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Patologie a fyziologie *de novo* syntézy purinů

Pathology and physiology of de novo purine synthesis

Disertační práce

Vedoucí závěrečné práce/Školitel: Ing. Marie Zikánová, Ph.D.

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Poděkování

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Mates

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 aktivitními 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 odlehlé 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.

Content

List of abbreviations

aa	Amino acid
Acetyl-CoA	Acetyl coenzyme A
ADA	*Adenosine deaminase*
ade$^-$B/D/E/F/H/I	Adenosine dependent CHO cell line type B/D/E/F/H/I
ADP	Adenosine diphosphate
ADSL	*Adenylosuccinate lyase*
ADSS	*Adenylosuccinate synthetase*
ADSLD	*Adenylosuccinatelyase* deficiency
AdoK	*Adenosine kinase*
AIC	Aminoimidazolecarboxamide
AICAR/r	Aminoimidazolecarboxamide ribotide/riboside
AICARFT	*Aminoimidazole carboxamide ribonucleotide formyltransferase*
AIR/r	Aminoimidazole ribotide/riboside
AIRC	*Phosphoribosyl aminoimidazole carboxylase*
AIRS	*Phosphoribosyl aminoimidazole synthetase*
AMP	Adenosine monophosphate
AMPD	*Adenosine monophosphate deaminase*
AMPK	*Adenosine monophosphate kinase*
APRT	*Adenosine phosphoribosyl phosphoribosyl transferase*.
ATIC	*Aminoimidazole carboxamide ribonucleotide formyltransferase/IMP cyclohydrolase*
BM	Bratton-Marshall
BUdR	5-bromodeoxyuridine
cADP	Cyclic adenosine diphosphate
CAIR/r	Carboxyaminoimidazole ribotide/riboside
cAMP	Cyclic adenosine monophosphate
Cas	CRISPR associated protein
cDNA	Complementary deoxyribonucleic acid
CE	Capillary electrophoresis
CEn	Collision energy
cGMP	Cyclic guanosine monophosphate
CHO	Chinese hamster ovary cells
CIP	Calf intestine phosphatase
CK2	Casein kinase II
CMP	Cytidine monophosphate
CRISPR	Clustered regularly interspaced short palindromic repeats
crRNA	CRISPR related ribonucleic acid
CSF	Cerebrospinal fluid
CXP	Collision cell exit potential
cN-I or cN-II	*Cytosolic 5'-nucleotidase-I* or *II*
DAD	Diode array detection
DNA	Deoxyribonucleic acid
DNPS	*De novo* purine synthesis
DP	Declustering potential

EMS	Ethyl methanesulfonate
EP	Entrance potential
FAD	Flavin adenine dinucleotide
FAICAR/r	Formamidoimidazolecarboxamide ribotide/riboside
FGAMR/r	Formylglycine amidine ribotide/riboside
FGAR/r	Formylglycinamide ribotide/riboside
GABA	Gamma-aminobutyric acid
GAR/r	Glycinamide ribotide/riboside
GARFT	*Phosphoribosyl glycinamide formyltransferase*
GARS	*Phosphoribosyl glycinamide synthetase*
GART	*Phosphoribosyl glycinamide formyltransferase*, *phosphoribosyl glycinamide synthetase*, *phosphoribosyl aminoimidazole synthetase*
GDA	*Guanine deaminase*
GDP	Guanosine diphosphate
GFP	Green fluorescent protein
GMP	Guanosine monophosphate
GMPS	*Guanosine monophosphate synthase*
GMPR	*Guanosine monophosphate reductase*
GTP	Guanosine triphosphate
HEK293	Human embryonic kidney cells
HeLa	Human epithelial carcinoma cell line
HepG2	Human hepatocellular liver carcinoma cell line
HGPRT	Hypoxanthine-guanine phosphoribosyl transferase
HPLC	High pressure liquid chromatography
HPLC-HRMS	High pressure liquid chromatography-high resolution mass spectrometry
HPLC-MS	High pressure liquid chromatography-mass spectrometry
HPLC-MS/MS	High pressure liquid chromatography-tandem mass spectrometry
HRMS	High resolution mass spectrometry
Hsp70/90	Heatshock protein 70/90
IMP	Inosine monophosphate
IMPCH	*Inosine monophosphate cyclohydrolase*
IMPDH	*Inosine monophosphate dehydrogenase*
IPTG	Isopropylthiogalactoside
KC	Primary human keratinocytes
kDa	Kilodalton
LB	Luria–Bertan
LoF	Loss of function
MBP	Maltose binding protein
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MS2	Product ion
mTOR	Mammalian target of rapamycin
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
OFP	Orange fluorescent protein (548 nm)

PAICS	*Phosphoribosyl aminoimidazole carboxylase* and *Phosphoribosyl aminoimidazole succinocarboxamide synthase*
PCR	Polymerase chain reaction
pCR4-TOPO-TA	Vector for PCR product cloning
PFAS	*Phosphoribosyl formylglycinamidine synthase*
PIF	precursor ion fingerprinting
pLI	Probability of gene loss of function intolerance
PNC	Purine nucleotide cycle
PNP	*Purine nucleoside phosphorylase*
PPAT	*Phosphoribosyl pyrophosphate amidotransferase*
PRA	Phosphoribosylamine
PRPP	Phosphoribosyl pyrophosphate
PRPS	Phosphoribosyl pyrophosphate synthase
PRPSS	Phosphoribosyl pyrophosphate synthetase superactivity
PurD	*Phosphoribosyl glycinamide formyltransferase*
*Pur*L	*Phosphoribosylformylglycinamidine synthase*
PurM	*Aminoimidazole ribonucleotide synthetase*
PurN	*Phosphoribosyl glycinamide synthetase*
Q1	First quadrupole
Q2	Second quadrupole (collision cell)
Q3	Third quadrupole
rib-5-P	Ribose-5-phosphate
RNA	Ribonucleic acid
SAdo	Succinyladenosine
SAICAR/r	Succinylaminoimidazolecarboxamide ribotide/riboside
SAICARS	*Succinyl aminoimidazole carboxamide ribonucleotide synthetase*
SAMe	S-adenosyl-L-methionine
SAMP	Succinyladenosine monophosphate
Saos-2	Sarcomaosteogenic cells
SF	Human skin fibroblasts
S.O.C. medium	Super Optimal Broth medium
SNP	Single nucleotide polymorphism
TLC	Thin layer chromatography
TMP	Thymidine monophosphate
trancrRNA	Trans-activating CRISPR related ribonucleic acid
UV	Ultraviolet
UMP	Uridine monophosphate
wt	Wild type
XMP	Xanthine monophosphate
XO	*Xanthine oxidase*

1 Introduction

1.1 Purines

Purines are organic compounds with miscellaneous functions found in all living organisms. They have been shown to be universal energy source and storage compounds. Aside from this they have key roles in signalling, alterations of enzyme activities and serve as coenzymes, neurotransmitters and as basic building blocks of DNA and RNA. The purine molecule consists of two heterocycles – the imidazole ring and the pyrimidine ring (Fig. 1A), which appears in an organism with various modifications, i.e. the addition of a ribose results in a formation of nucleosides (Fig. 1B), moreover, nucleosides can be phosphorylated to nucleotides (Fig. 1C) and their mono-, di- or triphosphate forms. Monoribosides appear also in a cyclic form (Fig. 1D).

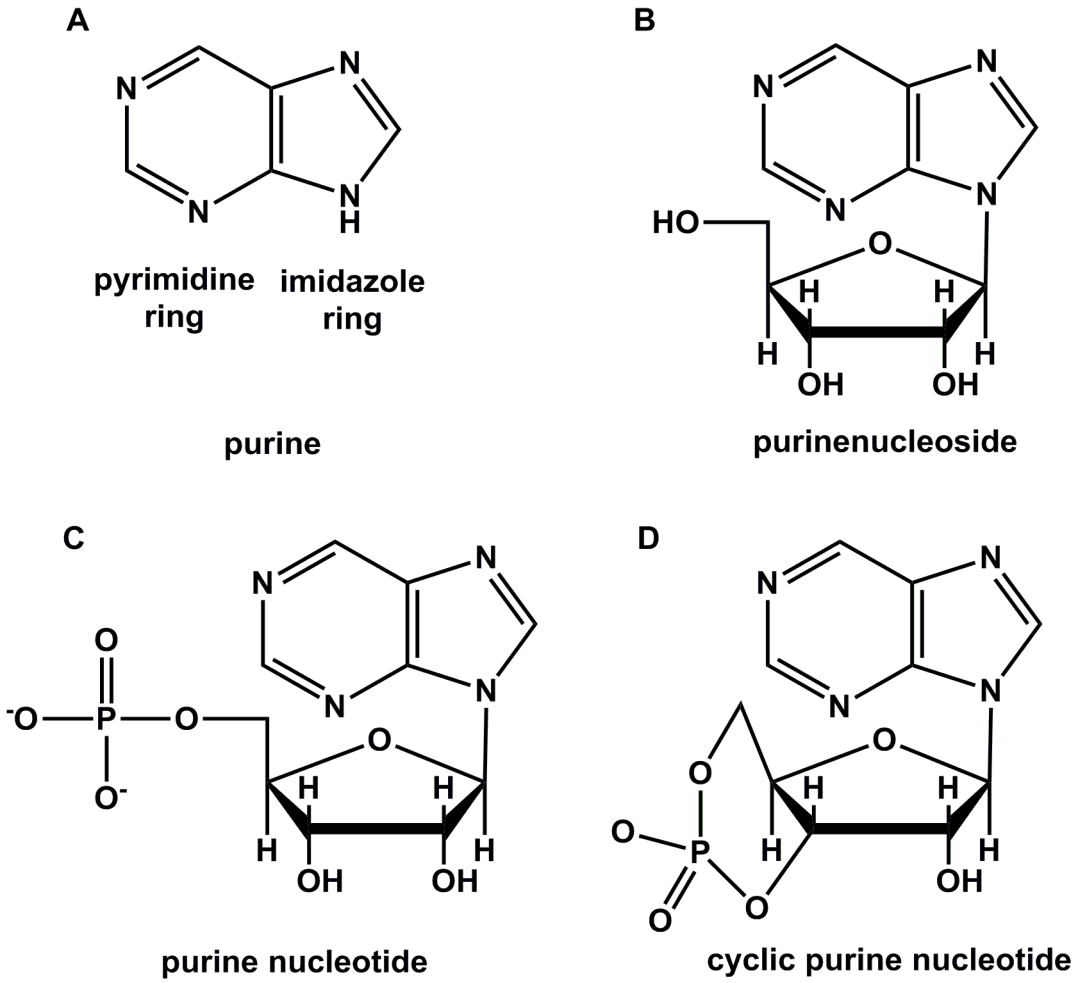

Figure 1. Basic types of purine containing compounds. The purine base (**A**) together with a bound ribose form a ribonucleoside (**B**), a ribonucleotide is formed after phosphorylation of the ribonucleoside (**C**) and the ribonucleotide may be either further phosphorylated to di- or triphosphate, or form a cyclic monoribonucleotide (**D**).

Naturally occurring species of purine bases, e.g. uric acid, hypoxanthine or adenine and guanine (Fig. 2A-D) are formed by addition of functional groups to the base molecule of purine.

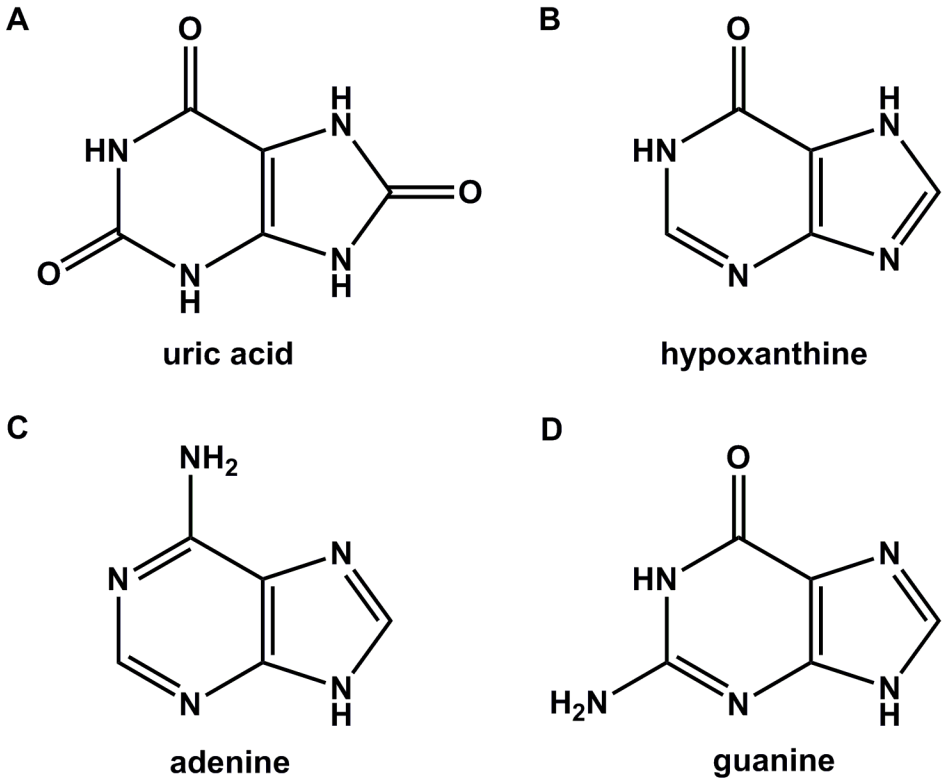

Figure 2. Selected representatives of naturally occurring purine bases. The first discovered purine base was uric acid (**A**). The close congeniality of purine bases is demonstrated by the structure of hypoxanthine **(B)**. The most important and metabolic active purines are those containing adenine **(C)** or guanine **(D)** base.

The first isolated purine base was the uric acid from urinary calculi by Carl Wilhelm Scheele (1742 – 1786) (Richet G.,1995). However, it was Emil Fisher (1852 – 1919, NB 1902) (Nagendrappa G.,2011), who showed that uric acid, caffeine and xanthine have similar chemical origin containing two nitrogenous heterocycles – the purine – and thus established brand new category of chemical compounds. John M. Buchanan (1917 – 2007) was next eminent contributor to the purine biochemistry. The fact that hypoxantine is produced by assembly of small molecules through the intermediate inosine (Greenberg G.R.,1950) motivated Buchanan and his group to trace small radioactively labelled molecules glycine and carboxamide added to pigeon liver homogenates. These experiments resulted in a discovery of a way the glycine and carboxamide are incorporated into newly produced molecules of hypoxantine (Schulman M.P. *et al.*,1952). Thus, the biochemical pathway producing new molecules of purines was suggested and it was later named as the *de novo* purine synthesis (DNPS) pathway (Levenberg B. *et al.*,1957).

An observation that the enzyme nucleotide phosphorylase catalyses reversible assembly of nucleotide base and ribose-1-phosphate in order to create the nucleotide molecule contributed to a description of the purine salvage pathways (Kalckar H.M.,1947). John M. Buchanan confirmed the existence of salvage pathways by a discovery that ribose and phosphate groups are connected directly to purine bases (Schulman M.P. *et al.*,1952) and proved that the purine base 4-amino-5-imidazolecarboxamide is converted into ribotide form after incubation with ribose-1-phosphate in pigeon liver protein extract (Korn E.D. *et al.*,1955).

1.1.1 Functions of purines

Purines provide energy for many metabolic processes, constitute the building blocks of deoxyribonucleic acid (DNA), ribonucleic acid (RNA) and many cofactors, i.e. nicotinamide adenine dinucleotide (NAD) (Fig. 3), flavin adenine dinucleotide (FAD) or acetyl coenzyme A (acetyl-CoA). In addition, they participate in cell signalling or function as neuromodulators and co-transmitters.

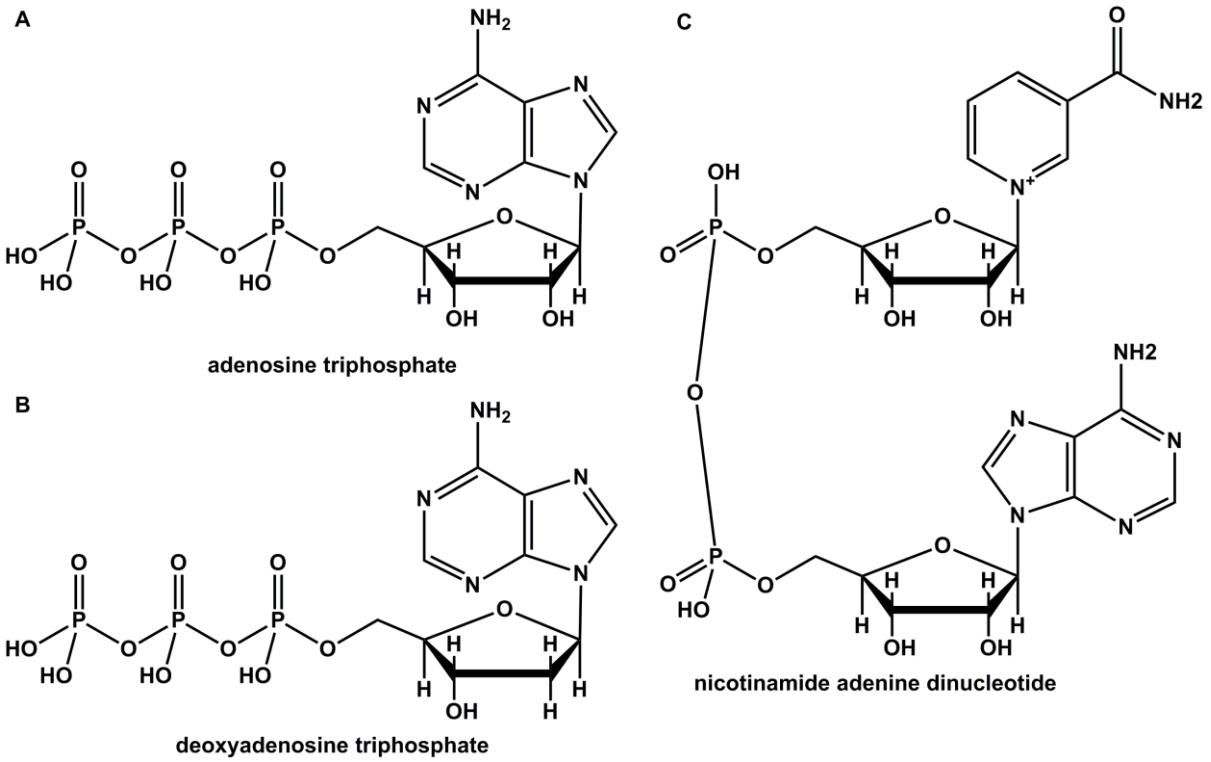

Figure 3. Structure of large biomolecules containing purine bases. The adenosine triphosphate **(A)** is used as a substrate in the RNA formation while the deoxyadenosine triphosphate **(B)** in the DNA formation. Adenosine monophosphate figures also as a prosthetic group in many cofactors, e.g. NAD **(C)**.

