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GENETIC ANALYSIS IN INHERITED METABOLIC DISORDERS – FROM DIAGNOSIS TO TREATMENT. OWN EXPERIENCE, CURRENT STATE OF KNOWLEDGE AND PERSPECTIVES

ANALIZA GENETYCZNA DZIEDZICZNYCH CHORÓB METABOLICZNYCH – OD DIAGNOZY DO LECZENIA. DOŚWIADCZENIA WŁASNE, AKTUALNY STAN WIEDZY I PERSPEKTYWY

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Abstract

Inherited metabolic disorders, also referred to as inborn errors of metabolism (IEM), are a group of congenital disorders caused by mutation in genomic or mitochondrial DNA. IEM are mostly rare disorders with incidence ranging from 1/50 000-1/150 000), however in total IEM may affect even 1/1000 people. A particular mutation affects specific protein or enzyme that improper function leads to alterations in specific metabolic pathway. Inborn errors of metabolism are monogenic disorders that can be inherited in autosomal recessive manner or, less frequently, in autosomal dominant or X-linked patterns. Some exceptions to Mendelian rules of inheritance have also been described.

Vast majority of mutations responsible for IEM are small DNA changes affecting single or several nucleotides, although larger rearrangements were also identified. Therefore, the methods used for the identification of pathogenic mutations are mainly based on molecular techniques, preferably on Sanger sequencing. Moreover, the next generation sequencing technique seems to be another prospective method that can be successfully implemented for the diagnosis of inborn errors of metabolism.

The identification of the genetic defect underlying the disease is not only indispensable for genetic counseling, but also might be necessary to apply appropriate treatment to the patient. Therapeutic strategies for IEM are continuously elaborated and tested (eg. enzyme replacement therapy, specific cells or organ transplantation or gene therapy, both in vivo and ex vivo) and have already been implemented for several disorders.

In this article we present current knowledge about various aspects of IEM on the basis of our own experience and literature review.

Key words: IEM, inborn error of metabolism, monogenic disorders, mutation, molecular diagnostics

Streszczenie

Dziedziczne choroby metaboliczne, zwane także wrodzonymi wadami metabolizmu są grupą chorób powodowanych przez mutacje w genomowym bądź mitochondrialnym DNA. Większość z nich występuje z niską częstością 1/50 000-1/150 000 urodzeń, ale szacuje się, że łącznie występują one nawet u jednej na tysiąc osób.

Mutacje powodują zaburzenie funkcji poszczególnych białek lub enzymów, co skutkuje nieprawidłowym działaniem określonych szlaków metabolicznych. Wrodzone wady metabolizmu, uwarunkowane monogenowo, mogą być dziedziczone w sposób autosomalny recesywny, rzadziej autosomalnie dominująco bądź w sposób sprzężony z chromosomem X. Opisywane są także przypadki odstępstw od praw Mendla.

Większość mutacji odpowiedzialnych za występowanie chorób z tej grupy to zmiany o charakterze punktowym, obejmujące jeden lub kilka nukleotydów, aczkolwiek identyfikowane są także rearanżacje o bardziej rozległym charakterze. Z tego względu do identyfikacji mutacji wykorzystuje się przede wszystkim metody molekularne, głównie sekwencjonowanie DNA metodą Sangera. Nową i perspektywiczną metodą diagnostyki wrodzonych wad metabolizmu wydaje się być również tzw. sekwencjonowanie następnej generacji.

Określenie defektu genetycznego jest nie tylko niezbędne dla celów poradnictwa genetycznego, ale także stanowi podstawę do włączenia odpowiedniego leczenia. Strategie terapeutyczne dotyczące dziedzicznych chorób metabolicznych są stale opracowywane i testowane (np. enzymatyczna terapia zastępcza, przeszczepy komórek i organów oraz terapia genowa, zarówno in vivo, jak i ex vivo) i już teraz są stosowane w leczeniu niektórych z nich. W niniejszej pracy prezentujemy aktualną wiedzę dotyczącą szeregu aspektów związanych z zagadnieniem wrodzonych wad metabolicznych w oparciu o doświadczenia własne i przegląd piśmiennictwa.

Słowa kluczowe: wrodzone wady metabolizmu, choroby monogenowe, mutacje, diagnostyka molekularna

DEV PERIOD MED. 2015;XIX,4:413-431

INTRODUCTION

Deoxyribonucleic acid (DNA), also referred to as "molecule of heredity", contains all information that are indispensable for any living organism to develop, live and reproduce (see Box 1 – fig. 1). The number of genes in the human genome is estimated at 20 000-30 000 and the coding part of DNA (exome) contain about 60 000 000 base pairs, which is only about 2% of whole genomic DNA [1, 2, www.ncbi.nlm.nih.gov].

According to current knowledge, these regions are the most important ones with respect to monogenic disorders. The function of the remaining part of the genome is only partially recognized. About 4% of human genome encodes RNA, further 45% accounts for various repetitive sequences that act in pairing of homologous chromosomes and recombination, influence chromosome structure and regulate gene expression. The function of the remaining part of the genome is less understood, but it was hypothesized that it played a major role in the regulation processes [3].

DNA mutations as a cause of disease

The DNA contains the information for every human cell and the genome of each cell thanks to the replication process is the copy of the genetic material that was present in a zygote. Depending on the cell type and developmental period specific set of genes are activated and this determines the cell morphology, function and behavior, including the response to external stimuli.

Since DNA contains the instruction for every human cell, one could say that all human disorders have a genetic component. It is almost true, as many of diseases are directly caused by the DNA alterations in germline (so called congenital disorders) or somatic cells (e.g. cancer). Also the complex diseases have multifactorial etiology and the genetic background together with environmental factors are important in their etiology. In this case, specific changes in DNA might predispose to the development of a particular disorder.

Changes in DNA can vary from large ones, comprising the abnormal number of chromosomes or their structural aberrations, to small ones, which can affect one nucleotide only. While the large alterations usually alter several genes, the smaller ones have an impact on a single gene and also might cause clinical symptoms. Disorders caused by mutations in a single gene are called monogenic disorders. There are more than 6000 different monogenic diseases known, of which over 500 are inborn error of metabolism (IEM), that are inherited metabolic disorders defined as a "group of congenital disorders caused by an inherited defect in a single specific enzyme that results in a disruption or abnormality in a specific metabolic pathway" (according to The American Heritage® Stedman's Medical Dictionary [4, 5]. Although all of IEM are rare[1] disorders, with a few ethnic specific exceptions only, their total number is high – taken together they can affect about 1/1000 people [5].

Most of the genes involved in monogenic disorders encode proteins, which primary structure is determined

[1]The criteria of rare disease: prevalence ≤ 5 affected people per 10 000 according to the "Regulation (EC) N°141/2000" of the European Parliament and of the Council of 16 December 1999 on orphan medicinal products

Box 1.

THE DNA – FROM NUCLEOTIDES TO CHROMOSOMES.

Chemically, DNA is a double-helix polymer composed of four different nucleotides. Each nucleotide contain monosaccharide - deoxyribose, a phosphate group and one unique nucleobase: adenine (A), guanine (G), cytosine (C) or thymine (T). The order of these four different nucleotides (so called sequence) in DNA molecule is crucial as it determines primary RNA and protein structure, as well as the regulation of gene expression.

The strands of DNA helix are complementary, which means that each nucleotide from one strand is paired with particular nucleotide on another: C with G and A with T. This feature enables exact DNA replication and the equal distribution of genetic material during cell division.

Normal human cell contains 46 molecules of DNA, called chromosomes, which can be easily visible and distinguished during cell division when they are highly condensed. The chromosomes can be classified as autosomes and sex chromosomes. Among 46 chromosomes in human cell, there are 22 pairs of autosomes (named 1-22) and one pair of sex chromosomes (X and Y). Autosomes are sex-independent, while sex chromosomes determine the gender: a pair of X chromosomes is specific for females, while males have one chromosome X and one chromosome Y. Therefore, X chromosome is always inherited from the mother, while a child can receive either X or Y chromosome from his father, which will determine his sex.

During cell division, DNA is replicated and doubled, so that finally both progeny cells and cells composing the whole human organism contain exactly the same genetic information packed in 46 chromosomes. This rule does not apply to the germline cells that undergo meiosis – a different division process, in which gametes, carrying only one chromosome of each pair, are produced. During fertilization two gametes, one from each parent, form so called zygote that contains 46 chromosomes again (one from each pair is derived from a different parent). Hence, genes of each child are inherited from both parents.

by three-letters nucleotide sequence – the genetic code. Each three subsequent nucleotides encode specific amino acids or "STOP" codon that is a signal to finish the protein synthesis. The coding parts of the gene are called exons that are splitted by non-coding parts called introns (fig. 1). Moreover, there are also regulatory regions located within the gene or in its neighborhood. Non-coding parts of the gene, intron and the regulatory ones, are involved in the regulation of timing and specificity of protein synthesis. Therefore, even small changes in gene sequence may affect the gene expression profile and the level or biochemical features of its product.

