS substrates detection methods were applied and resulted in diagnosis and description of new DNPS disorder, the PAICS deficiency (Pelet A. *et al.*,2019).

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# Seznam publikací doktoranda

1. publikace *in extenso*, které jsou podkladem disertace  
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Zikanova M, Krijt J, Skopova V, **Krijt M**, Baresova V, Kmoch S. *Screening for adenylosuccinate lyase deficiency using tandem mass spectrometry analysis of succinylpurines in neonatal dried blood spots*. Clin Biochem. **2015** Jan;48(1-2):2-7. doi: 10.1016/j.clinbiochem.2014.10.004. **IF = 2.729**

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\*These two authors contributed to the work equally.

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- b) bez IF

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