The most iconic role of purines adenosine monophosphate (AMP) and guanosine monophosphate (GMP) together with pyrimidines cytidine monophosphate (CMP) and thymidine monophosphate

(TMP), respectively uridine monophosphate (UMP), is as basic building blocks of DNA and RNA, the carriers of genetic information.

AMP is the molecule governing also cellular energy metabolism regulated by *AMP activated protein kinase* (AMPK), which utilizes AMP for maintaining the cellular homeostasis by phosphorylation of selected enzymes (Cameron K.O. and Kurumbail R.G.,2016). AMP may be phosphorylated to ADP and then to ATP, the universal energy carriers, according to actual demands.

AMP is present as prosthetic group in cofactor molecules like $NAD(P)^+$/NAD(P)H, FAD and acetylCoA. NAD^+/NADH are cofactors of oxidoreductases, the enzyme family responsible for electron transfer and that is also found in energy metabolism pathways, mainly in glycolysis. Together with enzyme poly-(ADP-ribose) polymerase 1 participates NAD^+ in DNA repair and cell death pathways (de Murcia G. and de Murcia J.M.,1994). Hereto serves NAD^+ as a substrate for sirtuin family of deacetylases (Smith B. *et al.*,2009) with the influence on transcription, apoptosis and inflammation (Preyat N. and Leo O.,2013). NAD^+ is also a precursor for cADP-ribose, which functions as calcium-mobilizing agent in astrocytes (Pawlikowska L. *et al.*,1996). NAD^+/NADH together with their phosphorylated forms $NADP^+$/NADPH exploits the redox potential in mitochondrial shuttles. Furthermore, $NADP^+$/NADPH are irreplaceable cofactors for glutathion reductase and thioredoxine reductase, which are the enzymes instrumental in responses to oxidative stress (Buettner G.R. *et al.*,2013). Additionally, $NADP^+$/NADPH contribute to biosynthesis of lipids, amino acids and nucleotides, in pentose phosphate pathway and to cell signalling by modulating Ca^+ channel (Lunt S.Y. and Heiden M.G.V.,2011).

FAD possesses similar properties as NAD^+ and in addition has even more positive reduction potential than NAD^+ (Finn R.D. *et al.*,2003). FAD is involved in citric acid cycle, mitochondrial electron transport chain, fatty acid oxidation, dopamine catabolism, niacine (vitamin B_3) synthesis and other metabolic processes (Lienhart W. *et al.*,2013; Mosegaard S. *et al.*,2020).

AMP is also part of acetyl-CoA, which contributes to metabolism of glucose, fatty acids and amino acids and on top of this influence regulatory mechanisms through DNA acetylation (Shi L. and Tu B.P.,2015). When AMP loses phosphate group and transforms into the ribonucleoside adenosine, it functions as a neuromodulator. In the hippocampus and basal ganglia, adenosine has important function in synaptic/non synaptic neuronal excitability (Sperlagh B. and Vizi E.S.,2011).

AMP together with GMP possess feedback inhibitory effect on *phosphoribosyl pyrophosphate amidotransferase* (PPAT) (Holmes E.W. *et al.*,1973) and enzymes converting inosine monophosphate (IMP) into the respective nucleotides (Holmes E.W. *et al.*,1974). In general, DNPS is regulated by end products of purine and pyrimidine synthesis, where AMP and GMP are the major contributors (Berg J.M. *et al.*,2002).

The next nucleotide, GMP, possesses an irreplaceable function as a second messenger in the form of cyclic GMP (cGMP) through cGMP dependent kinases. The cGMP mediates signal from captured

light to cation channel in retina (Iribarne M. and Masai I.,2017), contributes to vasodilatation of smooth muscles (Lincoln T.M., 1989), and participates in intracellular Ca^{2+} modulations (Lohmann S.M. *et al.*,1997), metabolic processes such as gluconeogenesis (Degerman E. *et al.*,2011) and apoptosis (Jeong S.O. *et al.*,2017). However, cyclic AMP (cAMP) also functions as a second messenger. There are many cellular processes involving cAMP via cAMP-dependent protein kinases (Francis S.H. and Corbin J. D.,1999).

1.1.2 Metabolism of purines

The natural balance of purine levels is maintained by their synthesis, recycling and degradation pathways. Food is a major source of purine molecules, where they appear typically in a form of DNA and RNA (He Y. *et al.*,1994). These molecules are digested into purine nucleotides in gastrointestinal tract. Of note is, that nucleotides cannot be transported through intestinal membrane and must be dephosphorylated to nucleosides in the small intestine by various enzymes with nucleotidase activity. Overall, enterocytes uptake purines mostly in the form of nucleobases, adenosine, guanine, xanthine and hypoxantine (He Y. *et al.*,1994), which is facilitated by nucleoside and nucleobase transporters (Pastor-Anglada M. *et al.*,2018). Internalized nucleobases then either immediately enter the salvage pathways in enterocytes, or they are transported through blood stream into other cell types (Uauy R. *et al.*,1994). In children, 75% of hypoxantine and guanine are recycled by *hypoxanthine-guanine phosphoribosyltransferase* (HGPRT) (Murray A.W.,1971). Purine mononucleotides may be utilized in various biological processes or undergo additional phosphorylation in order to enter the metabolism in their di- or tri- phosphate form.

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, the *de novo* purine synthesis (DNPS) is activated during conditions when there is a high purine demand. New molecules of IMP are produced from multiple precursors during DNPS. Eventually, IMP is further converted into AMP and/or GMP.

1.1.2.1 Salvage pathways

The purine nucleosides (adenosine, guanosine and inosine) may enter the degradation or salvage pathways. The salvage pathways in human enable the conversion of nucleosides into nucleotides and recycling of purine bases into nucleotides (Fig. 4) (Friedman T.,1969).

Only the nucleoside adenosine may be metabolised directly to AMP by adenosine kinase (AdoK) (Snyder F.F. and Lukey T.,1982). But AdoK has relatively low Km (approximately 1μM) compared

to *adenosine deaminase* (ADA, 20 - 120 μM) (Ipata P.L. *et al.*,2011), which preferentially converts adenosine into inosine (Fig. 4).

Inosine and guanosine as substrates for purine nucleotide phosphorylase (PNP) may be degraded to purine bases and ribose-1-phosphate (Canduri F. *et al.*,2004). Originated purine bases may proceed the degradation pathway or convert together with phosphoribosyl pyrophosphate (PRPP) to the IMP or GMP, respectively, in the reaction catalysed by HGPRT. Similarly, adenine phosphoribosyltransferase (APRT) catalyses the reaction of adenine with PRPP to form AMP (Fig. 4).

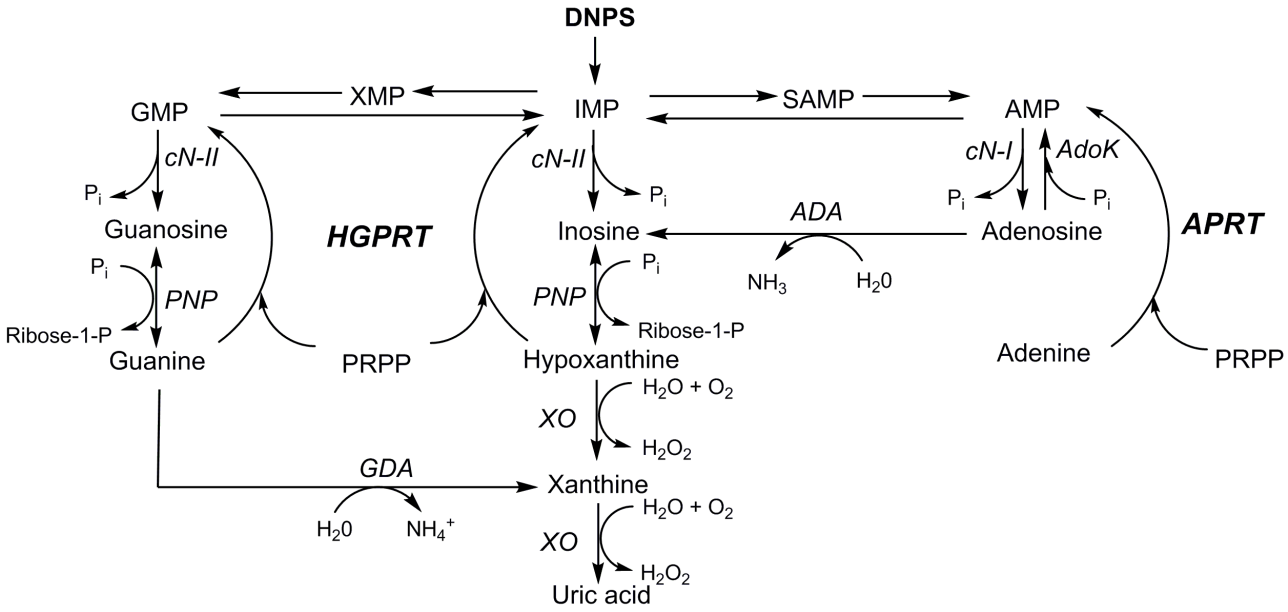

Figure 4. Salvage and degradation pathways of purine metabolites. Guanine, hypoxantine and adenine may be converted into nucleotides by salvage pathways or catabolized by degradation pathway into uric acid which is the end product of human purine metabolism.
cN-I or cN-II = *cytosolic 5'-nucleotidase-I* 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*.

1.1.3 Degradation pathway

The purine monophosphate degradation pathway begins with dephosphorylation of nucleotides to nucleosides by *cytosolic 5'-nucleotidases* (cN). The cN-I is specific for AMP and cN-II for IMP and GMP (Ipata P.L. *et al.*,2011). After dephosphorylation by PNP, guanine is deaminated and hypoxantine is oxidised to yield xanthine, which is further oxidised and converted into uric acid, the final product of purine degradation pathway in humans (Ihler G. *et al*.,1975) (Fig. 4). Uric acid is then eliminated from organism in urine. Other mammalian species possess enzyme *uricase*, which carries out an oxidation of uric acid to allantoin (Johnson W. *et al.*,1969). The side product of degradation

pathways, ribose-1-phosphate, may enter the pyrimidine salvage pathway or may be converted into ribose-5-phosphate (rib-5-P) by enzyme *phosphopentomutase*. Rib-5-P participates in various metabolic pathways. It can take part in pentose phosphate pathway, glycolysis or is activated into PRPP, which is the precursor in the salvage pathways and DNPS (Tozzi M.G. *et al.*,2006).

1.1.4 The de novo purine synthesis and purine nucleotide cycle (PNC)

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. 5).

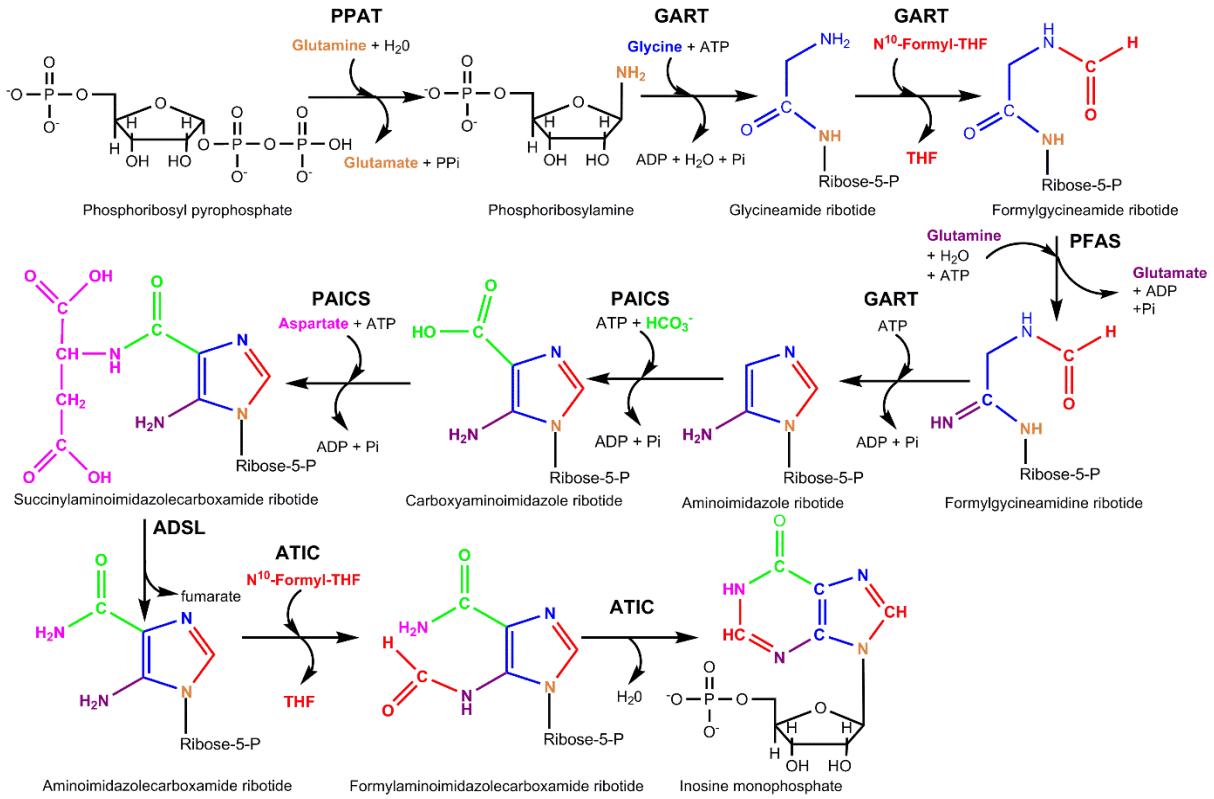

Figure 5. De novo purine synthesis. The metabolic flux through DNPS produces the IMP, which is assembled by stepwise addition of whole molecules or functional groups of organic, inorganic or amino acid origin to starting molecule of PRPP. The synthesis of IMP occurs in ten reactions catalysed by six enzymes (Tab 1).

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

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

The PRPP is synthesized from rib-5-P and ATP in a reaction catalysed by *phosphoribosyl pyrophosphate synthase* (PRPS, EC 2.7.6.1). In the first reaction of DNPS driven by *phosphoribosyl pyrophosphate amidotransferase* (PPAT, EC 2.4.2.14) PRPP releases pyrophosphate and gains amino group from glutamine. The product phosphoribosylamine (PRA) is very unstable with half-life of 5 s under physiological conditions (Rudolph J. and Stubbe J.,1995). The second, third and fifth reaction of DNPS are catalysed by the trifunctional enzyme GART (*phosphoribosyl glycinamide synthetase*, EC 6.3.4.13; *phosphoribosyl glycinamide formyltransferase*, EC 2.1.2.2; and *phosphoribosyl aminoimidazole synthetase*, EC 6.3.3.1). The first reaction of GART consumes one molecule of ATP to fuse the whole molecule of glycine with PRA and glycinamide ribotide (GAR) is created as a result. In the second reaction, GART catalyses addition of one-carbon residue from N^{10}-formyl tetrahydrofolate (N^{10}-fTHF) to GAR and formylglycine aminoribotide (FGAR) is formed. Next, in a reaction catalysed by another enzyme, *phosphoribosyl formylglycinamidine synthase* (PFAS, EC 6.3.5.3), the amino group from glutamine is used to exchange ketone group of FGAR for imino group and one molecule of ATP is consumed in order to produce formylglycine amidine ribotide (FGAMR). The following reaction catalysed again by GART that consumes one molecule of ATP to close the imidazole ring of aminoimidazole ribotide (AIR). The next two reactions are catalysed by bifunctional enzyme PAICS (*phosphoribosyl aminoimidazole carboxylase*, EC 4.1.1.21 and *phosphoribosyl aminoimidazole succinocarboxamide synthase*, EC 6.3.2.6) and both reactions consume a molecule of ATP. In the first

reaction, PAICS catalyses the addition of the whole molecule of bicarbonate to AIR and creates carboxyaminoimidazole ribotide (CAIR). In the second reaction, a whole molecule of aspartate is attached to CAIR to yield succinylaminoimidazole carboxamide ribotide (SAICAR). The production of aminoimidazole carboxamide ribotide (AICAR) is catalysed by bifunctional enzyme ADSL (*adenylosuccinatelyase* EC 4.3.2.2). ADSL catalyses an elimination of fumarate from SAICAR in DNPS, while the second catalytic activity of ADSL is exerted in the purine nucleotide cycle (PNC) (Fig. 6) during conversion of succinyladenosine monophosphate (SAMP) into AMP. Last two reactions of the DNPS are catalysed by a bifunctional enzyme ATIC (*phosphoribosyl aminoimidazole carboxamide formyl transferase*, EC 2.1.2.3 and *IMP cyclohydrolase*, EC 3.5.4.10). In the first reaction, the ATIC catalyses addition of one-carbon residue from N^{10}-fTHF to AICAR to form formylaminoimidazole carboxamide ribotide (FAICAR). In the second reaction, the pyrimidine ring is closed and finally a new molecule of IMP is produced. Additionally, IMP is converted to AMP and GMP (Fig. 6). The production of AMP is a two-step process. In the first step, *adenylosuccinate synthetase* (ADSS) consumes one molecule of GTP to add aspartate to IMP in order to get SAMP. In the second step, fumarate is cleaved from SAMP by ADSL and AMP is thus produced. AMP may be converted back to IMP by *AMP deaminase* (AMPD). The two-step synthesis of GMP begins with conversion of IMP into xanthine monophosphate (XMP) by *inosine monophosphate dehydrogenase* (IMPDH), which utilizes NAD^+ as the cofactor. In the second step catalysed by *GMP synthase* (GMPS), one molecule of ATP is consumed and glutamine provides an amino group in order to convert XMP to GMP. GMP may be metabolized by *GMP reductase* (GMPR), with NADPH as a cofactor, back to IMP. The production of purine monophosphates is in balance with the consumption of GTP in case of AMP production and ATP in case of GMP production, respectively.