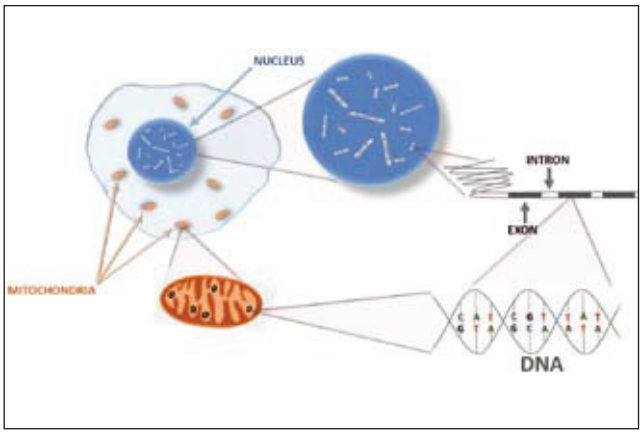

Fig. 1. DNA as a hereditary molecule – see description in a box 1 and 2.

There are several different types of mutations affecting single genes that lead to monogenic disorders. The vast majority of them localizes within the exons or at exon-intron junctions. Most of the mutations identified in monogenic disorders are nucleotide(s) substitutions that lead to amino acid change to another one (so called missense mutations) or STOP codon (nonsense mutations) (fig. 2). The other group of changes are small deletions or insertions, eventually complex changes that might affect the reading frame and lead to changes in amino acid sequence and formation of premature STOP codon. Substitutions and small deletions/insertions, especially those located within exon-intron junctions might cause disturbances in mRNA splicing (so called splicing mutations). The mutations can be also more complex and involve the deletion or duplication of a whole exon, several exons or even the whole gene.

According to Human Gene Mutation Database (HGMD), about 147728 pathogenic mutations in 6867 genes have been identified and published so far, but this number is probably much higher as there are no centralized repository for unpublished mutations for most of the genes. Depending on the gene, mutation type and its localization, the clinical effect of such alterations may be highly heterogeneous. The mutations causing IEM usually impair protein synthesis, function or stability. This further leads to the accumulation of toxic compounds, deficiencies in energy or product level or functional and structural defects of various cellular organelles [5].

Inheritance patterns in inborn error of metabolism

As stated above, most of IEMs are monogenic disorders and follow Mendelian rules of inheritance. They can be inherited either in autosomal (sex-independent) or in sex-dependent manner and can be both dominant or recessive. The majority of IEMs are autosomal recessive disorders and develop when the mutations occur in both alleles – copies of the gene inherited from the parents. Each parent, who

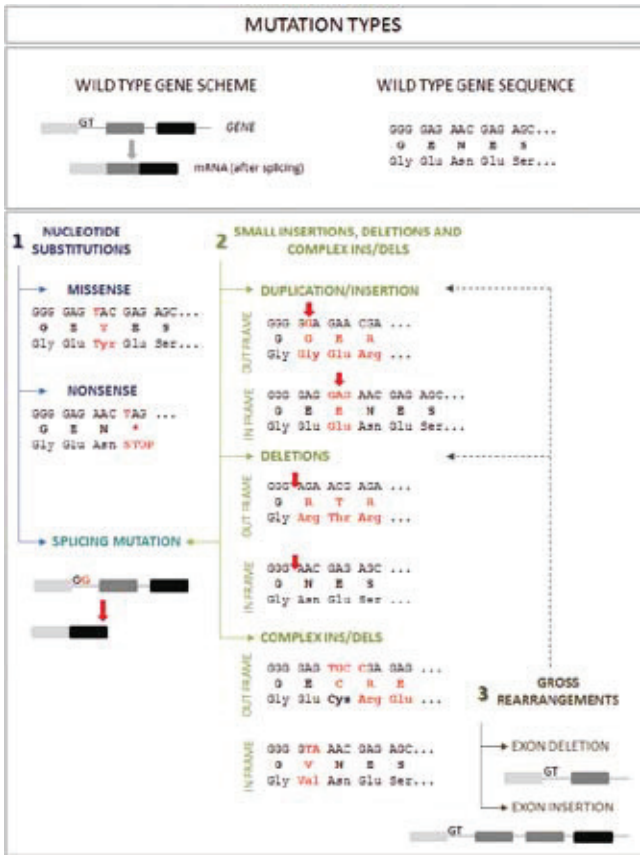

Fig. 2. Schematic representation of the most typical mutations causing monogenic disorders.
Solid lines refer to effect of particular mutations group 1. (nucleotide substitutions); 2. (small insertions (ins)/deletions (dels) and complex ins – ins/dels); 3. (gross rearrangements). Dotted lines show possible effect of gross rearrangements on DNA sequence. Red letters indicate mutations effect on nucleotides and amino acids. Red arrows indicate deletions sites.One and three letters code was used for amino acid description.Boxes in the gene scheme refer to exons, while narrow line refers to introns."GT" at the beginning of the first intron symbolizes conserved donor splicing site.

has one mutated allele, is an asymptomatic carrier and their chance of having an affected child is as high as 25% (tab. I, fig. 3).

In the case of autosomal dominant disorders, that are less common in the group of IEM, only one mutated allele causes the disease despite of the presence of a second non-mutated (wild type) allele. A person with a disease inherited in autosomal dominant manner has a 50% chance of having an affected child, although in many cases the mutation arises *de novo* in the patient (tab. I, fig. 3).

The majority of mutations leading to autosomal disorders are "loss of function" mutations that means they affect crucial protein function and/or activity. For example, biallelic mutations in *PAH* gene impair function of hepatic phenylalanine-4-hydroxylase, enzyme responsible for hydroxylation of phenylalanine to tyrosine, which leads to toxic phenylalanine accumulation and hypothyrosinemia.

In case of autosomal dominant disorders, the presence of "loss of function" mutation in one allele causes haploinsufficiency – the level or activity of the enzyme is too low for the proper cell functioning. However, in autosomal dominant disorders, mutations mainly have a "gain of function" character and lead to the hyperactivity of certain proteins. In this case, only one mutated allele is sufficient to trigger the disease symptoms. One of IEM caused by "gain of function" mutation is hyperinsulinism-hyperammonemia (HI/HA) syndrome, caused by mutation in *GLUD1* gene. The gene encodes glutamate dehydrogenase (GDH) – enzyme responsible for the oxidative deamination of glutamate to ammonia and α-ketoglutarate, which enters the tricarboxylic acid cycle and leads to insulin exocytosis. Mutations in *GLUD1* result in the synthesis of an altered GDH form that is less sensitive to the natural control mechanism – allosteric inhibition of the enzyme by GTP and ATP. Hence, the lack of negative control leads to the leucine-stimulated increased production of α-ketoglutarate and insulin oversecretion [6, 7]. What is interesting, in case of *GLUD1* the mutations responsible for the disease mainly appear *de novo* in the patients and the parents of affected children are unaffected [8].

There are also a few IEM that are inherited in X-linked recessive mode, where mutated genes are located on the X chromosome. The clinical symptoms of these disorders are generally present only in males as they have only one chromosome X. In females, the copy of the gene from the second chromosome compensates the effect of the mutation presence on the other one (tab. I, fig. 3).

Ornithine transcarbamylase (OTC) deficiency is a disease inherited in X-linked recessive manner, that is caused by mutation in *OTC* gene located on the X chromosome (Xp21.1). The disorder affects mainly the boys that have one *OTC* gene copy only. However, the disease can be also present in women, when the two copies of the gene are mutated or when the mutated *OTC* gene was not silenced during the process of X-chromosome inactivation. It was estimated that about 15% of heterozygous females might have the symptoms of ornithine transcarbamylase (OTC) deficiency [9]. Importantly, it has been observed that the percentage of women with clinical symptoms of various X-linked recessive disorders depends on the disease. The disease can be present in up to 70% of heterozygous females (e.g. Fabry disease), whereas in other cases affected women are very rarely observed (e.g. mucopolysaccharidosis type II (MPS II), i.e. Hunter Syndrome) [detailed review in 10].

There are also several diseases that are inherited in X-linked dominant manner (e.g. Rett syndrome), but this is uncommon in case of IEM. The diseases inherited according to this pattern, occurs in both sexes, although clinical symptoms in heterozygous females are milder than in males (tab. I, fig. 3). There are also certain disorders that can be restricted to females, as the presence of the mutation on the X chromosome might be lethal to the male fetuses and they are spontaneously

Table I. Summary of Mendelian modes of inheritance.

<table>
<tr><th>Type of inheritance</th><th>Autosomal recessive</th><th>Autosomal dominant</th><th>X-linked recessive</th><th>X-linked dominant</th></tr>
<tr><td>Affected sex</td><td colspan="2">Both</td><td>-Males
-Females
usually
have mild
symptoms</td><td>Both</td></tr>
<tr><td>When symptoms develop</td><td>Two alleles are mutated</td><td colspan="3">One allele is mutated</td></tr>
<tr><td rowspan="3">The risk of having an affected child*</td><td rowspan="3">For a pair of asymptomatic carriers: 25%</td><td>For an affected person: 50%</td><td colspan="2">For an affeted male:
none of sons will have a mutation;
but all daughters will inherit mutation</td></tr>
<tr><td rowspan="2">For a pair of asymptomatic parents of an affected child: low**</td><td>For a female carrier:</td><td>For an affected female:</td></tr>
<tr><td colspan="2">50% of children will inherit mutation</td></tr>
<tr><td colspan="5">Important note!
The table was prepared as an illustration of general data and in any case cannot be used for risk assessment without detailed molecular investigation in at risk family and genetic counseling. Note that de novo mutations and germline mosaicism, although the most common in autosomal dominant disorders can also apply to all the other types of inheritance.