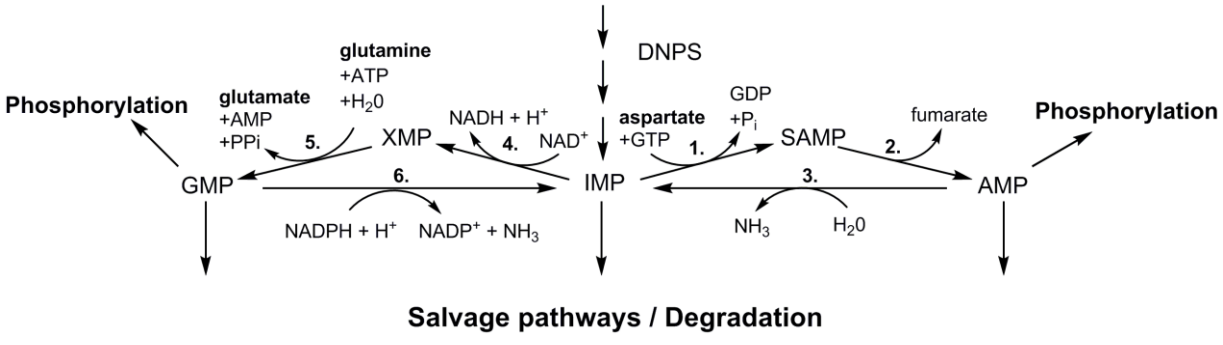

Figure 6. Purine nucleotide interconversions. IMP is converted into AMP and GMP in two steps. Synthesis of AMP requires GTP as substrate and vice versa, which contributes to equal production of both metabolites. **1.** ADSS = *adenylosuccinate synthetase*, **2.** ADSL = *adenylosuccinatelyase*, **3.** *AMP deaminase* (AMPD), **4.** *inosine monophosphate dehydrogenase* (IMPDH), **5.** *GMP synthase* (GMPS), **6.** *GMP reductase* (GMPR).

1.1.4.1 DNPS enzymes

1.1.4.1.1 PPAT

PPAT (Gene ID: 5471, EC 2.4.2.14) catalyses the first step of DNPS. The gene for human PPAT is located on chromosome 4 and encodes 517 amino acids (aa) long enzyme with the deduced molecular weight of 57398 Da (Iwahana H. *et al.*,1993). The active form of this allosteric enzyme consists of four identical subunits (Smith J. *et al.*,1994). A glutamine domain conserved through the amidotransferase family is characterised by a cysteine residue utilized for binding glutamine and a transferase domain specifically binds the PRPP. Irreversible conversion of PRPP into PRA is a rate limiting step of DNPS. Activation of tetrameric structure is initiated with high concentrations of PRPP, while PPAT is on the other hand regulated by a feedback inhibition of AMP, GMP and pyrimidine nucleotides (Holmes E.D. *et al.*,1974).

1.1.4.1.2 Trifunctional GART

The enzyme GART (Gene ID: 2618) is represented in human by protein with three enzymatic activities (Daubner S.C. *et al.*,1986). All three enzymatic activities catalysing the second, third and fifth reaction of DNPS pathway were first identified in a somatic model of CHO cell lines carrying defects in individual steps of DNPS pathway (Patterson D.,1975). The gene for human GART is located on the chromosome 21 (Patterson D. *et al.*,1981) and encodes 110 kDa trifunctional protein consisting of 1010 aa, as well as an alternative splicing variant of monofunctional enzyme GARS with molecular weight of 50 kDa, which is also metabolically active (Aimi J. *et al.*,1990; Brodsky G. *et al.*,1997). GART functional structure is assembled from two monomers (Welin M. *et al.*,2010) and catalyses second (GARS, EC 6.3.4.13), third (GARFT, EC 2.1.2.2) and fifth (AIRS, EC 6.3.3.1) step of DNPS. GART and GARS levels and functions were studied in patients with Down syndrome, in view of their location on chromosome 21. It was shown that in cerebellum of human patients, the monofunctional GARS remained active after birth, in contrast to the trifunctional GART (Brodsky G. *et al.*,1997). The enzyme GART is a target of anti-cancer drugs due to the connection with folate metabolism, which provides formyl groups also for DNPS (Sato Y. *et al.*,2018).

1.1.4.1.3 PFAS

PFAS (Gene ID: 5198, EC 6.3.5.3) also called *formylglycine aminoribotide amidotransferase* (FGARAT) or *phosphoribosyl formylglycinamidine synthase* (FGAMS) catalyses the fourth step of DNPS. The gene for human PFAS is located on chromosome 17 and codes a protein with molecular weight of 150 kDa (Barnes T.S. *et al.*,1994) consisting of 1371 aa (Patterson D. *et al.*,1999) and the active enzyme is assembled from four monomers (Mathews I.I. *et al.*,2006).

1.1.4.1.4 Bifunctional PAICS

The bifunctional enzyme PAICS (Gene ID: 10606) catalyses the sixth (AIRC, EC 4.1.1.21) and seventh (SAICARS, EC 6.3.2.6) step of DNPS. PAICS gene is located on chromosome 4 (Brayton K.A. *et al.*, 1994) and encodes 428 aa sequence (Li S.X. *et al.*,2007). PAICS dual function and molecular weight of 50 kDa was suggested by Patey and Shaw (Patey C.A.H. and Shaw G.,1973) and lately refined to 47 kDa (Li S.X. *et al.*,2007). The active PAICS forms an octamer consisting of identical subunits with molecular weight of 47 kDa creating a structure suitable for substrate channelling (Li S.X. *et al.*,2007). PAICS enzymatic activity was found to be reversible, thus enabling conversion of SAICAR to AIR (Tyagi A.K. *et al.*,1980; Firestine S.M. *et al.*,1994). PAICS was identified as an oncogene in lung adenocarcinoma (Goswami M.T. *et al.*,2015; Zhou S. *et al.*,2019), breast (Meng M. *et al.*,2018) and prostate cancer (Chakravarthi B.V.S.K. *et al.*,2017). Thus, several PAICS inhibitors (Chakravarthi B.V.S.K. *et al.*,2017; Goswami M.T. *et al.*,2015) and siRNA (Meng M. *et al.*,2018) were proposed, which significantly reduced cancer cell growth.

1.1.4.1.5 ADSL

The enzyme ADSL (Gene ID: 158, EC 4.3.2.2) is involved in two different but closely related biochemical pathways, DNPS and PNC. In DNPS, ADSL catalyses the eighth step while in PNC, this enzyme participates in AMP production. The ADSL gene was cloned (Stone R.L. *et al.*,1993) and mapped to chromosome 22q13.1-q13.2 (Fon E.A. *et al.*,1993). The ADSL gene encodes 52 kDa protein consisting of 459 aa, which forms a homotetramer in an active state (Stone R.L. *et al.*,1993; Kmoch S. *et al.*,2000).

1.1.4.1.6 Bifunctional ATIC

The bifunctional enzyme ATIC (Gene ID: 471) is represented by polypeptide chain with the activities of AICARFT (EC 2.1.2.3) and IMPCH (EC 3.5.4.10) (Ni L. *et al.*,1991; Ebbole D.J. and Zalkin H.,1987; Chopra A.K. *et al.*,1991). It catalyses last two steps of DNPS. The gene sequence for ATIC is spanned on chromosome 2 (Colleoni G. *et al.*,2000). The human ATIC cDNA codes 591 aa protein with molecular weight of 64.4 kDa (Ni L. *et al*.,1991; Rayl E. *et al.*,1996), which functions in homodimeric fashion (Cheong C.-G. *et al.*,2004). The AICARFT activity is located on C- terminus, whereas the IMPCH activity is on N-terminus (Rayl E. *et al.*,1996). Since one of enzymes' substrates is folate, ATIC, as well as GART, are inhibited by antifolate drugs such as methotrexate (Cao H. *et al.*,2018). The first product of ATIC, AICAR, is also a by-product of histidine synthesis (Baggott J.E. and Tamura T.,2015) and high levels of AICAR activate AMPK (Asby D.J. *et al.*,2015), which induces formation of PFAS granules resulting in DNPS blockage (Schmitt D.L. *et al.*,2016).

1.1.4.2 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, which 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 either green fluorescent protein (GFP) or orange fluorescent protein (OFP) (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 Baresova et al. (2012), who detected purinosome formation by confocal microscopy of immunolabelled DNPS endogenous proteins in various cell types cultivated in PR and PD medium. In all investigated cell lines, including HeLa cells, human hepatocellular liver carcinoma cell line (HepG2), sarcoma osteogenic cells (Saos-2), human embryonic kidney cells (HEK293), human skin fibroblasts (SF) and primary human keratinocytes (KC), the purinosome assembly was demonstrated in PD medium (Baresova V. *et al.*,2012). Then, an objection against the formation of purinosome after transient transfection with DNPS proteins was made and a theory of a protein aggregation was suggested (Zhao A. *et al.*,2013). The authors claimed, that formation of the protein aggregates was induced by a stress due to nutrient deprivation in the purine depleted medium and that the protein aggregate formation was supported by blocking protein degradation. These statements were in concordance with co-localization of transiently transfected purine enzymes with Hsp70, Hsp90 or ubiquitine (Zhao A. *et al.*,2013). However, a study of Pedley and co-workers proved that Hsp70 and Hsp90 sustain formation of purinosome (French J.B. *et al.*,2013). Finally, to support the formation of purinosome, a study was devised in with combination of transient transfection and immunolabeling of the endogenous proteins, which finally confirmed of the purinosome existence (Baresova V. *et al.*,2018).

Several contributors other than Hsp90 were identified to regulate purinosome formation. Purinosome disassembles under certain conditions which include the purine supplementation (An S. *et al.*,2008), inhibition of Hsp90 (French J.B. *et al.*,2013) or inhibition of microtubule polymerization (An S. *et al.*,2010a), mitochondrial network (French J.B. *et al.*,2016) and mutations in genes encoding DNPS enzymes, specifically ADSL and ATIC (Baresova V. *et al.*,2012). In particular, deficiency in ADSL, ATIC and GART leads to a complete loss of purinosomes, while PFAS and PAICS insufficiency results in partial but significant decrease in purinosome formation (Baresova V. *et al.*,2016). The protein-protein

mapping of proximity revealed that a purinosome core consists of the first three DNPS proteins PPAT, GART and PFAS, respectively (Deng Y. *et al.*,2012). Besides ADSL, PAICS and ATIC, enzymes ADSS and IMPDH from PNC were additionally identified as the members of purinosome (Zhao H. *et al.*,2015). Moreover, Hsp90 and *casein kinase II* (CK2) were found to promote the purinosome formation (French J.B. *et al.*,2013; An S. *et al.*,2010b) and G-protein-coupled receptor (GPCR) was shown to trigger purinosome assembly in HeLa cells (Fang Y. *et al.*,2013). The purinosome assembly is also stimulated in G1 and S phase of cell cycle (Chan C.Y. *et al.*,2015), by purine depletion in growth medium (An S. *et al.*,2008). Eventually, non-physiological conditions like HPRT deficiency as well as PRPS superactivity (PRPSS) compel the production of purines through DNPS pathway (Fu R. *et al.*,2015; Becker M.A. *et al.*,1987). It seems that PRPSS stimulates purinosome formation and arguably causes urate overproduction resulting in gout and, in some severe cases, nephrolithiasis and acute kidney disease (Zikanova M. *et al.*,2018).

An effort to describe purinosome spatial distribution by fluorescence microscopy showed its proximity to microtubule network (An S. *et al.*,2010a) and mitochondria (French J.B. *et al.*,2016). A disruption of microtubule network by administration of nocodazole (An S. *et al.*,2010a) or mitochondrial activity by antimycin A and oligomycin (French J.B. *et al.*,2016) affected the co-localization with purinosome and has decreased metabolic flux through DNPS. Mitochondria assist in supplying the DNPS with 5 ATP molecules required for production of one IMP molecule and they help by generating the cofactor N^{10}-fTHF responsible for transfer of formyl residue demanded by GARFT and AICARFT. Studies of mitochondrial contribution to purinosome localization and function revealed that mammalian target of rapamycin (mTOR) enhances the production of mitochondrial tetrahydrofolate (mTHF) cycle enzyme *methenylenetetrahydrofolate dehydrogenase 2* in nucleus through mTORC1-mediated activation of transcription factor 4 (Ben-Sahra I. and Manning B.D.,2017). Furthermore, it was shown that mTOR potentiates genes of pentose phosphate cycle and therefore contribute to PRPP biosynthesis (Duvel K. *et al.*,2010). In conclusion, the comprehensive investigation suggests novel alternatives for cancer therapeutic strategies through disruption of purinosome.

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 gnomaAD (Karczewski K.J. *et al.*,2020) show that there are no evolutionary constraints against loss of function (LoF) or missense

mutations in DNPS genes except that of PPAT. Data provided herein represent the range of genetic variability (z score) and the probability of gene loss of function intolerance (pLI) (Tab. 2).

Table 2. The probability of existence of disorders in DNPS. A number of *in silico* predicted mutations in genes coding DNPS enzymes and the number observed by sequenations resulted in calculation of z score and LoF and pLI values determining the evolutionary constraint against existence of individuals with defects in DNPS enzymes. The closer pLI is to 1, the lower the probability of occurrence is (URL: http://gnomad.broadinstitute.org) [June 2019 accessed].

Constraint from gnomAD	Expected no. variants	Observed no. variants	Constraint Metric
PPAT			
Synonymous	67.8	55	z = 0.97
Missense	164	73	z = 3.48
LoF	19.6	1	**pLI = 0.99**
GART			
Synonymous	125.1	136	z = -0.60
Missense	294.9	319	z = -0.69
LoF	33.7	9	**pLI = 0.06**
PFAS			
Synonymous	232.2	256	z = -0.97
Missense	544.2	506	z = 0.80
LoF	47.4	21	**pLI = 0.00**
PAICS			
Synonymous	32.7	36	z = -0.36
Missense	79.1	88	z = -0.49
LoF	12	4	**pLI = 0.07**
ADSL			
Synonymous	69.1	59	z = 0.75
Missense	190.3	164	z = 0.93
LoF	22.8	10	**pLI = 0.00**
ATIC			
Synonymous	94.8	108	z = -0.84
Missense	198.4	235	z = -1.27
LoF	23.6	21	**pLI = 0.00**

Z score was calculated as a difference between observed counts and expected numbers of synonymous and missense mutations. Genes with more observed variants than expected were predicted to have negative z score. On the other hand, positive z score with lower number of variants than expected signifies increased intolerance to variation. LoF determines the probability of tolerance to loss of function of a given gene. The closer pLI is to one, the more the gene seems to be LoF intolerant.

Therefore, individuals with biallelic mutations in *GART*, *PFAS* and *PAICS* should theoretically exist. Disorders, which these genes are associated with may manifest with similar symptoms as known DNPS disorders, such as nonspecific neurological and neuromuscular features and the accumulation

of specific DNPS substrates in body fluids. The only exception is suggested for a putative disorder involving the first catalytic function of *GART* due to an instability of the substrate PRA.

1.2.1 Adenylosuccinatelyase deficiency (ADSLD)

At first, ADSLD was described in 1984 in three patients suffering from psychomotor delay and autism. Routine biochemical analyses of these patients had not revealed any abnormalities. Afterwards, the thin layer chromatography (TLC) analysis showed two unusual spots which were later identified as succinylaminoimidazolecarboxamide riboside (SAICAr) and succinyladenosine (SAdo) by HPLC in urine, plasma and cerebrospinal fluid (CSF) (Jaeken J. and van den Berghe G.,1984). Based on this discovery Jaeken and van den Berghe have postulated a new disorder – the ADSL deficiency.

Diagnostic methods of ADSLD are primarily based on detection of accumulated succinylpurines SAICAr and SAdo (concentrations are in order of hundreds of μM) in urine, CSF or plasma. The TLC with Pauly reagent or Bratton-Marshall (BM) test usually served for this purpose in the past. Currently, there are more diagnostic techniques with higher specificity in the art, including HPLC coupled with UV or mass spectrometric detection. Alternatively, the ADSLD may be diagnosed and/or confirmed with genomic or cDNA sequencing and/or the enzyme kinetic assays.

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). Prenatal symptoms such as pulmonary hypoplasia, impaired intrauterine growth and foetal hypokinesia could also appear (Mouchegh K. *et al.*,2007). The patients pass away soon after birth. The most common mutations identified in neonatal fatal form of ADSLD is the heterozygous combination of c.340T>C and c.1277G>A resulting in substitutions of amino acids p.Tyr114His and p.Arg426His, respectively, which are located in an active site and a substrate channelling part of the ADSL enzyme

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 most common mutations identified in severe childhood form showed homozygous appearance of c.1277G>A, c.1312T>C, c.674T>C and c.1107A>C resulting in amino acid substitutions p.Arg426His, p.Ser438Pro, p.Met225Thr and p.Ile369Leu, which are located in the substrate channelling part of ADSL enzyme. Other mutations appear mostly solely

in heterozygous combinations and influence active site, substrate channelling, central helical region or substrate subunit of the ADSL enzyme.

The mild to moderate form (Type II) is defined with SAdo/SAICAr ratio larger than 2. The first reported case study was a patient with an abnormal concentration for SAdo (475 µM) compared to SAICAr with the SAdo/SAICAr ratio of 3.7 (Jaeken J. *et al.*,1988; Jurecka A. *et al.*,2008a). 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.*,2008a) or even up to the age 9 (Gitiaux C. *et al.*,2009). The most common mutations found in mild to moderate form is homozygous c.1277G>A and c.907C>T causing amino acid substitutions p.Arg426His and p.Arg303Cys in substrate channelling and active site of the ADSL enzyme, respectively. Other mutations occur independently in heterozygous combinations causing mutations in central helical region, active site or substrate channelling part of the ADSL enzyme (http://www1.lf1.cuni.cz/udmp/adsl/index.php?adsltable=orderbyphenotypedesc, November 2019).

The very mild form (Type III) was described in two individuals with SAdo/SAICAr ratio larger than 3.5 (Macchiaiolo M. *et al.*,2020). The first mild form of ADSL was diagnosed by whole exome sequencing showing mutation c.926G>A and splice site change c.1191+5G>C causing amino acid substitution p.Arg309His and shortening of Exome11 for 11 aa (Macchiaiolo M. *et al.*,2017). Both aberrations led to the enzyme instability and caused SAdo/SAICAr accumulation with the ratio slightly over 2, which was attributed to ADSLD Type II. However, this patient has shown very mild symptoms. The new type of ADSL was recently confirmed in two patients manifesting mild hypotonia, psychomotor and speech delays. No other symptoms or abnormal results from routinely carried out diagnostic methods were present until clinical exome sequencing revealed missense variants in the ADSL gene. The compound heterozygous combination of c.76A>T and c.1187G>A was detected leading to amino acid substitutions p.Met26Leu and p.Arg396His and functional studies confirmed that the aforementioned variants are the underlying cause of the pathology (Macchiaiolo M. *et al.*,2020).

Investigations of metabolic fate of radioactively labelled compounds related to DNPS have also revealed metabolic abnormalities in individual cases. The addition of ^{14}C-formate to patients' fibroblasts led to discoveries that a residual activity of ADSL as low as 3 % is sufficient for required production of AMP, but results in a massive accumulation of SAMP and SAdo, respectively (van den Bergh F. *et al.*,1993). The comparison of functional studies has revealed that the disease prognoses depend on factors like the residual enzyme activity and stability of the mutated enzyme, which could be hardly predicted from genetic sequence (Ariyananda L.D.Z. *et al.*,2009; Kmoch S. *et al.*,2000; Zikanova M. *et al.*,2010; Ray S.P.,2012). However, such relationship may be studied on recombinant mutant proteins (Jurecka A. *et al.*,2015).

Still, 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 (Slavic, Romany, Spanish, Italian, Dutch, Belgian and German) and the Mediterranean (Morrocan, Turkish) region followed by cases from Brazil and US (http://www1.lf1.cuni.cz/udmp/adsl/index.php?adsltable=orderbypatientasc, November 2019). The disorder may be underdiagnosed as it is probably panethnic (Kmoch 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, dysmorfic features and congenital blindness. All metabolic measurements including serum, urinary uric acid, cholesterol, free fatty acids were normal. Other values of diagnostic markers such as aminoacidophaties, sterol, biotin and protein glycosylation enzymes lied in physiological ranges too. Since not even a CT scan or a magnetic resonance imaging had brought it to any conclusions, it was not until the positive BM test showed the high SAICAr signal and thus pointed out the correct diagnosis. However, the HPLC analysis provided three additional unknown signals. Besides the elevated values of SAICAr and SAdo, the most intense peak proved to be AICAr by BM test (Marie S. *et al.*,2004). AICAr and AICAr base aminoimidazolecarboxamide (AIC) physiological values could be elevated in urine during deficiencies of vitamin B12 and folic acid (2.17 against 0.65 mmol AIC/mol creatinine in controls) (Middleton J.E. *et al*.,1965), in patients suffering from acute leukemia (in relapse 5.21 against 0.96 mmol AIC/mol creatinine/24 hours in controls) (Lulenski G. *et al*.,1970) or in hypoxanthine-guanine phosphoribosyl transferase deficiency (patients 12.5 against 1.25 mol AIC/mol creatinine in controls (Newcombe D.S.,1970), 2.82 mol AICAr/mol creatinine in patients against undetectable values in controls (Lopez J.M. *et al*.,2020)) and finally AICA-ribosiduria (280 mmol AICAr/mol creatinine in patients against undetectable values in controls) (Marie S. *et al.*,2004). Therefore, the set of experiments which confirmed the AICA-ribosiduria diagnosis involved the addition of AICAr to patient's aberrant and normal fibroblasts. Patient's aberrant fibroblasts revealed massive accumulation of AICAR

suggesting the dysfunction of enzyme ATIC. This fact was verified by enzymatic assays showing AICAR-formyltransferase activity under detection limit and IMP-cyclohydrolase activity lowered to 40 % of control values. The sequencing of the *ATIC* gene revealed the frame shift in exon 2 in one allele and point mutation in exon 13 in the second allele causing mutation c.1277A>G resulting in pathogenic protein variant p.Lys426Arg. Thus, a new inborn disease was identified and named AICA-ribosiduria (Marie S. *et al.*,2004). The current condition of the first patient with AICA-ribosiduria expose the disability of independent walking or standing at the age of 18. The anti-epileptic treatment (vigabatrin, valproate and clonazepam) is not effective and the patient suffers from severe irreducible scoliosis and feeding issues (Ramond F. *et al.*,2020).

The newly described cases were preceded with intrauterine growth retardation followed by neonatal hypotonia, scoliosis and dysmorphic features. Thereafter, delayed cognitive skills, ophtalmological disorders, basic or no spoken language were observed. The progression of the disease included uncontrollable vomiting episodes and epilepsy resistant to pharmacotherapy in two out of three cases (Ramond F. *et al.*,2020). At first and similarly to the first described AICA-ribosiduria patient, the general metabolic examination did not show any abnormalities besides slightly decreased levels of HDL and triacylglyceroles. Postnatal karyotype was normal too (Ramond F. *et al.*,2020). Finally, the exome sequencing revealed compound heterozygous variants in *ATIC* gene resulting in accumulation of AICAr (305 mmol AICAr/mol creatinine) and moderate elevation of SAICAr and Sado. The c.406G>A variant causes p.Ala136Thr in cyclohydrolase domain of *ATIC* and c.1654A>T is truncating (Ramond F. *et al.*,2020). The patient with damaged cyclohydrolase domain of ATIC manifest the milder symptoms and response to antiepileptic treatment when compared with the two new cases with pathogenic variants in transformylase domain caused by mutation c.1277A>G (p.Lys426Arg). The sequencing of the second allele did not reveal any variant. Therefore, it is probably a non-coding allele or its product is an unstable RNA (Ramond F. *et al*.,2020). Patients with mutation in transformylase domain of ATIC manifest severe symptoms and also accumulate AICAr (70 and 143 mmol AICAr/mol creatinine, respectively) and moderate levels of SAICAr and Sado (Ramond F. *et al.*,2020).

Similarly to ADSL deficiency, AICA-ribosiduria cytotoxicity is presumably caused by AICAr accumulation and disruption of purinosome (Baresova V. *et al.*,2012). The AICAr cytotoxicity was lately demonstrated by the ability to induce necrosis (Guo F. *et al.*,2016), cellular apoptosis (Morishita M. *et al.*,2017) and endoplasmic reticulum-stress (Nie J. *et al.*,2017). On the other hand, AICAr stimulates anti-tumoral properties in kidney cancer (Liang S. *et al.*,2018) and was proposed to treat chronic lymphocytic leukemia (van den Neste E. *et al.*,2010). Furthermore, anti-doping controls check AICAR (the phosphorylated form of AICAr) levels due to its ability to stimulate AMPK (Henin N. *et al.*,1995) and act as an exercise mimetic (Narkar V.A. *et al.*,2018).

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. The aim of treating epilepsy-like symptoms is to control or at least decrease seizure frequency with minimal side effects. Patients often require polypharmacy with the use of two or more anticonvulsants and the drug resistance is common. Another way of seizures reduction is a ketogenic diet (Freeman J.M. and Vining E.P.G.,1998; Lefevre F. and Aronson N.,2000). The ketogenic diet increases levels of adenosine, ATP, acetone (Masino A.S.,2008) and gamma-aminobutyric acid (GABA) (Yudkoff M. *et al.*,2007). Among the ketone bodies, acetone is the best *in vitro* and *in vivo* anticonvulsant (Masino A.S.,2008). The GABA has inhibitory neurotransmitter effects (Yudkoff *et al.*,2007). Adenosine is an endogenous neuroprotective molecule (Fredholm B.B.,1997) and together with ATP regulates neuronal signalling (Trams E.G.,1981). Moreover, ATP is an inhibitor of PPAT and may also be metabolized to GMP, respectively GTP, and demands on purine production via DNPS is thus decreased. Another alternative adenosine donor is S-adenosyl-L-methonine (SAMe). The supplementation of SAMe did not alter SAICAr and SAdo levels in ADSLD patients (van Werkhoven M.A. *et al.*,2013) and the same effect was shown for the administration of uridine (Salerno C. *et al*.,2000) and D-ribose (Salerno C. *et al.*,1999). Both compounds stimulate production of PRPP, which is not only the precursor of DNPS, but could also enter purine salvage pathways. The promising effect of D-ribose on frequency of seizures in one patient (Salerno C. *et al.*,1999) was not confirmed in later studies (Jurecka A. *et al.*,2008b; Perez-Duenas B. *et al.*,2012). Uridine has additional dopaminergic effects and positively influence neurologic symptoms (Page T. *et al.*,1997). An effort to increase nucleotide concentrations by adenosine and allopurinol was not successful (Jaeken J. *et al.*,1988). DNPS is also a target of anticancer drugs represented mostly by purine analogues or antifolates. Nevertheless, the methotrexate or mercaptopurine inhibiting DNPS in leukemic patients (Dervieux T. *et al.*,2002) were in case of ADSLD patients clinically not tested or the results were not published.

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 TLC and HPLC coupled with UV detection (Jaeken J. and van den Berghe G.,1984). Another method applied to screening of ADSLD was the BM test. The BM test was modified and established for screening

of DNPS substrates with a free aromatic amino group (AIR, CAIR, SAICAR, AICAR) (Laikind P.K. *et al.*,1986). In comparison with reverse phase chromatography including 1 hour gradient (Jaeken J. and van den Berghe G.,1984) and the column chromatography coupled with two dimensional TLC (de Bree P.K. *et al.*,1986), the modified BM test was significantly faster (10 min) and offered less demanding instrumentation.

As an alternative to BM test, three new TLC screening methods with emphasis on ADSLD were designed. Within the study 2642 subjects were tested, resulting in identification of three newly diagnosed cases in the Czech population (Sebesta I. *et al.*,1995). Proposed TLC methods were applied to identification of ADSLD due to their low cost, easy performance and sensitivity.

However, more specific, high throughput, but also more expensive methods, such as capillary electrophoresis (CE) (Adam T. *et al.*,1999; Friedecky D. *et al.*,2002; Hornik P. *et al.*,2007) or high-pressure liquid chromatography (HPLC), have been lately introduced. CE separates investigated compounds based on their electrophoretic mobility in capillary often filled with fused silica gel. CE is utilizing micro- or nano- fluidic system with introduction of nL volume of a sample. Sample is introduced hydrodynamically by pressure injections for a few seconds into aqueous buffer solution, followed by an electroosmotic flow created by high voltage power supply and brings separated anions and cations to the detector (Bory C. *et al.*,1998). CE allows to perform a gradient of pH to obtain better separation of some particular compounds. CE method for analysis of metabolites dysregulated during purine and pyrimidine disorders was developed for detection of substrates from the DNPS pathway (AIr, CAIr, SAICAr, AICAr and FAICAr) and one substrate from purine nucleotide cycle AMP synthesis branch (SAdo) (Adam T. *et al.*,1999; Friedecky D. *et al.*,2002; Hornik P. *et al.*,2007).

HPLC method transfers compounds of the sample on the principle of high pressure and separates individual components of the mixture on columns filled with diverse functional groups. First HPLC methods intended for detection of DNPS substrates included anion exchange column and a linear gradient of phosphate buffers (Simmonds H.A. *et al.*,1988). Nowadays, the reverse phase columns with C18 or NH_2 filling are used together with mobile phases containing water and organic solvents, which could perform isocratic or gradient elution of investigated compounds (Krijt J. *et al.*,1999; Madrova L. *et al.*,2018).

Based on scientific experience, it could be concluded that both separation methods, CE and HPLC, provide reproducibility over 90 %, retention time variability lower 5 % in case of HPLC and 2 % in case of CE. CE is 3-times faster, however, it is 5-times less sensitive then HPLC (Lee B.L. and Ong C.N.,2000). In general, both techniques are commonly coupled with UV diode array detection (DAD) (Lee B.L. and Ong C.N.,2000; Duval N. *et al.*,2013). The diode array detector enables to detect compounds in the region of 190 – 1100 nm. A major advantage of DAD is the ability to scan the whole spectrum of wavelengths at the same time, which enables to pick the maximum absorption wavelength

of a compound and distinguish or at least discriminate, if more than one compound is eluted within the same peak. The substrates of DNPS pathway containing imidazole ring, the first of which appear in the form of AIR or AIr, respectively, absorb UV light. Particularly, the absorption maximum of DNPS substrates lies between 245 and 275 nm. However, this is also the absorption region for many other biological compounds present in body fluids. Therefore, a further development of analytical tools ideal for studying DNPS pathway was required. Inconveniences with compounds that do not absorb the UV light were circumvented by use of a mass spectrometer (MS) as the detector. Nowadays, among the most suitable technique for detection of DNPS substrates is the high pressure liquid chromatography coupled with triple quadrupole mass spectrometer (HPLC-MS/MS) or high resolution MS (HRMS) technique utilizing the Orbitrap mass analyzer. Advanced metabolomics techniques enable to analyse dephosphorylated substrates of DNPS in urine, plasma/serum and CFS (Ito T. *et al.*,2000; Hartmann S. *et al.*,2006; van Werkhoven M.A. *et al.*,2013;Madrova L. *et al.*,2018). However, only PRPP, AICAR, SAMP and IMP may be purchased as standards. Optionally, samples of patients positively diagnosed by other methods may be assumed as positive controls for presence of accumulated DNPS substrates. In such case has to be taken into consideration that bacterial infection, could cause the deribosylation of DNPS substrates and thus false negative results in urine samples (Krijt J. *et al*.,2013). Therefore, commercially unavailable DNPS substrates had to be prepared for purpose of new diagnostic methods development.

1.3.1.1 Standards preparation

Several strategies may be applied to preparation of commercially unavailable DNPS substrates. There have been described organic procedures for synthesis of glycinamide ribotide (GAR), formylglycinamide ribotide (FGAR) and their ribosides. Another way of preparation of DNPS substrates is the enzymatic synthesis utilizing protein extracts or recombinant proteins.

1.3.1.1.1 Organic synthesis of GAR/r and FGAR/r

The very first step of synthesis of DNPS substrates is challenging, when PRPP or D-ribose, respectively, in case of an organic approach, is converted into phosphoribosyl amine (PRA), with half-life only 5 s (Rudolph J. and Stubbe J.,1995). The synthesis of glycinamide riboside (GAr) and the next metabolite formylglycinamide riboside (FGAr) starts with 2,3,5-tri-O-benzoyl-β-ribofuranose (Chu S.Y. and Henderson J.F.,1970), which is initially converted into amine form and then subsequently fused with glycine ethyl ester or formylglycin ester (Schrimsher J.L. *et al.*,1986). In order to produce GAr and FGAr, respectively, the benzoyl protecting groups are finally cleaved (Chu S.Y. and Henderson J.F.,1970). In case of preparation of the phosphorylated substrate GAR a ten-step procedure is

included. In short, the synthesis involves condensation of 2,3-O-isopropyliden-D-ribofuranosylamine p-toluensulfonate with N-(benzyloxycarbonyl)glycine. Phosphorylation is then achieved by 2-cyanoethyl phosphate and a product is deprotected (Chettur G. and Benkovic S.J.,1976).

1.3.1.1.2 Enzymatic synthesis using avian liver extract

Yet another strategy to prepare DNPS substrates utilizes the pigeon or chicken liver extracts (Buchanan J.M., Hartman S.C.,1959). The pigeon liver acetone powder was passed through ion exchange chromatography, dialyzed and lyophilized. The prepared protein extract was used for an *in vitro* preparation of DNPS substrates (Goldthwait D.A. *et al.*,1955). As the first were prepared GAR and FGAR (Buchanan J.M. *et al.*,1955). Developed procedures were modified and used for preparation of all DNPS substrates (Lukens L. and Flaks J.,1963) except FAICAR due to the bifunctional activity of enzyme ATIC, which irreversibly converts AICAR directly to IMP.

1.3.1.1.3 Enzymatic synthesis using recombinant proteins

Recombinant enzymes of DNPS pathway were produced using the pMAL™ Protein Fusion and Purification System (New England Biolabs Inc., USA). PCR products of genes from DNPS pathway (*PPAT, GART, PFAS, PAICS, ADSL, ATIC*) were inserted into pMAL-c2 vector downstream from malE gene coding maltose binding protein (MBP) maintaining the open reading frame. After a heat shock transformation, the vector was internalized into *E. coli* and the expression of an MBP fusion protein was induced with isopropylthiogalactoside (IPTG). The produced MBP fusion protein of interest was purified by affinity chromatography and verified by SDS electrophoresis. The prepared protein was then used in enzymatic assays to produce individual substrates for a purified enzyme in a mixture with a suitable buffer (Zikanova M. *et al.*,2005).

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). As for the rest of the described mutations, there is mostly only one single incidence or a few cases each (http://www1.lf1.cuni.cz/udmp/adsl/index.php?adsltable=orderbyphenotypedesc, November 2019).

In contrast to point mutations, there were identified cases with different types of modifications such as the 39 bp deletion caused by a c.620C>A mutation (Kohler M. *et al.*,1999; Marie S. *et al.*,1999), the case with an alternative stop codon due to a c.774_778insG mutation resulting in shortened 284 aa long protein (Chen B.C. *et al.*,2010) and in frame duplication of asparagine297 caused by c.889_891dupAAT (van Werkhoven *et al.*,2013). Moreover, five cases with mutation -49T>C in a promotor region significantly decreased the ADSL mRNA expression (Marie S. *et al.*,2002). The most important advantage of genetic diagnostics is the subsequent possibility of a prenatal diagnosis offered to the affected families, if a disease-causing mutation has been previously identified.

1.3.3 Function studies

1.3.3.1 Enzyme activities in cells

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. In case of ADSLD, there may be a difference between affinities to SAICAR and SAMP according to causal mutations (van den Bergh F. *et al.*,1993) or the activity is decreased for both substrates similarly (van den Bergh F. *et al.*,1991). The reduced activity of ADSL was described in kidney, liver (van den Bergh F. *et al.*,1991), lymphocytes (Jaeken J. *et al.*,1988), lymphoblasts (Barshop B. A. *et al.*,1989), fibroblasts and erythrocytes (Salerno C. *et al.*,1995). In contrast, the ADSL activity was almost normal in granulocytes and, in some cases, also in muscles (Jaeken J. *et al.*,1988). Similarly, in case of AICA-ribosiduria were detected lowered activities of ATIC in patients' fibroblasts (Marie S. *et al.*,2004).