* Only the most common scenarios are presented.
**According to the literature data the occurrence of de novo mutations or germline mosaicism varies highly between disorders</td></tr>
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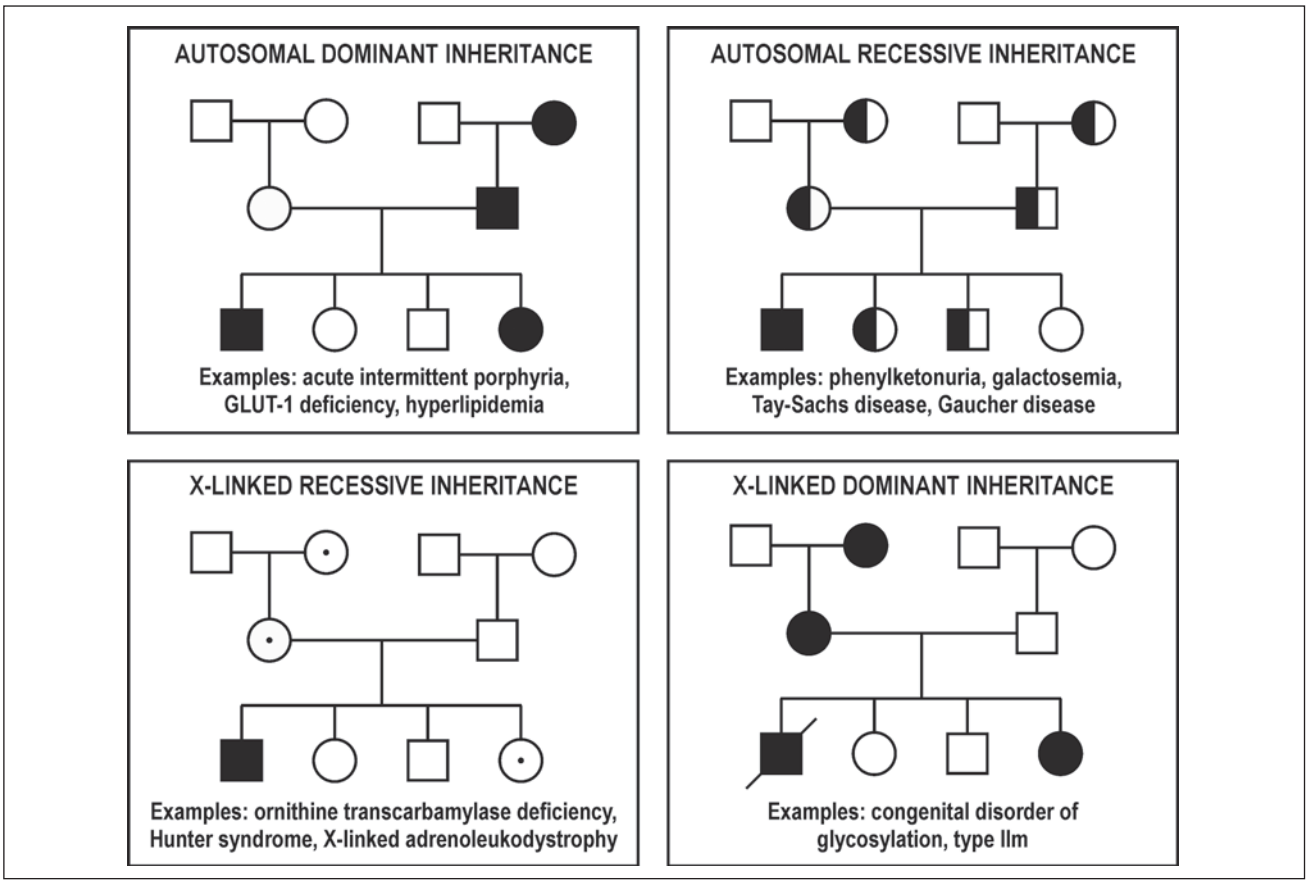

Fig. 3. The pedigree charts. Different modes of inheritance with examples of IEM (see description in the text). square – male, circle – female, the dark squares or circles – affected individuals; half-dark squares or circles – healthy disease carriers in autosomal recessive inheritance patterns; circle with a dot – healthy disease carriers in X-linked recessive inheritance pattern; crossed square – in X-linked dominant inheritance the presence of mutation on the X chromosome is usually lethal for male individuals.

lost during the pregnancy. A good example is congenital disorder of glycosylation (CDG) type SLC35A2 (i.e. UDP-galactose transporter deficiency also known as congenital disorder of glycosylation type II m) caused by mutations in *SLC35A2* gene, which lead to defective N-glycosylation of proteins and lipids. The main clinical symptoms of SLC35A2-CDG include: disease seizures, hypotonia, visual impairment and developmental delay [11, 12]. In contrast to other of around 60 congenital disorders of glycosylation, the data concerning molecular basis SLC35A2-CDG, although highly limited, strongly indicate that the disease is inherited in X-linked dominant manner. Hence, the only SLC35A2-CDG affected patients described so far are females, with two mosaic males exceptions only [13].

The last manner of inheritance observed in IEM is the exception from the classic Mendelian inheritance rules and is related to the presence of mutations in mitochondrial DNA (see Box 2). As the mitochondria are inherited from the mother, the disease can be observed in her progeny independently from the sex, but never can be inherited from the father. Moreover, the severity of clinical symptoms, penetrance and clinical outcome depend on the type of tissue that contain mutated mtDNA and the ratio of mutated to wild-type mtDNA (so called heteroplasmy) [3].

There are several mitochondrial disorders, which main manifestation is the lack of cellular energy caused by oxidative phosphorylation defects. Of note, only part of this disorders is due to mutations in mtDNA, while others result from mutations in nuclear genes encoding proteins indispensable for mitochondria function (these are inherited according to Mendelian rules). There are over 250 different mutations in mtDNA described so far, which are point mutations and DNA rearrangements, mainly deletions. Depending on their type and localization within the mtDNA, they can affect single gene encoding protein, or several ones including tRNA or rRNA genes, which further affect production of all mitochondrial proteins.

Although several mtDNA mutations are connected with particular disorder, some of them may also cause different clinical entities. For example, the m.8993T>G mutation is usually related to Neuropathy, Ataxia and Retinitis Pigmentosa syndrome (NARP), while if the mutation is present in >95% of mtDNA molecules, the Leigh syndrome develops. Similarly, the mtDNA deletions and their variable distribution within the tissues and the level of heteroplasmy are the major determinants of the clinical symptoms. This pleiotropy, together with the influence of other genetic and environment factors, result in a wide spectrum of clinical phenotypes [14]. Hence, due to the high complexity of mitochondrial disorders caused by mtDNA mutations both diagnostics and genetic counseling in at-risk families is very difficult.

The origin of mutation in monogenic disorders

The IEMs, as many other genetic disorders, usually occurs in families with a negative family history that brings up the question about the mutation origin. There can be many different factors contributing to DNA changes e.g. in the case of Down syndrome, the increasing maternal age contributes significantly to the risk of chromosome 21 trisomy. Also the exposition to the chemical or environmental factors may induce various alterations at the DNA level and cause genome instability and in consequence e.g. stimulate cancer development.

Nevertheless, the significant number of mutations occurs as a natural process during DNA replication. It is estimated that average germline point mutation rate per base pair per generation equals to $1.0\text{-}2.2 \times 10^{-8}$. In case of small insertions/deletions (up to 20bp) and larger variations (>20bp) the mutation rate is estimated at 2.94 and 0.16 per generation, respectively. In other words, each individual carries around 60 *de novo* point mutations that arose during gametes formation in their parents [15]. The distribution of *de novo* mutations is not homogeneous and there are regions in the genome that are more prone to mutation occurrence [15]. The germline mutations are inherited and passed to the offspring and this phenomenon is one of the evolutionary determinants of genetic heterogeneity. Although vast majority of mutations is clinically insignificant, some of them may be pathogenic as well. Hence, it is estimated that each individual has 15-60 deleterious mutations in various genes [16].

In case of mitochondrial DNA, the mutation rate is 10-17 times higher than observed in nuclear DNA. This is due to the presence of high levels of free radicals

Box 2. Mitochondria.

Mitochondria – subcellular organelles involved in aerobic cellular respiration and energy production as well as in the other metabolic and signaling pathways. Unlike any other organelle in mammalian cells, mitochondria contain their own DNA molecules (mitochondrial DNA, mtDNA), which are inherited exclusively from the mother, independently from nuclear DNA. Each mitochondrium contains around five mtDNA molecules, but the quantity of mitochondrium per cell varies considerably depending on the cell type and ranges from few hundreds to thousands [3]. Every mtDNA molecule consists of 16 568 base pairs and encodes 13 proteins, which are necessary for oxidative phosphorylation process, 22 types of tRNA and two types of rRNA molecules. In majority of cases mutations are present in a part of mtDNA molecules within a cell (which is defined as heteroplasmy). The ratio of mutated to wild-type mtDNA is one of determinants of disease severity. This value can change as mitochondria segregate randomly during mitotic cell divisions and clonal expansion of mutated mtDNA has been observed. Furthermore, different level of heteroplasmy seen in various members of same family is also caused by mtDNA bottleneck phenomena – the number of mtDNA molecules is highly reduced on random in maternal germ line cells and increases later during development [14]. Thus, the initial proportion of mutated mtDNA in fetuses of the same mother can differ.

in the mitochondria and therefore higher chance of DNA damage that is further fixed during the mtDNA replication. It was estimated that around 1/10 000 might have clinical symptoms resulting from mtDNA mutations.