1.3.3.2 Functional studies with recombinant mutated proteins

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). Race et al. studied eight recombinant ADSL proteins and found three mutations causing thermal lability, four mutations without thermal effect and one causing enzyme inactivity. Their results also confirmed the hypothesis: the higher the ratio SAdo/SAICAr is the milder the symptoms of the disorder are (Race V. *et al.*,2000). In concordance with these findings, the effect of point mutations causing equal decrease in ADSL activity for SAdo and SAICAR or even lowered activity for SAICAR was shown (Kmoch S. *et al.*,2000). Both studies could not present significant correlation between genotype and phenotype and came to agreement that excess of SAdo over SAICAr is beneficial (Kmoch S. *et al.*,2000). A comprehensive study compared biochemical and structural basis of 19 mutant proteins from 16 patients with neonatal fatal, Type I and Type II form of ADSLD. The study has revealed that

the phenotype severity is correlated with mutated enzyme activity and stability (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 (CHO-K1), which possess only 20 chromosomes with small degree of variability, 10 h generation time and low uncertainty of karyotype analysis, were selected to develop mammalian model for genetic studies (Kao F.T. and Puck T.T.,1968). Previously, a subclone of CHO cell line was established requiring proline for growth. This cell line represented a suitable cell model for biochemical pathways research (Ham R.G.,1963). Following an incubation of CHO cells in medium containing 5-bromodeoxyuridine (BUdR) and lacking certain nutrient (e.g. proline), only those cells proliferate, that possess the ability to circumvent the nutrient shortage, i.e. which incorporate BUdR into biomolecules. An irradiation with the near visible light causes BUdR cell death and selective survival of cell population dependent on the omitted nutrient (Kao F.T. and Puck T.T.,1968). The next step in development of the CHO cell lines dependent on the certain nutrient was the use of well established mutagens ethyl methanesulfonate and N-methyl-N'-nitro-N-nitrosoguanidine (Liwerant I.J. and Pereira Da Silva L.H.,1975), which produce single point mutations. This method was used in biochemical genetic studies of hybridization, metabolism analysis, gene localisation and nutritional analysis resulting in an isolation of series of CHO-K1 with aberrant DNPS metabolism and requiring adenine for growth (ade^-) (Patterson D. *et al.*,1974; Patterson D.,1975, Patterson D.,1976). The cell line CHO ade^-B was identified to accumulate FGAR (Patterson D. *et al.*,1974). Next, other adenine requiring subclones of CHO cells were investigated and the accumulation of GAR was found in ade^-E, AIR in ade^-D, AICAR in ade^-F (Patterson D.,1975), SAICAR and SAMP in ade^-I (Patterson D.,1976). Detection of DNPS intermediates was performed by TLC techniques after labelling the cell lines with radioactive ^{14}C-formate (Patterson D.,1975). In order to avoid disadvantages of working with radioactive compounds, modern methods used for detection of intermediates accumulated in ade^-I and ade^-D cell lines (deficient in ADSL, respectively PAICS enzyme) (Tu A. S. and Patterson D.,1977; Patterson D.,1975) were introduced, including a liquid chromatography with an electrochemical detection (Duval N. *et al.*,2013).

1.4.2 Genetically modified HeLa model cell lines

Yet another approach to develop stable cell lines with aberrant DNPS metabolism is the use of bacterial adaptive RNA guided immune system against invading bacteriophage and plasmid DNA named

CRISPR/Cas (Garneau J.E. *et al.*,2010). The Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) allied with CRISPR associated (Cas) proteins are endowed with an endonuclease activity. The CRISPR/Cas type II system consists of two RNAs guiding the Cas9 endonuclease to generate double stranded DNA (dsDNA) breaks (Jinek M. *et al.*,2012). The CRISPR RNA (crRNA) includes approximately 20 base pairs compatible with a specific region or the target dsDNA. The trans-activating RNA (tracrRNA) possess two functions. Firstly, it is responsible for crRNA maturation. Secondly, it is required for an activation of Cas9. Then it was shown that CRISPR/Cas9 system may be guided by single chimeric RNA (Jinek M. *et al.*,2012) consisting of crRNA, polylinker and tracrRNA suitable for genome editing of human cells (Jinek M. *et al.*,2013). 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 purchased. 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 of 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 (SRM) 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 organism simulating known and putative DNPS disorders. Such model organisms are represented by HeLa cell lines with defects in individual enzymes of DNPS (Baresova V. *et al.*,2016). The hypothesis was to verify 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

The selected strategy for preparation of DNPS substrates utilizes the enzymatic synthesis and purification by column chromatography. For the purpose of enzymatic synthesis, plasmids with sequences encoding specific bacterial or human enzyme from DNPS pathway have been already previously prepared. A culture of *E. coli* was transfected with the enzyme expression vector, which was then utilized in explicit enzyme production. Purified enzymes were used in enzymatic reactions resulting in production of individual DNPS substrates. DNPS substrates were purified and their concentration was determined spectrophotometrically, if possible. Next, a fraction of thus obtained DNPS substrates in form of nucleotides were dephosphorylated in order to get their corresponding nucleosides.

3.1.1 Expression vectors coding recombinant DNPS enzymes

Sequences coding human and corresponding bacterial recombinant enzymes from DNPS pathway were previously prepared in our laboratory (Kmoch S. *et al.*,2000; Baresova V. *et al.*,2016). The cDNA coding human genes *GART, PFAS, PAICS, ADSL* and *ATIC* and bacterial genes *purD* , *purN* , *purM* (each encodes the separate catalytic function of human trifunctional *GART*: *GARS*, *GARFT* and *AIRC*) and *phosphoribosylformylglycinamidine synthase (PurL)* (with function of human *PFAS*) were prepared, amplified and ligated into pMAL-c2 expression vector.

3.1.1.1 Preparation of cDNA

The cDNA coding genes of human DNPS enzymes were isolated from control sample of a pooled peripheral blood containing lymphocytes and the cDNA coding bacterial genes were obtained from control *E*. *Coli* TOP10 strain(Invitrogen) by established protocol (Chomczynski P. and Sacchi N.,1987) including total RNA isolation and reverse transcription. For isolation of poly(A)RNA, Oligotex Direct mRNA kit (Quiagen, Hilden, Germany) was used and cDNA was procured by the 1st-strand cDNA Synthesis kit (Clontech, Palo Alto, CA) and oligo(dT) primer. The amplification of cDNA coding ADSL was obtained with Stratagene (La Jolla, CA) and the amplification of cDNA coding other genes was carried out with TOPO®TA Cloning® Kit (Invitrogen).

3.1.1.2 cDNA cloning

The size and purity of cDNA RT-PCR product was verified by agarose gel electrophoresis. Next, a cloning of the PCR product into pCR4-TOPO-TA vector (Invitrogen) was performed according to manufacturer's protocol. After verification of a positive clone by sequencing, a sufficient amount of the vector was isolated (ZymoPURE Midiprep). Finally, the insert (cDNA sequence of individual enzyme) was cloned into pMAL-c2 vector using appropriate restriction endonucleases.

3.1.1.3 Transformation of competent E. Coli DH5αF'IQ strain

Competent *E. coli* DH5αF'IQ strain (Invitrogen) were transformed with pMAL-*DNPS* plasmids according to the manufacturer's protocol. Briefly, 10 ng of pMAL plasmid was incubated with cells for 30 min. The cells were heat-shocked for 45 s in 42°C water bath and placed on ice for 2 min. Afterwards, 0.9 mL of S.O.C. medium was added and a mixture was shaken for 1 h at 225 rpm. Finally, 200 µL was plated on LB supplemented with 50 µg/mL ampicillin and incubated overnight at 37 °C.

3.1.1.4 Plasmid isolation from E. Coli DH5αF'IQ strain (Mini-prep)

The bacterial colonies growing on LB plates were picked, inoculated in 2 mL of LB medium supplemented with 50 µg/mL ampicillin and a mixture was shaken at 37 °C. When the inoculum began to be opalescent, another 6 mL of LB medium supplemented with 50 µg/mL ampicillin was added and inoculum was incubated overnight. A mini-prep isolation protocol was then applied.

3.1.2 Expression and purification of recombinant proteins

For preparation of DNPS recombinant proteins, *E. coli* strain DH5αF'IQ transfected with pMAL-c2 plasmid was utilized. Expressed DNPS proteins are fused with MBP protein which serves for purification by affinity chromatography.

3.1.2.1 Preparation of bacterial culture

The bacterial culture was obtained from inoculum previously required for plasmid isolation. The inoculum was restored by adding fresh LB medium with ampicillin and incubated overnight. Then, the inoculum was added to 200 mL of Rich medium (LB medium with 0.2 % glucose and 50 µg/mL ampicillin). The bacterial growth was monitored by measurements of optical density (OD) at wavelength of 600 nm until the value reached 0.5 – 0.7. Then, the addition of 0.3 mM IPTG triggered the promoter and increased expression of enzyme coded by pMAL-c2 plasmid. After 3-6 hours

of incubation (at temperatures 18 - 37 °C based on selected plasmid), the bacterial culture was centrifuged at 4000 g for 10 min at 4 °C. The bacterial pellet was stored at -80 °C.

3.1.2.2 Preparation of bacterial cell lysate

The bacterial pellet was removed from freezer at -80 °C and thawed on ice after addition of 1 mL of appropriate buffer supplemented with protease inhibitors (Roche, 1 tablet per 10 mL of medium). After that, 100 µL of buffer were added per 1 mL of medium used for preparation of bacterial pellet followed by a lysozyme to a final concentration of 20 mg/mL. The lysate was incubated for 1 h at RT on Gyro Twister and then sonicated. The following sonication protocol was used: 4 times 15 s sonication at 40 % of 120 W amplifier with 1 min pause between cycles. The lysate was centrifuged at 9000 g for 30 min at 4 °C.

3.1.2.3 Affinity chromatography

The column for affinity chromatography was filled with 4 mL of prewashed amylose resin (New England BioLabs) per 100 mL of medium used for bacterial pellet preparation and washed with 5 volumes of the column buffer. Then, the lysate was applied and incubated for 30 min at 4 °C on Gyro Twister. Next, the sample was centrifuged at 800 g for 5 min at 4 °C, the supernatant was kept as sample "through" and another 5 volumes of column buffer were added. After 5 min of incubation, the sample was centrifuged at 800 g for 5 min at 4 °C, the supernatant was kept as sample wash 1. The last step was repeated to obtain a sample wash 2. In the next step, the volume equal to amylose resin of column buffer with 10 mM maltose was added. After 30 min of incubation, the sample was centrifuged at 800 g for 5 min at 4 °C, the supernatant was kept as sample elution 1. The last step with an altered incubation time of 10 min was repeated twice to yield elution 2 and 3. Finally, the amount of protein in each elution was determined by nanodrop spectrophotometer by absorbance measurements at 280 nm. Positive fractions were concentrated by centrifugation through Amicon® ultra filter unit with cut off appropriate to enzyme molecular weight. Aliquots from each step of affinity chromatography were verified by SDS-PAGE gel electrophoresis and the affinity column was regenerated by subsequent washing by 5 volumes of column buffer, 10 volumes of column buffer with 10 mM maltose, 1 volume of ddH_2O, 3 volumes of 0.1% SDS, 5 volumes of ddH_2O and finally 2 volumes of 20% EtOH, which also served as storage buffer.

3.1.2.4 SDS-PAGE gel electrophoresis

The SDS-PAGE gel electrophoresis was performed for the purpose of affinity chromatography evaluation. The 10% separating and 4.5% stacking polyacrylamide gels were prepared according to Table 3 A. and B. The voltage was set to 70 V for 1 h followed by 140 V for 3.5 h. The gel was stained by Coomassie Brilliant Blue for 45 min and then bleached with solution of methanol:acetic acid:H_2O in ratio 1:1:8.

Table 3 A. Composition of polyacrylamide separation gel.

	stock	**final**	**1 gel**	**2 gels**
Acrylamide	40 %	10 %	2.25 mL	4.5 mL
Separating buffer	4 x	1 x	2.25 mL	4.5 mL
APS	10 %	0.06 %	54 µL	108 µL
TEMED	100 %	0.06 %	5.5 µL	11 µL
ddH_2O			4.44 mL	8.88 mL

Table 3 B. Composition of polyacrylamide stacking gel.

	stock	**final**	**1 gel**	**2 gels**
Acrylamide	40 %	4.5 %	0.68 mL	1.35 mL
Stacking buffer	4 x	1 x	1.5 mL	3 mL
APS	10 %	0.06 %	36 µL	72 µL
TEMED	100 %	0.06 %	4 µL	8 µL
ddH_2O			3.79 mL	7.58 mL

3.1.3 Preparation of non-labelled DNPS substrates

We prepared human and bacterial recombinant enzymes for each step of DNPS and utilized them in synthesis of all DNPS substrates. The only exception was the preparation of FAICAR, which was prepared utilizing inorganic synthesis. The prepared and commercially available standards of nucleotides were subjected to the dephosphorylation in order to obtain corresponding nucleosides thereof.

3.1.3.1 PRA

The first reaction of DNPS pathway including conversion of PRPP to PRA by enzyme PPAT was bypassed by inorganic synthesis of PRA. The inorganic preparation of PRA is based on addition of ammonium

hydroxide to rib-5-P as a function of pH (Nierlich D.P. and Magasanik B.,1965). The resulting product of this reaction is unstable (Rudolph J. and Stubbe J.,1995) and PRA was therefore carried over to next enzymatic reaction and directly converted to GAR or FGAR.

3.1.3.2 GAR and GAr

GAR was prepared by enzymatic reaction of bacterial protein MBP-PurN (maltose binding protein fused with *phosphoribosylglycinamid synthetase*). The reaction mixture contained 5.7 mM rib-5-P, 0.7 mM ATP, 10 mM glycine, 10 mM ammonium hydroxide and 12.7 mM manganese chloride in 20mM phosphate buffer with pH 6. Reaction components were mixed in 1.5mL tube. As a next step, an appropriate amount of enzyme was added to reach the final enzyme concentration of 0.4 µg/µL. The reaction mixture was incubated for 4 hours at 37 °C. Then, the enzyme was removed by centrifugation through Amicon® ultra filter unit with the 3 kDa cut off. The enzyme was stored at – 80 °C. A fraction of the reaction product GAR was diluted 10 times and analysed by HPLC-MS/MS. The dephosphorylation of GAR to GAr was carried out by a mixture containing 1xNEB3 buffer and 1 U of calf intestine phosphatase (CIP). After 2 h of incubation at 37 °C GAR was converted into GAr and the enzyme was removed by centrifugation through Millipore filter unit with 3 kDa cut off.

3.1.3.3 FGAR and FGAr

FGAR was prepared by enzymatic reaction of bacterial proteins MBP-PurN and MBP-PurD (maltose binding protein fused with p*hosphoribosyl glycinamide formyltransferase*). The reaction mixture 5.7 mM rib-5-P, 0.7 mM ATP, 10 mM glycine, 10 mM ammonium hydroxide and 12.7 mM manganese chloride and 0.1 mM N^{10}-fTHF in 20 mM phosphate buffer with pH 6. Reaction components were mixed in 1.5mL tube. In the end, the enzyme was added into reaction to reach the final enzyme concentration of 0.4 µg/µL. The reaction mixture was incubated for 4 hours at 37 °C. Then the enzyme was removed by Amicon® ultra filter unit with 3 kDa cut off. The enzyme was stored at – 80 °C. A fraction of the reaction product FGAR was diluted 10 times and transferred into insert embedded in a HPLC vial and stored at -20 °C until the HPLC-MS/MS analyses. The dephosphorylation of FGAR to FGAr was carried out by a mixture containing 1xNEB3 buffer and 1 U of CIP. After 2 h of incubation at 37 °C FGAR was converted into FGAr and the enzyme was removed by Amicon® ultra filter unit with 3 kDa cut off (Baresova V. *et al.*,2016).

3.1.3.4 FGAMR and FGAMr

Formylglycine amidine ribotide was prepared by enzymatic reaction with bacterial protein 6H-PurL (6 histidines anchor fused with *phosphoribosyl formylglycine amidine synthetase*). The reaction mixture contained 200 µL of the FGAR sample supplemented with 2 mM glutamine, 2 mM ATP and 0.25 µg/µL of 6H-PurL. The reaction mixture was incubated for 4 hours at 37 °C. The enzyme was then removed by Amicon® ultra filter unit with 3 kDa cut off. The enzyme was stored at – 80 °C. A fraction of the reaction product FGAMR was diluted 10 times and transferred into insert embedded in HPLC vial and stored at -20 °C until the HPLC-HRMS analyses. The dephosphorylation of FGAMR to FGAMr was carried out by a mixture containing 1xNEB3 buffer and 1 U of CIP. After 2 h of incubation at 37°C FGAMR was converted into FGAMr and the enzyme was removed by Amicon® ultra filter unit with 3 kDa cut off (Madrova L. *et al.*,2018).

3.1.3.5 AIR and AIr

Aminoimidazole ribotide might be prepared by two enzymatic approaches – forward enzymatic reaction and reverse enzymatic reaction driven by substrates and products concentrations.

3.1.3.5.1 Forward enzymatic reaction

AIR was prepared by enzymatic reaction with bacterial proteins MBP-PurN, MBP-PurD, 6H-PurL and MBP-PurM (maltose binding protein fused with *aminoimidazole ribonucleotide synthetase)*. The reaction mixture contained 5.7 mM rib-5-P, 0.7 mM ATP, 10 mM glycine, 10 mM ammonium hydroxide and 12.7 mM manganese chloride and 0.1 mM N^{10}-fTHF in 20mM phosphate buffer with pH 6. All reaction components were mixed in 1.5 mL tube. In the end, the enzyme was added into reaction to reach the final enzyme concentration of 0.4 µg/µL. The reaction mixture was incubated for 4 hours at 37 °C. The enzyme was then removed by Amicon® ultra filter unit with 3 kDa cut off. The enzyme was stored at – 80 °C. A fraction of the reaction product AIR was diluted 10 times and transferred into insert embedded in HPLC vial and stored at -20 °C until the HPLC-MS/MS or HPLC-UV analyses.

3.1.3.5.2 Reverse enzymatic reaction

AIR was prepared by enzymatic reaction with bifunctional human recombinant enzyme MBP-PAICS. The reaction mixture contained 6 mM SAICAR, 6 mM ADP, 35 mM $MgCl_2$, 100 mM sodium phosphate (pH 6), and 0.5 µg/µL MBP-PAICS. After 5 h of incubation at 37 °C, the reaction mixture was analysed by HPLC-DAD. The reaction mixture was added to a top of activated Strata X-AW (33 µm; 200 mg/3 mL)

column (Phenomenex) prewashed with 100mM sodium acetate with pH 5.1. Under selected conditions and unlike SAICAR, ADP and ATP, AIR eluted readily from the column with 100mM sodium acetate with pH 5.1. AIR positive fractions were concentrated under a stream of nitrogen. The concentration of AIR was determined at 250 nm using a NanoDrop spectrophotometer (Thermo Scientific) and calculated using an extinction coefficient of 3830 M^{-1} cm^{-1} (Meyer E. *et al.*,1992). The AIr was prepared from the 3 mM AIR solution in the standard reaction mixture of 1xNEB3 buffer and 1 U CIP via incubation for 2 h at 37 °C (Baresova V. *et al.*,2016).

3.1.3.6 CAIR and CAIr

CAIR was prepared by enzymatic reaction with bifunctional human recombinant enzyme MBP-PAICS. The reaction mixture contained 50 mM Tris-Cl (pH 8), 1.3 mM $MgCl_2$, 500 mM $NaHCO_3$, 3 mM AIR and 0.5 µg/µL human recombinant MBP-PAICS. After 4 h of incubation at 37 °C, the reaction mixture was analysed by HPLC-DAD. The reaction mixture was added to a top of activated Strata XL-A (100 µm; 200 mg/3 mL) column (Phenomenex). Under selected conditions, CAIR was captured by the column, washed with 3 mL of 100 mM ammonium acetate (pH 8.2) and 3 mL of methanol. The column was then dried under vacuum and CAIR was eluted with 5% formic acid in methanol. The concentration of CAIR was determined at 250 nm using a NanoDrop spectrophotometer (Thermo Scientific) and calculated using an extinction coefficient of 10580 M^{-1} cm^{-1} (Meyer E. *et al.*,1992). The CAIr was prepared from the 3 mM CAIR solution in the standard reaction mixture of 1xNEB3 buffer and 1 U CIP via incubation for 2 h at 37 °C (Baresova V. *et al.*,2016).

3.1.3.7 AICAr

AICAr was prepared by enzymatic reaction from the 10 mM AICAR (Sigma Aldrich) solution in the standard reaction mixture of 1xNEB3 buffer and 1 U CIP via incubation for 2 h at 37 °C. The concentration of AICAr was determined at 265 nm using a NanoDrop spectrophotometer (Thermo Scientific) and calculated using an extinction coefficient of 12500 M^{-1} cm^{-1} (Bazurto J.V. *et al.*,2015).

3.1.3.8 SAICAR and SAICAr

SAICAR was prepared by enzymatic reaction with human recombinant ADSL. The reaction mixture contained 10 mM Tris-Cl (pH 8), 10 mM KCl, 2mM EDTA, 70 mM fumaric acid, 6 mM AICAR and 50 µg/mL human recombinant ADSL. After 3 h of incubation at 37 °C, the reaction mixture was analysed by HPLC-DAD. The reaction mixture added to a top of activated Strata XL-A (100 µm; 200 mg/3 mL) column (Phenomenex). Under selected conditions, SAICAR was captured by the column,

washed with 3 mL of 100 mM ammonium acetate (pH 8.2) and 3 mL of methanol. The column was then dried under vacuum and CAIR was eluted with 5% formic acid in methanol (Baresova V. *et al.*,2016). The concentration of SAICAR was determined at 265 nm using a NanoDrop spectrophotometer (Thermo Scientific) and calculated using an extinction coefficient of 13100 M^{-1} cm^{-1} (van den Bergh F. *et al.*,1991). The SAICAr was prepared from the 10 mM SAICAR solution in the standard reaction mixture of 1xNEB3 buffer and 1 U CIP via incubation for 2 h at 37 °C.

3.1.3.9 FAICAR and FAICAr

Due to the fact that enzyme ATIC of prokaryotic and eukaryotic origin possess bifunctional activity and the reaction catalysed by ATIC is irreversible, was the most suitable option for generating DNPS substrates FAICAR and FAICAr the inorganic synthesis.

The inorganic synthesis of FAICAR exploits previously published formylation conditions (Lukens L. and Flaks J.,1963). The reaction mixture contained 10 mg of AICAR, 11 mg of NaOH, 136 µL of formic acid and 250 µL of acetic anhydride for 1 h at 37 °C. FAICAr was prepared by the similar reaction but AICAr was substituted for AICAR (Vyskocilova P.,2011).

3.1.3.10 SAdo

SAdo was prepared by enzymatic reaction from the 10 mM SAMP (Sigma Aldrich) solution in the standard reaction mixture of 1xNEB3 buffer and 1 U CIP via incubation for 2 h at 37 °C (Zikanova M. *et al.*,2005). The concentration of SAdo was determined at 265 nm using a NanoDrop spectrophotometer (Thermo Scientific) and calculated using an extinction coefficient of 19200 M^{-1} cm^{-1} (van den Bergh F. *et al.*,1991).

3.1.4 Preparation of isotopically labelled substrates

The developed procedures for DNPS substrates preparation described in previous chapter could be extended by replacing small molecules in the aforesaid reactions by their multiple isotopically labelled alternatives. The most suitable compounds are glycine-$^{13}C_2$ and glycine-$^{13}C_2$,^{15}N, glutamine-^{15}N and fumarate-$^{13}C_4$. The only undescribed method is preparation of SAMP- and SAdo-$^{13}C_4$ from AMP.

3.1.4.1 SAMP- and SAdo-$^{13}C_4$

SAMP-$^{13}C_4$ was prepared by a reverse enzymatic reaction when the human recombinant ADSL transformed AMP into SAMP-$^{13}C_4$ by addition of fumarate-$^{13}C_4$. The reaction mixture contained 10 mM Tris-Cl (pH 8), 10 mM KCl, 2mM EDTA, 70 mM AMP, 6 mM fumaric acid and 50 µg/mL of the human

recombinant enzyme ADSL. After 4 h of incubation at 37 °C, the reaction mixture was concentrated by a nitrogen evaporation. SAMP-$^{13}C_4$ was separated on PEI-TLC plates (CEL 300 PEI/UV254, 10 cm x 20 cm; Macherey-Nagel) eluted with 1 M ammonium acetate, identification was performed under UV254 (RF 0.02) and the crude product was extracted into a solution of 2 M ammonium hydroxide. The sample was centrifuged at 5000 g for 10 min in order to avoid solid PEI cellulose. The supernatant was then concentrated by the nitrogen evaporation, analysed by HPLC-DAD. The final concentration of SAMP-$^{13}C_4$ determined at 269 nm using a NanoDrop spectrophotometer (Thermo Scientific) and calculated using an extinction coefficient of 19200 $M^{-1}cm^{-1}$ (van den Bergh F. *et al.*,1991). The SAdo-$^{13}C_4$ was prepared in the standard reaction mixture of 1xNEB3 buffer and 20 U CIP via incubation for 1 h at 37 °C and characterized by the same methods as SAMP-$^{13}C_4$ (Zikanova M. *et al.*,2015).

3.2 Analytical methods for detection of DNPS substrates

3.2.1 HPLC-UV

The DNPS pathway could be divided in two groups of substrates from the perspective of UV detection. The first group, which does not absorb the UV light, of DNPS includes substrates such as PRPP, PRA, GAR, FGAR and FGAMR. The second group, which does absorb the UV light, of DNPS includes substrates such as AIR, CAIR, SAICAR, AICAR, FAICAR and IMP. Methods for detection of these substrates and their nucleotides were previously developed in our group and used in this study. SAICAR, SAICAr, SAMP and SAdo were separated on Prontosil 120-3 C18-AQ column (200 * 4 mm, 3 µm; Bischoff Chromatography) with gradient elution in mobile phases containing 0.1 M KH_2PO_4 and 5 mM tetrabutylammonium hydrogensulfate with pH 3 (mobile phase A) and 30% acetonitrile, 0.1 M KH_2PO_4 and 5 mM tetrabutylammonium hydrogensulfate with pH 3 (mobile phase B) at a flow rate 0.7 mL/min (Zikanova M. *et al.*,2015). Substrates AIR and AIr were separated on Prontosil 120-3 C18-AQ column with gradient elution in mobile phases containing 0.1 M KH_2PO_4 and 5 mM tetrabutylammonium hydrogensulfate with pH 1.5 (mobile phase A) and 30% acetonitrile, 0.1 M KH_2PO_4 and 5 mM tetrabutylammonium hydrogensulfate with pH 1.5 (mobile phase B) at a flow rate 0.7 mL/min (Baresova V. *et al.*,2016). Substrates CAIR and CAIr were separated on Prontosil 120-3 C18-AQ column with gradient elution in mobile phases containing 5 mM KH_2PO_4, 75 mM K_2HPO_4 and 10 mM tetrabutylammonium bromide with pH 8.1 (mobile phase A) and 30% acetonitrile, 5 mM KH_2PO_4, 75 mM K_2HPO_4 and 10 mM tetrabutylammonium bromide with pH 8.1 (mobile phase B) at a flow rate 1 mL/min (Baresova V. *et al.*,2016). The advantage of HPLC-UV against HPLC-MS is the ability to utilize buffers as mobile phases. The major drawback is that the first group of DNPS substrates cannot be assessed by UV detection due to the absence of chromophore in their structure. Therefore,

an application of more sophisticated methods including HPLC coupled with tandem mass spectrometry detection (HPLC-MS/MS) was desired.

3.2.2 HPLC-MS/MS

The suitable tool for analysis of all DNPS substrates is high pressure liquid chromatography coupled with triple quadrupole (HPLC-MS/MS). The detector based on triple quadrupole at first captures the parental mass of chosen molecule, the parental molecule is then fragmented in the second quadrupole by collisions with nitrogen gas into smaller fragments, which are detected in the third quadrupole. The connection of parental mass with created fragments establish a specific transition, which is used in SRM analysis. This approach is applied to all selected masses and transitions. The available system was composed of the 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). The separation was performed on Prontosil 120-3 C18-AQ column (200 * 4 mm, 3 µm; Bischoff Chromatography) tempered 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 was 300 µL/min, and the injection sample volume was 5 µL (Baresova V. *et al.*,2016).

3.3 BIOLOGICAL SAMPLE PREPARATION

3.3.1 Urine

Urine samples were stored at – 20 °C. After thawing, the samples were spun down on mini centrifuge in order to remove proteins. Next, the supernatant with volume of minimum 30 µL or maximum of 200 µL was pipetted into insert and embedded into HPLC vial to be analysed by HPLC-MS/MS.

3.3.2 Cell medium and lysates

A pellet including 1 million of HeLa cells was washed in 1 mL of PBS and centrifuged at 2500 g for 5 min at 4 °C. The supernatant was discarded and 50 µL of ADSL buffer was added. The sample was sonicated twice with a sequence consisting of 15 s pulse and 45 s pause. The lysate was centrifuged at 17000 g, for 20 min at 4 °C. The supernatant was transferred into a new tube and deproteinized. For deproteinization, 30 µL of cell lysate supernatant or 30 µL of cell culture media was used. Firstly, 16.64 µL of 5% perchloric acid was added. After 5 min of incubation at 4 °C, the sample was spun down on mini centrifuge. Next, 30 µL of the supernatant was transferred into a new tube and 3.2 µL of 2.5 M $KHCO_3$ was added. After 10 min of incubation at 4 °C, the sample was spun down on mini centrifuge.

Finally, the pH was adjusted to an optimum range of 6 – 9 and the sample was pipetted into insert and embedded into HPLC vial to be analysed by HPLC-MS/MS.

3.3.3 Plasma

The plasma sample was deproteinized and prepared for HPLC-MS/MS analyses using the same procedure as was described above for the cell culture media (chapter 3.3.2).

3.3.4 DBS

Three 3mm diameter discs were punched from each DBS sample and they were placed in 100 µL of extraction buffer containing acetonitrile:methanol:water (1:1:1 ratio) with 100 nM Sado-$^{13}C_4$ as the internal standard. Samples were incubated for 15 min in ultrasonic bath. An 80 µL aliquot of extract was transferred to a clean tube and centrifuged at 8000 g for 5 min at RT. The supernatant was evaporated to dryness under the stream of nitrogen, dissolved in 30 µL of ddH_2O and embedded into HPLC vial to be analysed by HPLC-MS/MS.

4 RESULTS

4.1 Expression and purification of human and bacterial recombinant enzymes

Sequences of human or bacterial genes coding individual DNPS enzymes were cloned into expression vectors. Within the expression vectors, DNPS enzymes were fused with MBP or 6H (6 histidines) tag, which served for later purification by an affinity chromatography. A special strain DH5αF'IQ of *E. coli* was transfected and used in production of DNPS enzymes. These bacterial enzymes were prepared using this approach: 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 were produced by the forward or reverse enzymatic reactions catalysed with human or bacterial recombinant enzymes. The obtained ribonucleotides were converted into ribonucleosides by dephosphorylation performed with the enzymatic reaction with CIP. The exceptions in substrate preparation were the inorganic synthesis of PRA, FAICAR and FAICAr.

4.2.1 PRA

PRA was prepared by the inorganic reaction of ammonium hydroxide which was previously described (Nierlich D.P. and Magasanik B.,1965). PRA is unstable hence this reaction constitutes the first part in the two-stepped process in the enzymatic synthesis of GAR or FGAR.

4.2.2 GAR and GAr and GAR-$^{13}C_2$ or GAR-$^{13}C_2$,^{15}N

GAR was produced by the two downstream reactions including the inorganic preparation of PRA and the enzymatic reaction catalysed by MBP-GARS. The obtained product was either analysed by MS or underwent the dephosphorylation with CIP into GAr and was analysed afterwards by MS techniques. The substitution of commonly used glycine with glycine-$^{13}C_2$ or glycine-$^{13}C_2$,^{15}N resulted in a preparation of stable multiple isotopically labelled GAR-$^{13}C_2$ or GAR-$^{13}C_2$,^{15}N and their corresponding ribosides. The purity of prepared compounds was 98 % and the concentration calculated from HPLC-MS intensities was 124 µmol/L (Madrova L. *et al.*,2018).

4.2.3 FGAR and FGAr and FGAR-$^{13}C_2$ or FGAR-$^{13}C_2$,^{15}N

FGAR was produced by three downstream reactions including the inorganic preparation of PRA and the enzymatic reaction catalysed by MBP-GARS and MBP-GARFT. The obtained product was either analysed by MS or underwent the dephosphorylation with CIP into FGAr and was analysed afterwards by MS techniques. The substitution of commonly used glycine with glycine-$^{13}C_2$ or glycine-$^{13}C_2$,^{15}N resulted in a preparation of stable multiple isotopically labelled substrates. The purity of prepared compounds was 93 % and the concentration calculated from HPLC-MS intensities was 82 µmol/L (Madrova L. *et al.*,2018).

4.2.4 FGAMR and FGAMr and FGAMR-$^{13}C_2$,^{15}N

FGAMR was produced by the enzymatic reaction including addition of 6H-PurL to the reaction mixture of FGAR. The obtained product was either analysed by MS or underwent the dephosphorylation with CIP into FGAMr and was analysed afterwards by MS techniques. The substitution of glutamine with glutamine-^{15}N resulted in a preparation of stable multiple isotopically labelled substrate (FGAMR-$^{13}C_2$,^{15}N) which was detected after cyclization of imidazole ring in form of AIR-$^{13}C_2$,^{15}N. The purity of prepared compounds was not determined. The concentration calculated from HPLC-MS intensities was 57 µmol/L (Madrova L. *et al.*,2018).

4.2.5 AIR and AIr and AIR-$^{13}C_2$,^{15}N or AIR-$^{13}C_2$,$^{15}N_2$

AIR was produced either by four downstream reactions including the inorganic preparation of PRA and the enzymatic reaction catalysed by MBP-GARS, MBP-GARFT and 6H-PurL or by the reverse conversion of SAICAR by MBP-PAICS. The obtained product was either analysed by MS or underwent the dephosphorylation with CIP into AIr, which was then analysed by MS techniques. The substitution of glycine with glycine-$^{13}C_2$ or glycine-$^{13}C_2$,^{15}N and glutamine with glutamine-^{15}N resulted in a preparation of stable multiple isotopically labelled substrates (AIR-$^{13}C_2$, AIR-$^{13}C_2$,^{15}N or AIR-$^{13}C_2$,$^{15}N_2$). The purity of the prepared compounds determined by HPLC-DAD reached 84 % for AIR and the concentration of AIR was at least 3 mmol/L (Baresova V. *et al.*,2016).

4.2.6 CAIR and CAIr

CAIR was produced by the conversion of AIR by MBP-PAICS. The obtained product was either analysed by MS analysis or underwent the dephosphorylation with CIP into CAIr and was analysed afterwards

by MS techniques. The purity of the prepared compounds determined by HPLC-DAD reached 60 % for CAIR and the concentration of obtained CAIR was at least 3 mmol/L (Baresova V. *et al.*,2016).

4.2.7 SAICAR, SAICAr and SAICAr-$^{13}C_4$

SAICAR was prepared from AICAR in the reaction catalysed by human MBP-ADSL ran in a reversed direction due to excess of ADP. The obtained product was either analysed by MS or underwent the dephosphorylation with CIP into SAICAr and was analysed afterwards by MS techniques. The substitution of fumarate with fumarate-$^{13}C_4$ resulted in a preparation of stable multiple isotopically labelled substrates (SAICAR-$^{13}C_4$ and SAICAr-$^{13}C_4$). The purity as determined by HPLC-DAD (Zikanova M. *et al.*,2015) or HPLC-MS reached 96-99 % and the concentration of SAICAR was at least 10 mmol/L.

4.2.8 FAICAR and FAICAr

FAICAR was prepared by the inorganic formylation of commercially supplied AICAR. For the preparation of FAICAr, AICAR was dephosphorylated in the beginning into AICAr, which was added into the formylation mixture afterwards. Obtained products were analysed by MS techniques. The purity of prepared compounds was 50 %. The concentration calculated from HPLC-MS intensities was 73 µmol/L (Madrova L. *et al.*,2018).

4.2.9 SAMP-$^{13}C_4$ and SAdo-$^{13}C_4$

SAMP-$^{13}C_4$ was prepared from AMP in the reaction catalysed by human MBP-ADSL ran in a reversed direction due to excess of ADP. The obtained product was either analysed by MS techniques or underwent the dephosphorylation with CIP into SAdo-$^{13}C_4$ and was analysed afterwards. The purity as determined by HPLC-DAD (Zikanova M. *et al.*,2015) or HPLC-MS reached 96-99 % and the concentration was at least 10 mmol/L.