Importantly, there are several repair mechanisms within the cells that might correct the random mutations and maintain the stability of the genome. Moreover, mutations in the genes encoding key proteins of DNA repair systems can cause various systemic disorders (eg. Aicardi-Goutières syndrome) or increase cancer risk (e.g. Lynch Syndrome) [17, 18]. It is worth mentioning, that the Nobel prize in chemistry in 2015 was awarded to professors Tomas Lindahl, Paul Modrich and Aziz Sancar for the research on DNA repair mechanisms.

The frequency of mutations in different genes within and between population varies and is modulated by several factors, of which the most important ones are the selection pressure and the population isolation that determines the rate of consanguineous relationships. It should be mentioned that the fixation of the mutation in the population might take a long time (several generations), even if the genetic change has a selective advantage.

A good example of the impact of selection pressure on genetic fixation of DNA changes are mutations in *HBB* gene encoding β-globin that cause sickle cell anemia when present in both alleles. However, their presence in one allele protects the heterozygous individuals from malaria. In some populations, the resistance to the malaria infection is a positive selection pressure and favors people who are mutation carriers. In this way, the mutations in *HBB* gene were preserved and the carrier frequency in some Mediterranean populations can reach 15% [19]. Another example is cystic fibrosis (CF) and cholera. One of the theories concerning high frequency of *CFTR* gene mutations focuses on the mechanism of constitutive activation of CFTR-chloride channel by *V. cholerae'* toxin. According to this hypothesis, heterozygotes (*CFTR* mutation carriers) could benefit from mutation in a single allele, as they would be less susceptible to cholera toxin – induced dehydration. Nevertheless, it is also suggested that other bacteria eg. *Escherichia coli* or *Salmonella typhi*, might also use CFTR channel during infection processes, which could also contribute to selective pressure towards heterozygotes [20, 21].

The consanguineous relationships explain high incidence of mutations in some populations. The good example is the presence of mutations in *HEXA* gene, that cause autosomal recessive Tay-Sachs disease in Ashkenazi Jews of Eastern European origin population. It was calculated that 1 per 27 Jew individuals living in the USA is a mutation carrier in this gene, while in the general population the risk of being a carrier is 1/250 [22]. Similarly, in the Northern Pomeranian province in Poland, historically inhabited by Kashubian ethnic group, the incidence of isolated long-chain 3-hydroxyacyl-CoA dehydrogenase deficiency (LCHADD) is 1:16 900, which is around 7-times more than in other regions of the country. Of note, one per 73 persons in this region is a carrier of c.1528G>C mutation in *HADHA* gene in comparison to 1/217 in other regions of Poland, being also the highest rate of carrier status reported for this disorder worldwide [23].

Another example of the disease that is more frequent in Central Europe, especially in Poland (1/2300 to 1/3937), is a Smith-Lemli-Opitz syndrome (SLOS) inborn error of metabolism inherited in autosomal recessive manner. In comparison, its worldwide incidence was estimated at 1/10 000 to 1/70358. What is interesting, only two mutations of *DHCR7* gene, c.452G>A (p.Trp151Ter) and c.976G>T (p.Val326Leu), account for about 65.2% of all observed mutations in Polish SLOS patients [24]. Such changes that account for the majority of cases of particular disease are called "recurrent mutations".

The recurrent mutations not only cause diseases that are more common in some populations, but might be responsible for less frequent disorders. About 80% of patients with medium-chain acyl-CoA dehydrogenase deficiency (MCADD, incidence: 1-10000-1/27000) of European origin are homozygous for the p.Lys329Glu mutation in *ACADM* gene [25]. The haplotype analysis and the inter-population epidemiology studies suggest that this mutation derives from a single ancestor, has possibly appeared in a Neolithic era and was brought to Europe by IndoEuropean-speaking people [26]. This example shows that once appeared mutation can be preserved for thousands years. Similarly, the results of *ARSB* gene analysis in mucopolysaccharidosis VI (MPS VI) patients from Poland, Belarus, Lithuania and Estonia showed that p.Arg152Trp mutation was present in 50% of all cases, what also suggests the founder effect [27]. Other examples include galactosemia (disease frequency in Poland: 1:35000 [28] and phenylketonuria (PKU, incidence 1/8000 in Poland) [29]. Galactosemia is usually caused by *GALT* gene defects, where two mutations p.Gln188Arg and p.Lys285Asn only are present in 79% of disease associated alleles [unpublished results of Department of Medical Genetics, Institute of Mother and Child, DMG, IMiD Warsaw]. In phenylketonuria, there is a panel of recurrent mutations in *PAH* gene, which in Polish population accounts for about 63%-82% of mutated alleles and contains, besides the most prevalent mutation p.Arg408Trp (51%-61.8% of mutated alleles), eleven other mutations that are found in more than 1% of PKU patients: c.1066-11G>A, p.Arg158Gln, p.Pro281Leu, c.1315+1G>A, p.Ala300Ser, p.Ala403Val, p.Arg261Gln, p.Tyr414Cys, p.Ile306Val, p.Arg252Trp and p.Arg169His [30 and unpublished results of DMG IMiD, Warsaw]. However, in a group of 1286 Polish PKU patients analyzed by Bik-Multanowski, 84 other rare or "private" mutations were found in approx. 14% of alleles, while the total number of *PAH* mutations ever reported is 787 (according to the HGMD database) [30]. Indeed in several autosomal recessive disorders, there is various percentage of "private" or sporadic mutations, which often arose independently in ancestors of each parent. This has important implications for genetic diagnostic procedures, which are described later in the text.

Another condition related to the occurrence of autosomal recessive disorders is a uniparental isodisomy (UPD) – a state when two identical chromosomes of the

same pair are inherited from one parent only. In such cases, the corresponding (third – additional) chromosome from the other parent may be removed from zygote, so that total number of chromosomes is correct. In theory, this mechanism has a protective effect and might prevent of possible trisomy. However, in certain cases might also have the clinical effect. The uniparental disomy is a cause of so called imprinting disorders caused by the abnormal expression of genes that are specifically transcribed from paternal or maternal allele. Alternatively, the UPD may be the cause of recessive disorders, including IEM, that may develop when child inherits two identical copies of the chromosome harboring mutated allele from a heterozygous parent, who is the, mutation carrier. Such cases are extremely rare, but have been already described e.g. in GM1 gangliosidosis, Tay-Sachs disease, Morquio A syndrome (MPS IVA), Gaucher disease Type 3 [31-34].

Another phenomena related to mutation origin is the postzygotic mosaicism – a state when at least two population of the cells (for example one with disease causing mutation) are present in one individual. Depending on the time of mutation occurrence and its potential influence on the cell biology, the quantity and distribution of the mutated cells, will be different and may include either both germline and somatic cells, only one of these lines, selected germ layers or selected organs. Depending on this, also the severity of disease caused by the mosaic (somatic) mutation presence might differ significantly. A good example, is again congenital disorder of glycosylation type SLC35A2. The disease is lethal for male fetuses, but Ng et al. have identified somatic mutations in *SLC35A2* gene in two unrelated boys, who had congenital disorder of glycosylation. Due to concurrent presence of a wild type gene in a subset of cells, both boys survived the pregnancy [13].

The phenomena of mosaicism has an important significance for genetic counseling and clinical practice. In germline mosaicism, germline cells subset of proband's parent can also have a mutation. In such case the risk of having another affected child for a couple will increase. Precise estimations are impossible however, as risk values vary between disorders – they were calculated for around 0.2% in the case of achondroplasia and 4.3-8.6% for Duchenne and Becker muscular dystrophy [35]. Germline mosaicism is mostly seen in autosomal dominant disorders, however it was also observed in some X-linked or, rarely, in autosomal recessive disorders [36, 37].

Cytogenetic and molecular diagnosis – the most common methods

As mentioned above, the different types of mutations are responsible for the clinical presentation in the patient. Generally, in many cases the clinical diagnosis may be based on patient phenotype carefully analyzed during the physical examination and the pedigree analysis. Importantly, clinical symptoms of the patients may highly overlap despite different molecular defects. Moreover, in several disorders, e.g in various congenital disorders of glycosylation, there is no enzymatic assay developed to confirm the clinical diagnosis, though the deficient enzyme is known. Thus, molecular testing remains the only diagnostic option in such cases. Altogether, the exact diagnosis of inherited disorders should be confirmed with genetic testing, which is also indispensable with respect to genetic counseling.

Depending on the mutation type that causes the disease distinct methods can be used to identify genetic background of the specific disease. Briefly, these methods can be divided in two groups: cytogenetic ones, that include traditional karyotype analysis and molecular, that include applications based on molecular biology techniques. Nowadays however, due to the tremendous progress in the development of analytical methods the distinction between cytogenetics and molecular genetics becomes less prominent. Many molecular techniques were successfully implemented to the cytogenetic analyses and even the term "molecular karyotyping" was proposed to describe the method that allow to identify chromosomal imbalances with molecular technique – array comparative genomic hybridization [38].

First method, that was proposed for the diagnosis of genetic disorders in 1956, was the karyotype analysis – the analysis of the number and structure of metaphase chromosomes stained with Giemsa dye (so-called G banding). This methods allows to identify chromosomal poliploidies and aneuploidies – changes in chromosome number as well as large deletions, duplications, inversions, translocations and other structural changes [3, 39]. The main disadvantage of this method is the necessity of cell culture to obtain metaphase plates analyzed for the chromosomal aberrations and the possibility of detecting only large chromosomal alterations. However, despite the implementation of molecular methods to the cytogenetic analysis, the karyotype analysis still remains the reference method in certain clinical situations (e.g. prenatal diagnosis).