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 Csientific, MA, USA) coupled with Orbitrap Elite (ThermoFisher Scientific, MA, USA) hybrid mass spectrometer operated with MassFrontier 7.0.5.09 SP3 software (HighChem, SK).

4.3.1 HPLC-MS/MS

The ability of API4000 (AB Sciex) triple quadrupole to tune a precursor compound and its transitions after fragmentation in SRM regime was applied to the development of precise detection methods of DNPS substrates. A protocol for determining the product ions optimization was followed which encompassed direct infusion of target compound standard and tuning of measurement parameters. The optimization was established in both polarities.

Initially, the mass of an analysed compound parental ion (MS1) was determined within the expected mass range on the first quadrupole (Q1). The parental ion was then fragmented by interaction with N_2 gas in a collision cell (Q2) and the fragments`ions were finally detected with third quadrupole (Q3). Subsequently, all measurement parameters were tuned including declustering potential (DP), entrance potential (EP), collision energy (CEn) and collision cell exit potential (CXP). Method parameters were built for five most intense product ion (MS2) peaks, which provided unique transitions for purposes of detection method preparation. Obtained parameters were set in acquisition method suitable for detection of DNPS substrates by API 4000 triple quadrupole.

The separation of DNPS substrates was performed on Prontosil C18 reverse phase column with gradient elution of phase A containing 0.1% formic acid in water and phase B containing 0.1% formic acid in acetonitrile. The upgraded settings were used in all further analyses (Tab. 4).

Table 4. Parameters used in HPLC-MS/MS analysis of nucleosides detection.

name	Q1	Q3	time [ms]	DP [V]	EP [V]	CEn [V]	EXP [V]	RT [min]
GAr1	207.1	73	100	35	10	25	5	4.51
GAr2	207.1	75.1	100	61	10	15	5	4.51
GAR1	287.1	73	100	35	10	25	5	6.1
GAR2	287.1	75.1	100	61	10	15	5	6.1
FGAr1	235.1	86.1	100	41	10	25	4	5.48
FGAr2	235.1	103.1	100	41	10	11	6	5.48
FGAR1	315.1	103	100	41	11	25	6	5.65
FGAR2	315.1	86	100	41	11	25	6	5.65
FGAMr1	234.2	85.1	100	41	10	11	6	4.99
FGAMr2	234.2	102	100	41	10	11	6	4.99
AIr1	216.3	84	100	35	10	25	5	5.01
AIr2	216.3	94	100	35	10	25	5	5.01
AIR1	296.2	84	100	40	10	25	5	5.58
AIR2	296.2	94	100	40	10	25	5	5.58
CAIr1	260.2	110.2	100	35	10	25	5	6.89
CAIr2	260.2	128.2	100	35	10	25	5	6.89
CAIR1	340.2	128.2	100	35	10	25	5	10.62
CAIR2	340.2	110.2	100	35	10	25	5	10.62
SAICAr	375.2	243	100	35	6,2	22	18	11.31
SAICAR	455.2	243	100	35	10	22	6.2	11.73
AICAr1	259.2	110	100	20	4.5	18	4	8.52
AICAr2	259.2	127.2	100	20	4.5	18	4	8.52
AICAR1	339.2	127.2	100	35	10	22	6.2	8.97
AICAR2	339.2	110.2	100	35	10	22	6.2	8.97
FAICAr1	287.2	138.2	100	35	10	25	5	6.54
FAICAr2	287.2	155.2	100	35	10	25	5	6.54
FAICAR1	367.2	138.2	100	35	10	25	5	7.59
FAICAR2	367.2	155.2	100	35	10	25	5	7.59
SAdo	384.2	252.2	100	34	4.5	26	4.5	13.54
SAMP	464.2	252.2	100	35	10	22	6.2	13.35
Hx	137.1	110.1	100	35	6.2	22	18	7.17
X	153.1	110.1	100	35	6.2	14	18	7.39

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 (Baresova V. *et al.*,2016).

CRISPR/Cas9 model cell lines with individual defective enzymes GART, PFAS, PAICS, ADSL and ATIC were analysed for the accumulation of their substrates by MS techniques.

4.4.1 Accumulation of DNPS substrates

The developed detection methods were applied to analysis of cell lysates and media prepared from CRISPR/Cas9 model cell lines. The amounts of accumulated substrates (ranging from 0.38 µM AIr to 5.9 µM AICAr) for deficient enzymes was detected in cell growth medium in dephosphorylated form apart from unstable PRA, which is a substrate for the enzyme GART. The other CRISPR/Cas9 model cell lines confirmed the accumulation of dephosphorylated substrate for deficient enzyme caused by mutation in the respective gene (Fig. 7) (Baresova V. *et al.*,2016). These sets of experiments established first human model cell lines for each individual step of DNPS suitable for further investigation.

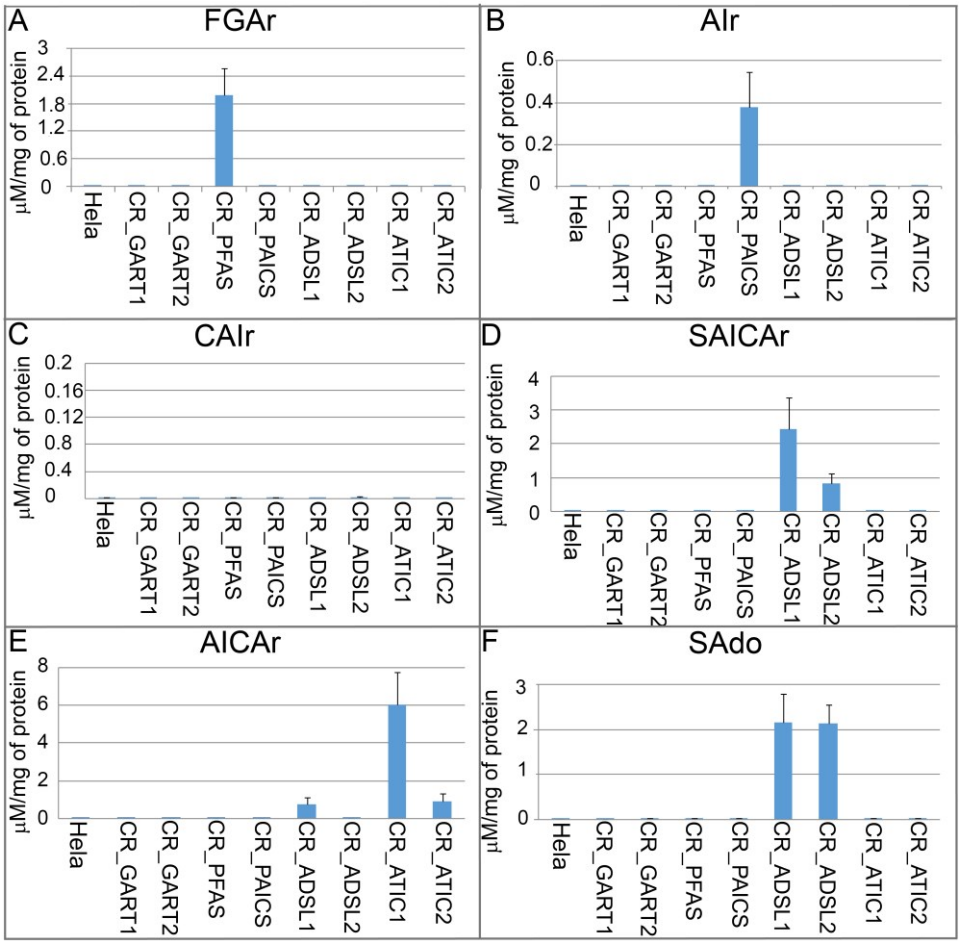

Figure 7. HeLa cell lines mutated with CRISPR/Cas9 technique in individual genes coding DNPS enzymes. The target mutation of individual DNPS genes resulted in cell lines with characteristic elevation of dephosphorylated substrates of defective enzyme in cell growth medium. HPLC-MS/MS analysis detected elevated FGAr (**A**) in CR_PFAS cells, elevated AIr (**B**), but not CAIr (**C**) was observed in CR_PAICS, high concentrations of SAICAr (**D**) and SAdo (**F**) were detected in CR_ADSL and AICAr (**E**) was accumulated in CR_ATIC cells.

4.4.2 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_ADSL and CR_ATIC cells grown 24 h in PD medium were analysed for presence of accumulated substrates of defective enzymes by HPLC-MS/MS before and after transfection with construct encoding appropriate wt protein. Samples of cell lysates prepared from CR_PFAS cell line shown decrease in concentration of FGAR and FGAr after transfection. Similarly, was observed decrease in AIR and AIr concentration in samples of CR_PAICS, SAICAR, SAICAr, SAdo and SAMP concentration in CR_ADSL and AICAR and AICAr concentration in CR_ATIC (Tab. 5) after transfection (Baresova V. *et al.*,2018).

Table 5. 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 in all model cell lines.

	HeLa	CR_PFAS		CR_PAICS		CR_ADSL		CR_ATIC	
metabolite	µmol/L	non TR µmol/L	TR µmol/L	non TR µmol/L	TR µmol/L	non TR µmol/L	TR µmol/L	non TR µmol/L	TR µmol/L
FGAr	nd	12.1±2.1	0.32±0.22	0.28±0.06	nd	nd	nd	nd	nd
FGAR	nd	2.1±0.9	0.07±0.06	0.13±0.04	nd	nd	nd	nd	nd
AIr	nd	nd	nd	1.0±0.1	0.004±0.001	nd	nd	nd	nd
AIR	nd	nd	nd	2.93±0.16	0.33±0.01	nd	nd	nd	nd
SAICAr	nd	nd	nd	nd	nd	0.40±0.04	0.072±0.002	0.05±0.01	≈LOQ
SAICAR	nd	nd	nd	nd	nd	0.21±0.03	nd	nd	nd
SAdo	≈LOQ	≈LOQ	≈LOQ	≈LOQ	≈LOQ	10.5±1.1	1.9±0.2	≈LOQ	≈LOQ
SAMP	≈LOQ	≈LOQ	≈LOQ	nd	nd	22.7±2.5	nd	nd	nd
AICAr	nd	nd	nd	nd	nd	nd	nd	0.09±0.03	nd
AICAR	nd	nd	nd	nd	nd	nd	nd	1.2±0.4	nd

nd – not detected, ≈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 dried blood spots were analysed.

4.5.1 Screening of urine samples

The urine samples of 40 controls and a group of 1447 patients were normalised to concentration of 1 mM creatinine and analysed by HPLC-MS/MS. First, physiological values of FGAr, AIr, CAIr, SAICAr, AICAr and SAdo in control urine samples were established (Tab. 6). Next, the levels of metabolites were determined in patients' urine samples. Slightly elevated levels of AIr were detected in 3 samples and one extremely elevated FGAr in one sample (Fig. 8) (Krijt M. *et al.*,2019).

Table 6. Ranges of DNPS metabolites detected in control urine samples by HPLC-MS/MS.

metabolite	URINE [µmol/mmol creatinine]			[µmol/L]	
	min	max	median	LOD	LOQ
FGAr	0.00	0.32	0.098	0.001	0.004
AIr	0.00	0.43	0.068	0.033	0.109
CAIr	0.00	0.17	0.052	0.019	0.062
SAICAr	0.02	0.48	0.098	0.001	0.004
AICAr	0.03	0.40	0.120	0.002	0.004
SAdo	0.00	2.58	0.819	0.001	0.004

LOD – Limit Of Detection, LOQ – Limit Of Quantification

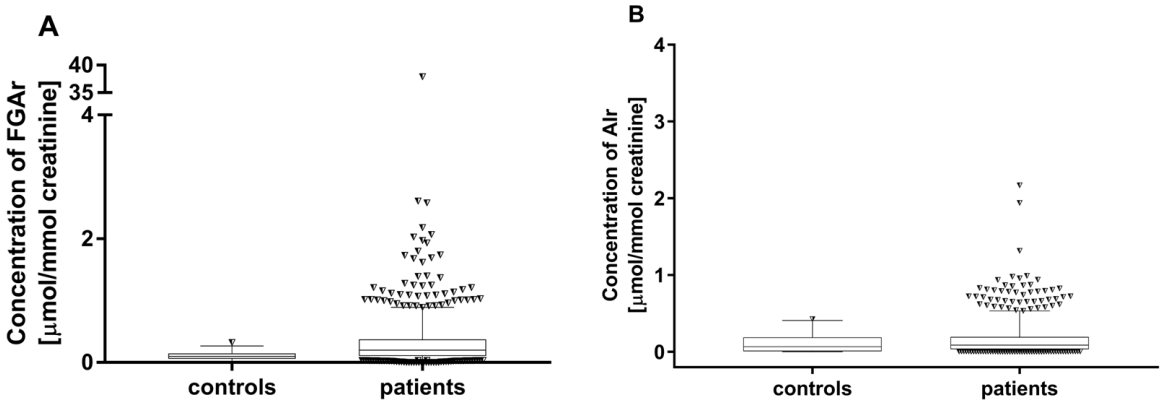

Figure 8. Urine analysis from patients with neurological impairment. Urine samples analysed with HPLC-MS/MS provided physiological range of FGAr, AIr and CAIr in 40 control samples and the screening of 1447 randomized patients' samples showed one extremely increased level of FGAr in one case **(A)** and three moderately upregulated concentrations of AIr **(B)**. The box extends from the 25th to 75th percentiles with plotted median by the horizontal line, and the whiskers mark the 5th and 95th percentiles.

4.5.2 Analysis of DNPS metabolites in DBS samples

4.5.2.1 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-$^{13}C_4$ and SAdo-$^{13}C_4$ were utilized as internal standards in the precise detection and quantification of studied metabolites. Control samples revealed physiological range 0-0.026 µmol/L for SAICAr and 0.06-0.14 µmol/L for SAdo (LOD 0.004 and 0.002 µmol/L, respectively). Higher levels of SAICAr and SAdo in group of patients' samples were observed, 0.03-4.7 µmol/L in case of SAICAr and 1.5-21.3 µmol/L in case of SAdo (Fig. 9) (Zikanova M. *et al.*,2015).

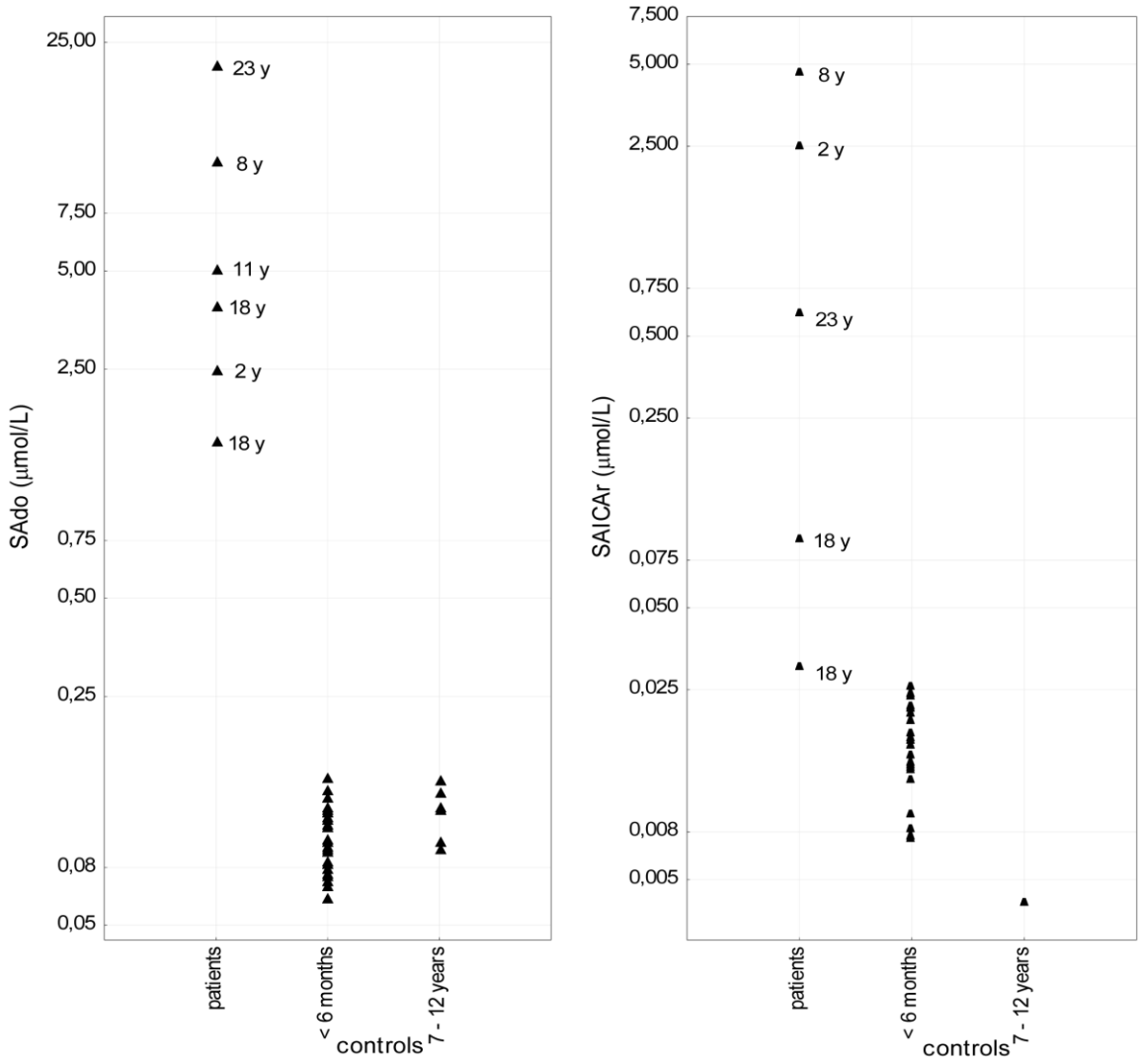

Figure 9. Detection of SAICAr and SAdo in DBS of ADSL-deficient patients by HPLC-MS/MS. Samples of ADSL-deficient patients prepared from DBS stored for 2 and up to 23 years revealed elevated concentrations of SAICAr and SAdo when compared to control samples.

4.5.2.2 Screening of DNPS substrates in DBS

The DBS samples of 50 controls and a group of 365 patients' samples were prepared by standard protocol and analysed by HPLC-MS/MS. Physiological values of FGAr, SAICAr, AICAr and SAdo were established in control DBS (Tab. 7) and compared with values from group of DBS patients' samples. The signals of AIr and CAIr were under detection limit. No increased values of DNPS substrates were detected in 365 DBS samples.