The other techniques used in the modern diagnostic lab are based on molecular methods and the characteristic attribute of the DNA – the formation of complex structures between complementary sequences. These methods can be grouped to hybridization-based and DNA amplification based (polymerase chain reaction (PCR) – based) techniques. The first group requires the use of specific oligonucleotide probes that are complementary to the sequences of the gene or genes which are analyzed. Such probes can be labeled with fluorescent dyes and used to cytogenetic analysis called fluorescent in situ hybridization (FISH) that allows to detect chromosomal alterations of the smaller size then in traditional karyotype analysis. Moreover, the analysis can be performed on both metaphase and interphase nuclei and needs less time to obtain informative results. However, the FISH methods allows to analyze only a defined number of *loci* in one experiment due to limited capacities of fluorescent microscopes and this is the main disadvantage of the method in the era of genomic medicine [40].

The limitations of karyotype and FISH techniques seem to be partially resolved by the application of array comparative genomic hybridization (aCGH)

to the cytogenetic analysis. This technique uses the oligonucleotide probes fixed on glass or plastic slides (microarrays). The patient and control DNA are labeled with fluorescent dyes and hybridized to the microarrays. The analysis of fluorescent signal after the hybridization reaction allows to detect chromosomal imbalances – deletions or duplications, even the small ones (aCGH resolution up to: 5-10kb, median 60kb) and to specify exactly the genes that are altered in the patient. The methods has one main limitation – it does not allow to detect polyploidies and balanced translocations, possible to detect with conventional karyotyping [38, 41].

Chromosomal imbalances are not common in rare, monogenic disorders, therefore the cytogenetic techniques are not the first method of choice in their genetic diagnosis. For this purpose rather PCR-based methods are used [42, 43]. These methods use the polymerase enzyme and a pair of oligonucleotide primers that are complementary to the analyzed region of the DNA. The product of the PCR reaction can be used to detect particular mutation responsible for the disease. It can be digested with restriction enzyme (so called restriction fragment length polymorphism, RFLP) if the specific mutation cause gain or loss of the restriction site, or assessed with sensitive screening methods that detect changes in DNA conformation caused by the mutation presence (e.g. Denaturing High Pressure Liquid Chromatography – DHPLC, High Resolution Melting – HRM) [44]. A modification of the PCR reaction by the addition of fluorescently labelled oligonucleotide probes specific for the defined alleles can also be used to detect specific mutation (so called allele discrimination assay with Real-Time PCR) [45]. Moreover, PCR product can be used in the hybridization reaction with oligonucleotide probes specific for wild and mutated alleles. These probes are usually immobilized on a specific carrier and complexes formed by the PCR products and specific probes can be detected with color reaction (e.g. Inno-lipa tests). Many of the methods dedicated to the detection of specific mutation has CE-IVD certification and were carefully validated in the clinical laboratories [46]. However, their limitation to detect particular changes in specific genes is in the same time their main disadvantage – we cannot predict whether in the analyzed DNA fragment there is no other mutation.

Therefore, another method that uses the PCR product and was successfully implemented for the diagnosis of the genetic conditions was the direct sequencing with the classic Sanger sequencing method. This technique uses specific, fluorescently labelled dideoxynucleotides together with non-modified deoxynucleotides for the synthesis of the new DNA strand on the PCR template. The incorporation of dideoxynucleotides stops the synthesis of the new strand and generates a set of DNA fragments of different length, labelled with a fluorochrome specific for each dideoxynucleotide [46]. These DNA fragments are separated by the capillary electrophoresis in automatic sequencers and dedicated software deducts the sequence of the analyzed fragment. The analysis of the nucleotide sequence, sometimes supported with a special software (e.g. Mutation Survyeor, SeqNext) for mutation detection, allows to detect known and new mutations related to the disease. The sequencing technique can be used to detect point mutations: nucleotide substitutions or small deletions and insertions [47]. Larger aberrations such as single exon deletion/duplication will not be detected with Sanger sequencing.

For this purpose another useful technique – Multiplex Ligation-dependent Probe Amplification (MLPA) can be applied. This semi-quantitative method uses oligonucleotide probes, specific for the examined and control regions. For each examined region (gene fragment, exon, chromosomal *loci*) two probes were designed that are located next to each other. Both probes have a part that is complementary to the analyzed region and another fragment that is used in subsequent PCR reaction. After the hybridization reaction part, the ligase enzyme is added to the samples and hybridized probes are ligated to each other. Subsequently, the ligated probes are amplified in the PCR reaction with fluorescently labelled primers and the reaction products are separated by the capillary electrophoresis. The analysis of the amount of the normalized reaction product for the analyzed region in comparison to the control sample gives the information about the deletion/duplication presence. The MLPA technique has higher resolution then the whole genome aCGH as it is dedicated to the specific regions of interest and therefore can detect even single exon deletion/duplication. However, it is again worth to mention that such aberrations are not common in rare, monogenic disorders [48, 49].

How to find a gene that mutation is responsible for the genetic disease?

When the first molecular tests for the genetic conditions were performed, they were based on the analysis of specific genetic markers that were inherited in the families together with the disease. For example, this method was used to perform the diagnosis in families with Fragile X syndrome or cystic fibrosis and assess which family members might be the carriers of the disease. The analyzed markers were located near the genes that were found to be mutated in patients and therefore were inherited together with the mutation [3].

This approach was also applied in further studies on the identification of new genes related to the pathogenesis of other genetic conditions with the method called linkage analysis. This method was especially useful in case of multi-generational families with many affected family members. The analysis of polymorphic makers in all affected and unaffected members of the family allowed to find specific markers that were inherited together with the disease and to identify chromosomal *locus* that may contain gene related to the clinical phenotype. After the locus identification, the sequencing of the candidate genes was performed and causative mutations could be found [50, 51].

The main problem with the linkage analysis is the necessity of the analysis of several affected family members. However, in many cases of rare diseases, the proband is the only affected person within the family

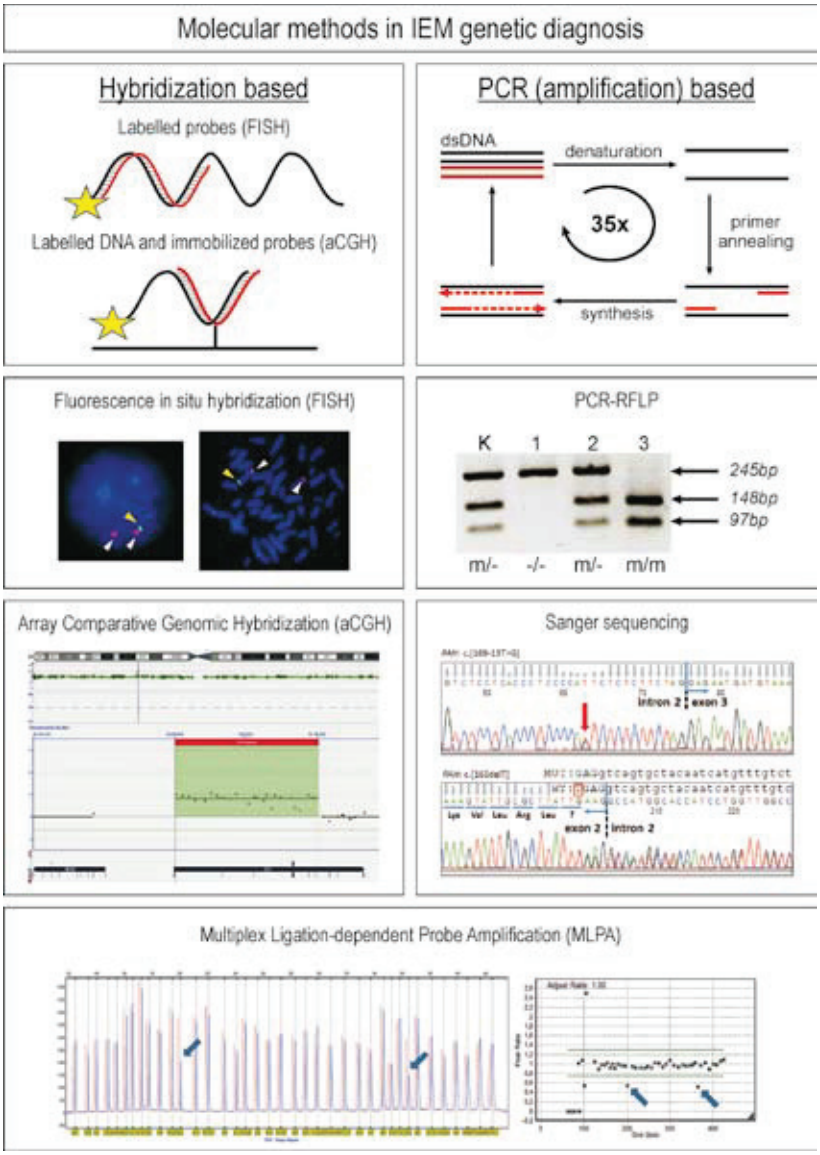

Fig. 4. Selected molecular methods used in genetic testing.

There are two groups of molecular methods that can be applied in genetic testing of inborn errors of metabolism: hybridization based and amplification (PCR) based. The first approach uses oligonucleotide probes (red color) that are complementary to the specific DNA fragment (black). Depending on the method, both probe or DNA can be labelled with fluorescent dyes. The second approach is based on the amplification of specific DNA fragment in a PCR reaction that consists of 30-35 cycles of subsequent denaturation, primer annealing and synthesis. In one 35-cycle PCR reaction, one DNA fragment is amplified to about 34 billions copies.

The Fluorescence in situ hybridization method (FISH) is used to detect deletion or duplication of specific regions with the application of *locus* specific fluorescently labelled oligonucleotide probes. The interphase and metaphase nuclei are shown from a patient with a deletion of a small region within the chromosome 7 (7delq33q35). The probes labelled with green dye are specific to the deleted region and labelled with red dye are specific to chromosome 7 centromeric region.

The array comparative genomic hybridization (aCGH) method is used to detect chromosomal imbalances using oligonucleotide probes immobilized on the slide. The example shows the duplication of the fragment of *OTC* gene in a patient with ornithine transcarbamylase (OTC) deficiency.

The PCR-RFLP technique is used to detect point mutations – the presence of specific sequence change leads to the loss or gain of a restriction site for a specific restriction enzyme. The example of the analysis of p.Arg408Trp mutation in *PAH* gene is presented. The amplification (PCR) product (length: 245bp) was digested with StyI restriction enzyme. The nucleotide change specific for this mutation leads to the creation of the restriction site for the StyI enzyme and the PCR product is digested into 148 and 97bp fragments. The analysis of the restriction pattern allows to identify double mutant alleles (only 148 and 97bp fragments), mutation carriers (245, 148 and 97bp fragments) and individuals without the mutation (245bp fragment only).

The Sanger sequencing is the method used for the detection of point mutations. The examples show point mutations detected in *PAH* gene in a patient with phenylketonuria.

The MLPA technique is used as an additional method for the detection of deletion/duplication in specific regions /genes. The technique allows to detect single exon deletion. The example shows the analysis of *PAH* gene - in the patient the deletion of exon 3 is present.

All of the above pictures were reproduced courtesy of the diagnosticians from the Department of Medical Genetics of the Institute of Mother and Child.

and classic linkage analysis for the gene identification cannot be applied. In such cases, the direct sequencing of candidate genes can be performed. Such genes are chosen based on the role that their protein product plays in the cellular, developmental or metabolic processes. For example, if biochemical tests show alteration of the level of a specific metabolite, we can look for the mutation in genes encoding the enzymes involved is its synthesis or further metabolism. Using this approach many genes involved in monogenic disorders were found, although much time and effort was needed [50, 51].

Today, thanks to the development of molecular techniques, the linkage analysis is rather historical method for pathogenic gene identification. A new technique called next generation sequencing (NGS) can replace direct sequencing of candidate genes one by one and was successfully implemented to the research on molecular basis of monogenic disorders [52, 53, 54]. The next generation sequencing, with alternative description massive parallel sequencing, allows the simultaneous analysis of many genes in one reaction. Depending on the necessities of the user, the next generation sequencing can be applied to analyze whole genome (so called whole genome sequencing, WGS) or only its selected regions e.g. only coding sequences (all exons, so called whole exome sequencing, WES) or only selected genes (targeted or panel sequencing) [55]. Whole genome sequencing generates much data on coding and non-coding sequences of human DNA and is a huge challenge in interpretation as there are still many questions regarding the function of particular genome regions [56]. Therefore, the research on genetic conditions is mainly focused on the exome sequencing as the data generated from coding sequences is easier to interpret. However, it is worth to mention that next generation sequencing, even focused on the exome, generates much data. For individual patient, about 450 000 variants are identified, from which about 1000 are not polymorphic and are passing the filters applied in the interpreting lab. From this variants, about 200 are individual for the patient (seen only in this person) and might be analyzed in relation to the disease with respect to the gene function and prediction of alterations caused by the variant presence (bioinformatic functional analysis of the variant). Therefore, the proper interpretation of the data needs the strict cooperation between the molecular biologist, bio-IT specialist and the clinician [52, 53, 57].

During the last years, new pathogenic variants in many genes have been identified in relation to disease development and several approaches were used to interpret the data (see fig. 5, [reviewed in 57]). However, the identification of a variant in a new gene not always means that this specific gene is causative for a disease, especially if the variant was found in a single patient. The role of such gene should be confirmed by functional studies in cell cultures or animal models and if possible the gene should be analyzed in the independent, follow-up cohort of the patients with specific clinical phenotype.

The exome sequencing is an excellent tool for research studies, but due to the difficulties with the data interpretation, it might be challenging to apply this method for diagnostic purposes [58, 59]. To overcome these problems, a targeted next generation sequencing seems to be a good solution. In this approach only the panels of selected genes related to a specific clinical phenotype are analyzed with NGS technique (e.g. cardiac disease panels, autism panels, epilepsy panels, inborn errors of metabolism panel etc.). However, the design and sequencing of such panels can be quite expensive if only single patients need to be analyzed. A good option is the sequencing of clinically relevant genes only (so called clinical exome sequencing) as variants that are identified can be directly interpreted in relation to phenotypes. Although such clinical panels give the information about many genes, the bioinformatic analysis can be restricted only to a specific set of them related to clinical observations for the patient [60].

Why it is so important to find a disease-related mutation? From gene to diagnosis and personalized treatment

The identification of disease causing mutation is not only the research goal, but has some other consequences for the patient and its family. First, the mode of inheritance of the disease can be assessed and therefore the risk of having another affected child for the parents can be calculated. This is important for genetic counseling not only for the parents, but for other family members as they might be the carriers of rare, recessive disorders and have a higher risk of having an affected child (see tab. I). Second, the identification of the mutation is important from a psychosocial point of view – the patient and more importantly his parents know the cause of the disease. This a little bit facilitates their functioning in the medical and social care system as it reduces any doubts about the patient disease.

Third, the identification of specific mutation might be necessary to apply appropriate treatment to the patient (fig. 6). In case of inborn errors of metabolism, the presence of mutation usually leads to the improper catalytic activity of particular proteins (enzymes) that can result in enzyme substrate accumulation, reaction product insufficiency or synthesis of other possibly toxic products by the alternative catalytic pathway that might cause disturbances of organism development. Many different methods can be used to overcome these negative effects and one of the oldest treatments include diet modification [61]. The application of the special diet might overcome the effect of the presence of a metabolic defect. A good example is a phenylketonuria (PKU), where the negative effect of the *PAH* mutation presence might be overcame with a low phenylalanine diet and this treatment is routinely used in PKU patients. Also special diet supplements might exert positive effect on disease course. For example, the supplementation of the diet of the patients suffering from glutaric aciduria type I with the carnitine leads to faster glutaric acid clearance that probably contributes to the lower level of