Table 7. Ranges of DNPS metabolites detected in control DBS samples by HPLC-MS/MS.

metabolite	DBS [µmol/L]			[µmol/L]	
	min	max	median	LOD	LOQ
FGAr	0.24	0.55	0.35	0.05	0.017
AIr	nd	nd	nd	nd	nd
CAIr	nd	nd	nd	nd	nd
SAICAr	0.006	0.030	0.014	0.008	0.025
AICAr	0.004	0.051	0.01	0.002	0.006
SAdo	0.126	0.522	0.299	0.001	0.004

LOD – Limit Of Detection, LOQ – Limit Of Quantification, nd – not detected

4.6 Description of new DNPS disorder

A networking tool GeneMatcher (GM) (Sobreira N. *et al.*,2016) is a website connecting researchers who are interested in the same genes. We initiated collaboration through GM, which resulted in establishing the collaboration with colleagues from Université de Paris (France) to investigate two cases suspected of mutations in PAICS gene. The probands were children of consanguineous parents from Faroe Islands having three healthy male and one female descendants. One pregnancy was terminated at week 13 with spontaneous abortion and two children were born, but manifested numerous malformations including craniofacial dysmorphism, progressive hypotension and hypoxia. The first proband succumbed to cardiorespiratory failure after 28 h and the second one on day 3 (Pelet A. *et al.*,2019).

4.6.1 Identification of genetic origin

Our colleagues from the Universite de Paris in France used the Affymetrix 250K NspI 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).

4.6.2 Protein analysis and functional studies

The application of genetic data in combination with crystal structure of PAICS (available at PDB 4JA0, http://www.rcsb.org/pdb/home/home.do, March 2020) revealed the destabilization of the mutant protein which caused alteration in structure of catalytic pocket resulting in lower enzyme activity. 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).

4.6.3 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 AIr, respectively. We expected similar results as in our previous study when CRISPR edited HeLa cells (CR_PAICS) showed elevated levels of AIr in a growth medium (Baresova V. *et al.*,2016) and lysates (Baresova V. *et al.*,2018). However, neither AIR nor AIr 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

Purines represent versatile molecules participating in reproduction and take part in various biochemical processes and functions. The production of purines is sustained by DNPS and salvage pathways. The difference between these two pathways is that salvage pathways recycle purine bases by assembly with PRPP while the DNPS build up a new molecule of purine nucleotide from PRPP in ten subsequent reactions catalysed by six enzymes forming the purinosome, which enables the substrate channelling. Purines attract a lot of attention and DNPS has been intensively studied due to its irreplaceable role during organism development, as the therapeutic target and for the potential to discover new disorders related to DNPS.

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). Also due to the current absence of effective treatment for DNPS disorders, is the priority to test treatable disorders manifesting with uncontrolled seizures in the differential diagnosis (i.e. pyridoxine-responsive seizures, pyridox(am)ine-5'-phosphate oxidase deficiency, folinic acid-responsive seizures, biotinidase and holocarboxylase synthetase deficiency, glucose transporter defect, serine biosynthesis disorders) (Saudubray J.M. *et al.*,2006; Jurecka A. *et al.*,2015; Sharma S. and Prasad A.N.,2017). Furthermore, diagnostic methods of DNPS disorders are demanding and not routinely performed. This all contributes to neglect of the DNPS disorders. Over time the screening methods for DNPS disorders were included among routine laboratory tests resulting in detection of four new cases with ADSL deficiency (Castro M. *et al.*,2002) and lately the introduction of novel diagnostic methods caused rapid increase of newly diagnosed patients (van Werkhoven M.A. *et al.*,2013). The initial screening method for DNPS inborn errors was the BM test based on spectrometric detection of SAICAr derivate. Another rapid and inexpensive method for detection of DNPS substrates is TLC. More advanced and semiautomatic method introduced for measurement of DNPS substrates represents HPLC with UV detection, lately upgraded to HPLC coupled with MS detection. HPLC-MS detection provides more accurate analysis and possibility of software assisted evaluation. 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 commercially obtained. 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). DNPS and PNC substrates could be prepared by organic synthesis (Camarasa M.J. *et al.*,1980; Schrimsher J.L. *et al.*,1986; Chu S.Y. and Henderson J.F.,1970), which includes problematic phosphorylation step (Chettur G. and Benkovic S.J.,1976). Alternative procedure is biochemical synthesis of DNPS substrates in reactions catalysed by protein extracts (i.e. from pigeon liver) (Schulman M.P. *et al.*,1952) or recombinant enzymes (Zikanova M. *et al.*,2005). Purified human enzymes expressed in *E. coli* were used in our laboratory in synthesis of individual DNPS substrates – GAR, FGAR, FGAMR, AIR and CAIR, SAICAR. The enzymatic synthesis of DNPS substrates enables to replace some of the reaction compounds with their multiple isotopically labelled analogues. Multiple isotopically labelled substrates of DNPS provide an alternative to radioactive labelling and serve as internal standards for refinement of detection methods, contribute to precise evaluation of obtained results and may be used exclusively in tracking the metabolic flux (Madrova L. *et al.*,2018). The dephosphorylation of prepared nucleotides to nucleosides was performed by the enzymatic reaction catalysed by CIP. The exception from enzymatic preparation of DNPS substrates was the inorganic preparation of PRA (Nierlich D.P. and Magasanik B.,1965) and formylation of AICAR resulting in production of FAICAR (Lukens L. and Flaks L.,1963). All prepared DNPS substrates were brought into play in development of detection methods.

The methods exploiting HPLC-DAD for analysis of AIr, 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 GAr, FGAr and FGAMr. For this purpose, was utilized HPLC coupled with tandem mass spectrometry detection. Detection of DNPS substrates with HPLC-MS/MS was performed in SRM regime, which increases the evaluation confidence due to the additional analysis fragments generated from parental compound of interest. Methods for detection of GAr, FGAr, AIr, CAIr, SAICAr, AICAr, FAICAr, SAdo and their ribotides were established and used for the detailed studies of DNPS (Baresova V. *et al.*,2016). Subsequently, multiple isotopically labelled standards were analysed, which provided expected signals in terms of retention time and SRM transitions. All mentioned compounds with the addition of FGAMR and FGAMr, which were not possible to identify by our HPLC-MS/MS system due to the interference with FGAr, were detected and evaluated in cooperation with our colleagues from University Palackeho in Olomouc by HPLC-HRMS technique utilizing the Orbitrap mass analyzer (Madrova L. *et al.*,2018). Established detection methods enabled to test the hypothesis that putative DNPS disorders will biochemically manifest alike known DNPS disorders by accumulation of substrate of defective enzyme.

Prokaryotic and eukaryotic organisms were previously studied to determine effects of mutations in DNPS genes. Across many prokaryotic species were established knockout models of initial DNPS steps in *E. coli* causing accumulation of GAR (Andersen-Civil A.I.S. *et al.*,2018). The essentiality of loss-of-function of DNPS genes was investigated in eukaryotic models represented by yeast and C. elegans models (Chen P. *et al.*,2016). The developmental impact of mutations in individual DNPS genes were established in Drosophila melanogaster (Johnstone M.E. *et al.*,1985; Tiong S.Y.K. and Nash D.,1990; O'Donnell A.F. *et al.*,2000) and zebrafish (Ng A. *et al.*,2009). Despite the unsuccessful effort to produce a mouse DNPS knockout models of DNPS (Dickinson M.E. *et al.*,2016), the data from international human exome databases (Karczewski K.J. *et al.*,2020) indicate existing individuals with defective DNPS enzymes. The only exception is PPAT, the first enzyme of DNPS pathway. Although there is a mammalian cell model represented by CHO cell lines deficient in individual steps of DNPS (ade$^-$ B/D/E/F/H/I) (Patterson D. *et al.*,1974; Patterson D.,1975; Patterson D.,1976; Tu A.S. and Patterson D.,1977; Duval N. *et al.*,2013), there was a need of human cell models simulating known and putative DNPS disorders. To experimentally verify claims from genome databases and effects of known and putative DNPS disorders, HeLa cells genetically edited with CRISPR/Cas 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). Prepared cell lines were characterized by genetic sequencing, protein activity assays and determination of DNPS substrates presence in cell medium and lysates (Baresova V. *et al.*,2016). All CR cell lines with exception of CR_GART showed accumulation of the dephosphorylated substrate of defective enzyme in cell medium. In case of CR_ADSL, concentrations of both substrates were elevated and in case of CR_PAICS and CR_ATIC, only the first substrate of defective enzyme was elevated as expected. Thus, the first human model simulating DNPS disorders was established and verified (Baresova V. *et al.*,2016).

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. First and foremost, physiological values of DNPS substrates detectable in urine (40 control samples) and DBS (50 control samples) were established. All DNPS substrates were detectable in the urine by our HPLC-MS/MS methods except FAICAr and FGAMr. With regard to the DBS samples, they provided signals of FGAr, AICAr, SAICAr and SAdo. 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 AIr in three samples and accumulation of FGAr in one sample. The accumulation of AIr 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 for retrospective diagnosis of ADSLD. Samples stored at RT from 2

to 23 years were still applicable for the detection of SAICAr and SAdo (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.

Detection of elevated concentrations of DNPS substrates in body fluids is highly predictive for DNPS disorder diagnosis. However, such result must be confirmed by genetic sequencing for presence of mutation(s) and enzyme activity assays proving low enzyme activity also lead to final verification and full diagnosis of DNPS disorder.

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 NGS and Sanger sequencing revealed mutation on chromosome 4 in exon 2 of *PAICS* gene causing 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, AIr. 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).

DNPS disorders belong among rare diseases. Therefore, the determination of the diagnosis provides necessary background for the adjustment of treatment strategy. More importantly, the diagnosis of DNPS disorder enables genetic counselling and prenatal examination in case of subsequent pregnancy. Regardless of several attempts, no effective treatment was found for DNPS disorders. The endeavour to decrease concentration of accumulated DNPS substrates by inhibition of DNPS or by increased production of nucleotides aiming to indirectly attenuate the DNPS was not successful. The administration of D-ribose, uridine, S-adenosyl-L-methionine, antifolate drugs or allopurinol did not significantly improved patients' outcome (Salerno C. *et al.*,1999; van Werkhoven M.A. *et al.*,2013; Jurecka A. *et al.*,2015). With that said, the current treatment includes the amelioration of seizures by anticonvulsive drugs (Jurecka A. *et al.*,2015). Unlike the modulators of DNPS, the drugs used in controlling seizures target the neuronal synapse ion transporters in order to induce membrane hyperpolarization and decrease signal transmission. The administration of Clobazam (Sankar R.,2012)

and phenobarbital (Mattson R.H.,1989) activate γ amino butyric acid receptor A for chloride anions, carbazepine (Ambrosio A.F. *et al.*,2002) and phenytoin (Yaari Y. *et al.*,1986) block sodium channels, valproic acid (Ghodke-Puranik Y. *et al.*,2013) and topiramate (Mula M. *et al.*,2006) effect both synapse ion transporters and levetiracetam has multiple effects on synapse signal transmission (Abou-Khalil B.,2008). The current knowledge suggests the enzyme GART is the most suitable therapeutic target. Inhibition of the first reaction catalysed by GART should cause in essence the spontaneous degradation of PRA to rib-5-P and ammonia (Schendel F.J. *et al.*,1988), which propose an enormous potential to target some types of cancer by controlled impairment of purine molecules production.

The mechanism of DNPS disorders pathology has not yet been fully described, despite the fact that studies investigating DNPS in brain has offered several hypotheses. The initial idea of insufficient production of purines was disproved when the normal concentrations of AMP and GMP in various tissues from patients with ADSL deficiency were detected (Jaeken J. and van den Berghe G.,1984). Later on, it was described that the severity of ADSL deficiency correlates with SAICAr/SAdo ratio (Jaeken J. *et al.*,1988; Mouchegh K. *et al.*,2007; Jurecka A. *et al.*,2008a). Thus, it was proposed that the pathology of DNPS disorders is caused by toxic effects of accumulated substrates. The administration of SAICAr, SAdo and adenosine confirmed that SAICAr is causing degeneration of pyramidal neurones in rat brain (Stone T.W.,1998). However, the biological action of SAICAr is not yet understood. The neurotoxic effect of AICAr was demonstrated by increased apoptosis in undifferentiated neuroblastoma cells (Garcia-Gil M. *et al.*,2003). The detrimental effects of DNPS disorders onset during embryonic development and organogenesis, when the organism relies on DNPS rather on salvage pathways (Micheli V. *et al.*,2011). As was reported, GART enzyme remains active in patients with Down syndrome even though its expression in human brain normally fades after birth (Brodsky G. *et al.*,1997). Lately, enzymes PFAS, PAICS and ATIC were detected in rat hippocampal neurons. All enzymes were localized adjacent to mitochondria suggesting the close relationship of DNPS with energetic metabolism (Williamson J. *et al.*,2017). The brain consumes approximately 20 % of total glucose suggesting the extreme energetic demands (Jolivet R.,2009) and the impaired metabolism of purine synthesis probably induces malnutrition or metabolic disbalance (Micheli V. *et al.*,2011). The sustained abnormal brain maturation and function may be caused by altered blood brain barrier permeability through adenosine, respectively adenosine receptor (Bynoe M.S. *et al.*,2015) or alternatively launched by apoptosis triggered by AMP-kinase (AMPK) signalization. AMPK is also closely related to DNPS metabolism (Corton J.M. *et al.*,1995) by its inhibitory effect on mTOR, the serine/threonine protein kinase regulating cell growth (Camici M. *et al.*,2018). The mTOR suppression causes disruption of purinosome formation and spatial arrangement (French J.B. *et al.*,2016). The purinosome localization is related to microtubule network (An S. *et al.*,2010a; Chan C.Y. *et al.*,2018)

and mitochondria (French J.B. *et al.*,2016), which concerts with the DNPS requirement of 5 ATP molecules for the IMP production (Pedley A.M. and Benkovic S.J.,2017).

In order to study all described mechanisms and hypothesis in more detail and further investigate DNPS pathway the original methods for DNPS substrates preparation were introduced, detection methods were developed and the first human cellular model simulating known and putative DNPS disorders was established. Our effort resulted in description of the new DNPS disorder herein referred to as the PAICS deficiency (Pelet A. *et al.*,2019) and also in capturing suspicious proband with PFAS deficiency (Krijt M. *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

Most of the DNPS substrates are commercially unavailable, which makes the development of their detection methods extremely difficult. Therefore, we have developed procedures for preparation and purification of DNPS substrates GAR, FGAR, FGAMR, AIR, CAIR, SAICAR and FAICAR and their dephosphorylated analogues GAr, FGAr, FGAMr, AIr, CAIr, SAICAr, AICAr, FAICAr and SAdo in vitro by enzymatic reactions. Our procedures for preparation of DNPS substrates allow to exchange enzyme substrates for their isotopically labelled variants and we have produced stable isotopically labelled compounds GAR- and GAr-$^{13}C_2$ or -$^{13}C_2$,^{15}N, FGAR- and FGAr-$^{13}C_2$ or -$^{13}C_2$,^{15}N, FGAMR- and FGAMr-$^{13}C_2$ or -$^{13}C_2$,^{15}N, or -$^{13}C_2$,$^{15}N_2$, AIR- and AIr-$^{13}C_2$ or -$^{13}C_2$,^{15}N, or -$^{13}C_2$,$^{15}N_2$, CAIR- and CAIr-$^{13}C_2$ or -$^{13}C_3$,^{15}N, or -$^{13}C_3$,$^{15}N_2$, SAICAR- and SAICAr-$^{13}C_3$,$^{15}N_2$, -$^{13}C_3$,^{15}N or -$^{13}C_4$, AICAR- and AICAr-$^{13}C_3$,$^{15}N_2$ and SAMP- and SAdo-$^{13}C_4$.

6.2 Development of detection methods

Prepared DNPS substrates have served as standards for development of detection methods based on HPLC separation coupled with tandem mass spectrometry (Baresova V. *et al.*,2016) or high-resolution mass spectrometry (Madrova L. *et al.*,2018). The identification of DNPS substrates (GAR, FGAR, FGAMR, AIR, CAIR, SAICAR, AICAR, FAICAR, SAMP) and their dephosphorylated forms (GAr, FGAr, FGAMr, AIr, CAIr, SAICAr, AICAr, FAICAr and SAdo) including detection in positive mode, electrospray ionisation and application of the selected reaction monitoring in both methods has been established. Additionally, the HRMS has enabled to capture fragmentation spectra for DNPS nucleotides and nucleosides by collision induced dissociation 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

Developed methods for qualitative and quantitative analysis of DNPS substrates have been utilized in the studies with models of known and putative DNPS disorders in human cell model represented by 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, Baresova V. *et al.*,2018; Madrova L. *et al.*,2018). The hypothesis, that putative disorders of DNPS will manifest by accumulation of substrate

of defective enzyme, has been verified. The exception is represented by CR_GART, due to the instability of GART substrate PRA, which was thus not detected in cell lysate or growth medium (Baresova V. *et al.*,2016; Baresova V. *et al.*,2018). The transient transfection of individual defective cell lines with wt enzymes has resulted in decrease of accumulated DNPS substrate concentration and restoration of purinosome formation in PD medium (Baresova V. *et al.*,2018).

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

The confirmation of hypothesis claiming that dephosphorylated substrates of defective enzyme were accumulating in known and putative DNPS disorders encouraged us to screen of patients lacking a diagnosis and suffering with unspecific neurological symptoms. Therefore, we analysed 1447 urine and 365 DBS samples 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). Developed procedures may be adopted and utilized in diagnosis of known and putative DNPS disorders as has been demonstrated after introduction of HPLC-UV methods and recent identification of new cases of ADSLD (Kohler M. et al. 1999; van Werkhoven M.A. *et al.*,2013; Castro M. *et al.*,2014).

6.5 PAICS patient

Modern networking in scientific community allowed the unique connection of research teams and resulted in description of new DNPS disorder. Genetic data indicating the presence of PAICS deficiency were carefully studied. After recognition of homozygous SNP, verified investigative methods were applied including molecular genetic methods, protein functional and kinetic studies, purinosome visualisation and DNPS substrates detection methods for detailed description of DNPS pathway. All applied methods and techniques comprehensively enabled to confirm conditions of suspected patients and resulted in diagnosis and description of new DNPS disorder, the PAICS deficiency (Pelet A. *et al.*,2019).

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