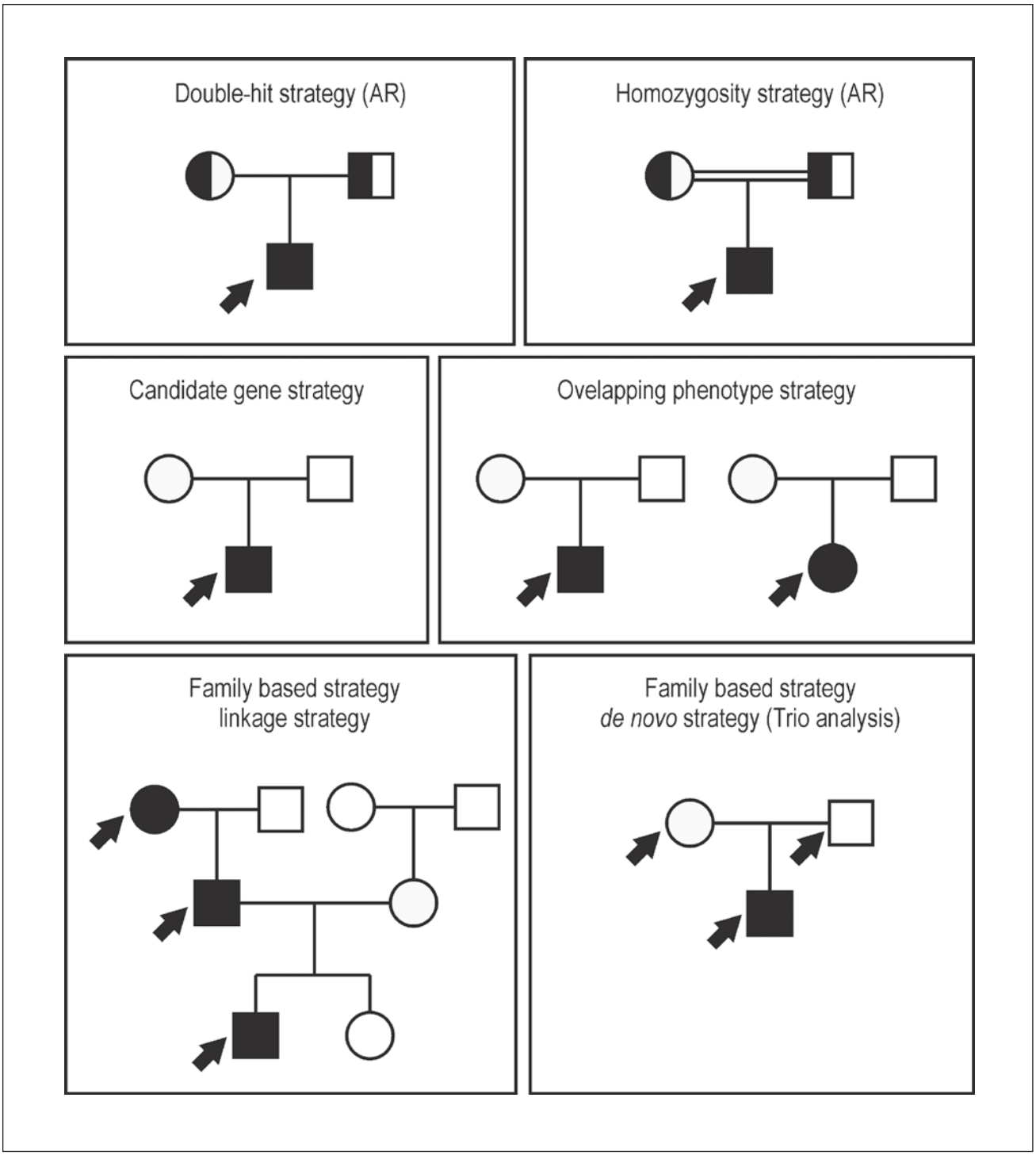

Fig. 5. Strategies for new gene identification with next generation sequencing.
In case of autosomal recessive diseases, genes might be selected based on the presence of homozygous (e.g. if the parents were consanguineous) or compound heterozygous mutations in a single patient (homozygosity strategy or double-hit strategy). In case of autosomal dominant disorders and several affected members in the family, the common heterozygous mutation can be looked for (family based/linkage strategy). In case of single affected family members, the group of patients with similar phenotype can be analyzed and a common mutated gene is selected as a candidate for further studies (overlapping phenotype strategy). If there is only a single patient – the genes can be selected according to their function and relation to the clinical observations (candidate gene strategy). Another commonly used option in such situation can be the sequencing of affected proband and unaffected parents (family based, de novo strategy / trio analysis) and searching for the de novo mutation that might be causative for the disease [based on 57]. Arrows point at individuals that are sequenced in each approach. AR – autosomal recessive inheritance.

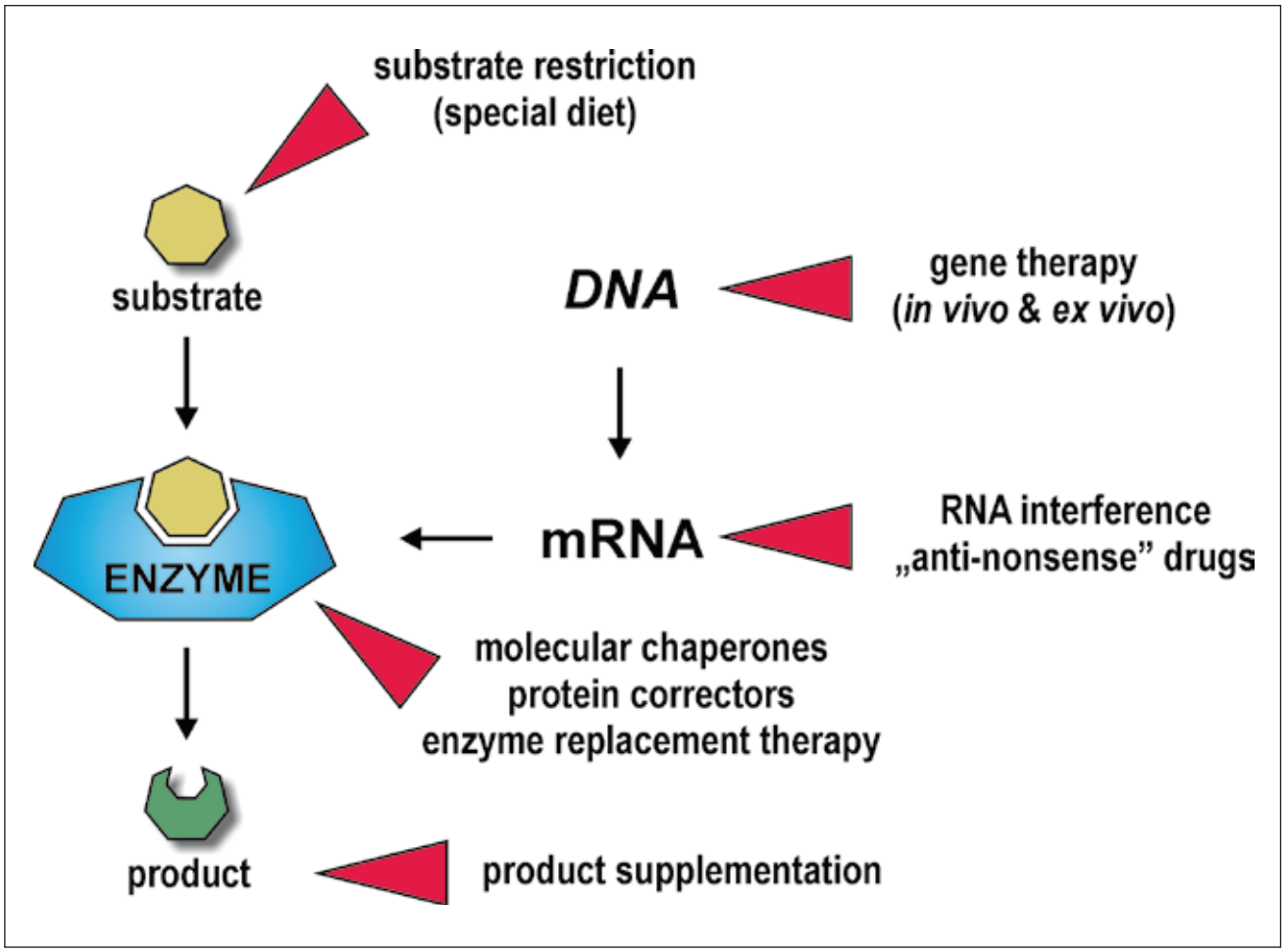

Fig. 6. Therapeutic approaches used in inborn errors of metabolism.
In case of IEM, different therapeutic approaches can be used to treat the patients depending on the type of affected metabolic pathway. First, the patient diet can be modified by the substrate dietary restriction or product supplementation. Second, the altered enzyme activity can be regulated by the application of molecular chaperons, protein correctors or by enzyme replacement therapy. Third, the ex vivo or in vivo gene therapy or other approaches influencing the gene transcript can be applied to overcome the effect of the mutation presence in particular gene [based on 67].

neurological complications observed in these patients [62].

In case of other disorders, when the diet cannot be applied as a possible treatment, the correct genetic diagnosis might give the opportunity to administer other specific drugs that will directly target the processes influenced by the mutation presence [detail reviewed in 63].

One of such treatments is the enzyme replacement therapy, for example used to treat lysosomal storage diseases like: Gaucher disease, Fabry disease, Pompe disease and mucopolysaccharidoses [64, 65]. In this kind of therapy, patients with absent or deficient enzymes receive the intravenous injections containing the active enzyme. The disadvantage of such therapy is the fact that the enzyme is unable to penetrate blood-brain barrier or bone or cartilage tissues. Therefore, the studies on the application of enzymes modified with a small peptides that in tissue specific manner will target the therapeutic molecules, are held. For example, in hypophosphatasia treatment, the alkaline phosphatase enzyme can be linked with deca-aspartate motif to target bone tissue with higher affinity [66]. In case of lysosomal diseases, the specific enzyme can be modified with an apolipoprotein LDLR binding domain to target the central nervous system [67].

Another option in the therapy of inborn errors of metabolism is the use of pharmacological chaperone treatment [65, 68]. This strategy has been developed for diseases caused by mutations that result in protein loss of function due to the defect in protein folding. Even a substitution of a single amino acid can affect the three dimensional structure of the protein or the formation of its complex (multimer). The improper protein folding may also lead to the formation of protein aggregates and premature protein degradation. Pharmacological chaperones are small molecules, which lower protein misfolding rate and stabilize its structure, hence enabling to restore the residual protein activity. One of the first molecular chaperones that have been proved to be beneficious in the clinic is tetrahydrobiopterin (BH4), the natural cofactor of

PAH enzyme. Its derivate, sapropterin dihydrochloride (trade name: Kuvan), has been registered by FDA[2] and EMA[3] as an orphan drug for PKU. Although not all PKU patients are BH4-responsive and can benefit from this treatment, still the therapy has a positive effect in around 60% of them [69].

Protein misfolding is also involved in the pathology of several other IEMs (eg. Fabry disease, Gaucher disease, GM1 and GM2 gangliosidosis, Pompe disease, MPS IIIB, medium-chain acyl-CoA dehydrogenase deficiency (MCADD)) for which many substances are under investigation as a potential pharmacological chaperones [69, 70]. Importantly, mutations can have entirely different effect on the same protein, hence effectiveness of pharmacological chaperones therapy depends mostly on a molecular defect and its location within protein [70]. Thus, identification of a molecular defect in affected individual is crucial in terms of the patient qualification to the targeted therapy. Nevertheless, in some cases such knowledge is not enough and detailed *in vitro* studies need to be performed [71].

Similar strategy has also been developed for the cystic fibrosis, although in this case so called correctors were used. The treatment was first developed for the most common mutation in *CFTR* gene – p.Phe508del that affect the protein folding. The corrector molecule not only acts on CFTR folding, but also functions as a proteostasis regulator, and influence the mutated protein processing by the modulation of the activity of cellular quality-control mechanisms. The second class of CFTR-protein modulators are called potentiators and act on the cell membrane to restore CFTR canal function. In 2012, potentiator called ivacaftor (Kalydeco, Vertex Pharmaceuticals) was approved by FDA to treat CF patients with the mutation p.Gly551Asp [72]. Currently, this drug can be used for the treatment of CF in patients who have one of the following mutations: p.Gly551Asp, p.Gly1244Glu, p.Gly1349Asp, p.Gly178Arg, p.Gly551Ser, p.Ser1251Asn, p.Ser1255Pro, p.Ser549Asn, p.Ser549Arg or p.Arg117His (http://www.kalydeco.com). Although monotherapy with ivacaftor is not effective in CF patients, who are homozygous with the p.Phe508del mutation, it can be beneficious while used in combination with one of the CFTR correctors – lumacaftor. Only recently (July 2015), an orphan drug - Orkambi (composed of lumacaftor and ivacaftor) dedicated especially to the treatment of p.Phe508del homozygous CF patients, gained the FDA approval and can be used for patients therapy (http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm453565.htm).

Special experimental therapies e.g. gene and/or cell therapy might be a promising method in improving affected people existence. Thanks to the advances in the development of molecular biology and cell culture techniques it is now possible to use patient or donor cells for the therapy as well as to prepare so called "therapeutic genes" that can be delivered to the diseased tissue. Treatment options that were or are applied in clinical trials for monogenic disorders can be divided to: cell based therapy (somatic or stem cell therapy) and gene therapy [73].

In the first case, patient or donor cells are cultured *ex vivo* in special conditions with cytokines, antibodies etc. and then delivered to the patient. Cells that can be used for such treatment include human umbilical cord blood derived stem cells (e.g. in hereditary ataxia), differentiated embryonic stem cells (d-ESC, e.g. ESC differentiated to retinal pigmented epithelial cells in Stargardt's disease or age related macular degeneration), tissue stem cells (e.g. cultured limbal stem cells in limbal stem cells deficiency, myoblasts in muscular dystrophy), differentiated cells (e.g. pancreatic islet cells in diabetes mellitus type I, hepatocytes in the liver diseases like Crigler-Najjar syndrome or urea cycle disorders, organic acdemias) or hematopoietic stem cells (e.g. muscular dystrophy, lysosomal storage disorders, X-linked adrenoleukodystrophy) [64, 73, 74].

In addition, cells used for cell therapy can be genetically modified and can be transformed with a "therapeutic gene". For example, cells derived from the bone marrow (e.g. CD34+ cells) of the patient are selected and cultured *in vitro* under special conditions. A therapeutic gene is administered to these cells with a special therapeutic vector (e.g. adeno-associated virus, AAV) and cells are given back to the patient as an autologous transplant. Such form of therapy was first successfully used to treat X-linked severe combined immunodeficiency (SCID) – hematopoietic CD34+ cells of the patients were transformed with retrovirus vector carrying non-mutated *IL2RG* gene and used for the transplant. Although patients responded positively to this treatment, after some time adverse effects were observed and several of the patients developed leukemia [75, 76]. Since then a big effort was put to develop vectors that can be safely used for patients therapy. Such "safe" vector with therapeutic genes were used in several clinical trials for immune system deficiencies (X-linked SCID, adenosine deaminase deficiency, Wiskott-Aldrich syndrome, chronic granulomatous disease), metabolic disorders (X-linked adrenoleukodystrophy) or blood disorders (β-thalassemia). Another cells that can be used for *ex vivo* gene therapy, especially in case of genetic skin disorders with disrupted genes coding collagen, keratin or laminin, are skin cells [73]. Corrected with a therapeutic gene in a retroviral vector, modified keratinocytes were successfully used for therapy of Netherton syndrome and epidermolysis bullosa patients [77, 78]. Also the hepatocytes were used as a possible carrier of the therapeutic gene – the treatment was investigated in a patient with familial hypercholesterolemia. The patient hepatocytes were transformed *ex vivo* with the correct *LDLR* gene in a retroviral vector and re-introduced successfully to the patient whose cholesterol levels significantly decreased although the effect of the treatment was only transient [79].

Another promising approach in patients treatment is *in vivo* (or *in situ*) gene therapy – the vector with a therapeutic gene is directly administered to the damaged tissue of the patient. In this kind of therapy mainly adeno-associated viruses are used as their specific serotypes show specific tropism towards different types of cells.

[2]FDA – Food and Drug Administration, USA.
[3]EMA – European Medicines Agency.

Box 3. Helpful links.

Organisation/DATABASE	Website
Online Mendelian Inheritance in Man (OMIM)	www.omim.org
ORPHANET	www.orpha.net
EURORDIS	www.eurordis.org
Metabolic Information Centre: MIC	www.metagene.de
RAMEDIS, the Rare Metabolic Diseases Database	agbi.techfak.uni-bielefeld.de/ramedis
Human Gene Mutation Database	www.hgmd.cf.ac.uk
ClinicalTrials.gov	clinicaltrials.gov
The EU Clinical Trials Register	www.clinicaltrialsregister.eu
FDA Office of Orphan Products Development (OOPD)	http://www.fda.gov/forindustry/developingproductsforrarediseasesconditions/default.htm
Society For Inherited Metabolic Disorders	www.simd.org
Society For The Study Of Inborn Errors Of Metabolism	www.ssiem.org
Polish Society of Human Genetics	www.ptgc-med.pl/
Polish Society Of Congenital Inborn Errors Of Metabolism	pediatriametaboliczna.pl/polskie-towarzystwo-wrodzonych-wad-metabolizmu/
Polish National Consultant in Pediatric Metabolic Medicine	pediatriametaboliczna.pl/

The first medicine approved for the treatment based on *in vivo* gene therapy is Glybera® (http://www.uniqure.com) dedicated to the therapy of patients with familial lipoprotein lipase deficiency caused by mutation in *LPL* gene. The treatment of many other conditions is under development or in clinical trials and include metabolic disorders (adrenoleukodystrophy, Canavan disease, mucopolysaccharidoses), childhood blindness (e.g. Leber congenital amaurosis, achromatopsia, X-linked retinitis pigmentosa) or haemophilia [80]. One of them is also aromatic L-amino acid decarboxylase (AADC) deficiency – an autosomal recessive disease caused by mutations in *AADC* gene, encoding DOPA decarboxylase. Patients with AADC deficiency, due to the impaired synthesis of catecholamines (dopamine, norepinephrine, epinephrine) and serotonin, manifest, usually since infancy, severe motor, behavioral and autonomic dysfunctions: developmental retardation, hypotony, hypokinesia, oculogyric crises and extraneurological signs (hypersalivation, hyperhidrosis, nasal congestion, temperature instability, sleep disturbances and hypoglycaemia) [81, 82].

This devastating neurodegenerative disease has been, until now, hardly treated using multiple pharmacotherapies. Though many different medicinal products have been tried in order to improve patients' clinical condition, therapeutic efficacy in AADC deficiency is still very limited. According to JAKE database (containing records of the patients with diagnosis of AADC deficiency, http://www.biopku.org/home/jake.asp) – there is no response or unsatisfactory response to pharmacotherapy in over 80% patients. Therefore, a gene therapy has been attempted recently. An adeno-associated virus viral vector, expressing the human AADC protein, was injected to the putamen of selected children with AADC deficiency. The therapy was well tolerated and clinical improvement was reported in all treated patients [83]. These promising initial trials will be continued as an open-label dose escalation safety and efficacy study of adeno-associated virus encoding human AADC (AAV2-hAADC), which will be administered by MR-guided infusion into the midbrain in pediatric patients with AADC deficiency [personal information from prof. Krzysztof Bankiewicz, UCSF].

The gene therapy can also be based on non-viral vectors. For example, a special plasmid expressing *CFTR* gene was used in the therapy of cystic fibrosis patients with respiratory problems. The treatment includes repeating inhalations of the plasmid complexed with special nanoparticles that improve the delivery of the "therapeutic gene" to the affected tissues [84].

SUMMARY

Inborn errors of metabolism are rare disorders, although taken together they affect a significant number of patients worldwide. The progress in molecular techniques enables the application of new methods to the diagnosis of IEM, which also lead to identification of other genes associated with a particular diseases. Knowledge about the exact disease-causing molecular defect is essential for the development of new drugs that specifically target the impaired genes, proteins or metabolic pathways. It has already been proved that the proper and early diagnosis is crucial - as the appropriate and personalized

treatment can be applied, often preventing serious clinical complications. It seems that in the next years technical progress combined with increasing knowledge about molecular pathology of rare disorders, will revolutionize healthcare systems. Novel diagnostic techniques involved in newborn screening programs will enable rapid diagnosis of several disorders and immediate introduction of proper treatment. Importantly, this will impact not only on individual families, but also will have huge socioeconomic significance, as taken together rare genetic disorders are frequent and affect millions of people worldwide.

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Conflicts of interest/Konfliktinteresu
The Authors declare no conflict of interest.
Autorzy pracy nie zgłaszają konfliktu interesów.

Received/Nadesłano: 20.10.2015 r.
Accepted/Zaakceptowano: 04.11.2015 r.

Published online/Dostępne online

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