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Molecular-genetic aspects of familial hypercholesterolemia

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Abstract. Familial hypercholesterolemia (FH) is the world's most abundant and the most common heritable disorder of lipid metabolism. The prevalence of the disease in general population is 1:500. Therefore the approximate number of FH patients all over the world is 14 million. From the genetic point of view the disease originates as a result of mutations in genes affecting the processing of LDL particles from circulation, resulting in an increase in LDL cholesterol and hence total cholesterol. These are mutations in genes encoding LDL receptor, apolipoprotein B, proprotein convertase subtilisin/kexin 9 and LDL receptor adaptor protein 1. Cholesterol depositing in tissues and blood vessels of individuals creates tendon xanthoma, xanthelesma and arcus lipoides cornae. Due to the increased deposition of cholesterol in blood vessels, atherosclerosis process is accelerated, what leads to a significantly higher risk of premature cardiovascular diseases. Therefore, early clinical diagnosis confirmed by the DNA analysis, and effective treatment are crucial to reduce the mortality and high risk of premature atherosclerotic complications.

Introduction

Familial hypercholesterolemia (FH) is one of the most common autosomal dominantly inherited disorders of lipid metabolism in humans. There are two clinical manifestations: milder heterozygous form and more severe homozygous form. The heterozygous form has been generally thought to affect 1 out of 500 individuals, but recent studies show that the prevalence appears to be higher 1:200 – 1:500. The prevalence of the homozygous form is rare only 1 individual in a million. FH is caused by genetic defects resulting in elevated LDL cholesterol (LDL-C) levels from birth, putting patients at high risk for premature coronary disease (CAD). The disease was first clinically described in 1938 by Norwegian scientist Carl Muller as elevated levels of serum cholesterol together with tendon xanthomas and coronary artery lesions.

FH is primary caused by almost 2 000 defects in the gene for LDL receptor (*LDLR*). Mutations in apolipoprotein B (*APOB*), proprotein convertase subtilisin/kexin type 9 (*PCSK9*) and LDL receptor adaptor protein 1 (*LDLRAP1*) genes can produce similar phenotypic consequences to classic FH. But still in 10% to 40% of FH cases no defect in these genes has been identified. This may be the cause of yet unknown genetic defect or polygenic, epigenetic or other hereditary defects. The genetics of currently known defects causing FH is discussed in further chapters of the article.

Prevalence

Worldwide population of FH patients is estimated for 14 to 51 million persons. Homozygous (or compound heterozygous) form occurs with a prevalence

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of 1:1,000,000 and heterozygous form of FH occurs in population with frequency of about 1:500. However, this prevalence was calculated based on clinical background from FH patients, who already overcame myocardial infarction (Goldstein et al. 1973), but there were no studies that calculated the prevalence directly from unselected general population. An exception is a study from Danish authors from 2012, who calculated the FH prevalence in general population of 69016 persons for 1:137, and later recalculated the prevalence for 1~200 (Benn et al. 2012). Based on the prevalence of 1:200 to 1:500, the estimated number of patients from the world population is somewhere between 1.4 – 5.4 million individuals in Europe. From the Slovak population of 5.4 million inhabitants, the approximate number of FH patients would be around 11 to 27 thousand persons.

The data from small isolated populations point to much higher prevalence of FH (more than 1:500) due to founder effect. These populations include French Canadians with prevalence of 1 in 270 individuals (Moorjani et al. 1989) and two other populations from one region in South Africa with extremely high prevalence of FH: Ashkenazi Jews 1:67 and Afrikaners from Transvaal Province 1:100 (Seftel et al. 1980; 1989).

Recent study on homozygous form of FH from Netherland also shows that the prevalence might be much higher 1:300,000 than previously estimated (Sjouke et al. 2015).

According to Consensus Statement of European Atherosclerosis Society based on the number of identified FH patients, familial hypercholesterolemia is still underdiagnosed (Nordestgaard et al. 2013). In most European countries there is only less than 1% patients already diagnosed. As an exception from the above mentioned is the higher detection rate in Netherlands (71%), Norway (43%), Czech Republic (30%), Iceland (19%), Switzerland (13%), Great Britain (12%) and Spain (6%) (http://www.athero.cz/cze/projekt-medped-prolaiky/projekt-medped.php; Nordestgaard et al. 2013). In Slovakia, 4% of patients are clinically diagnosed (Nordestgaard et al. 2013), what is higher than in most European countries. Nevertheless, the aforementioned detection rate is still not sufficient for establishment of an effective care of all FH patients in Slovakia.

Genetic causes of familial hypercholesterolemia

Presently there are four known genes involved in LDL cholesterol metabolism (*LDLR, APOB, PCSK9* and *LDLRAP1*) which cause the clinical phenotype of FH (Futema et al. 2013). However, there are still about 10% to 40% of FH patients (depending on diagnostic criteria), without a detected mutation in one of these genes (Civeira et al. 2008; Motazacker et al. 2012; Alves et al. 2014). These patients may inherit other yet unknown monogenic, polygenic or epigenetic defects. An example of monogenic cause of FH might be a variant in *STAP1* gene (signal transducing adaptor family member 1) which was identified in a Netherland study. This variant was identified in FH family and other unrelated FH patients without any defect in *APOB*, *LDLR* or *PCSK9* genes. The phenotype of the patients was similar to individuals with an *APOB* mutation. The authors stated that *STAP1* mutation was a novel locus for autosomal dominant FH. However, the exact function of STAP1 is unknown (Fouchier et al. 2014). On the other hand, another study showed that most of the mutation negative patients have inherited more than an average number of frequent variants of small effect that raises plasma lipid levels. They suggest a polygenic character of the disease, what is the most probable explanation for elevated cholesterol levels in FH patients without a mutation in currently known FH genes (Talmud et al. 2013).

Familial hypercholesterolemia due to mutations in *LDLR* gene

Classic familial hypercholesterolemia which is caused by mutations in *LDLR* gene is the most common form of monogenic hypercholesterolemia. The disease is inherited in autosomal dominant form, while homozygotes have more severe clinical phenotype compared to heterozygotes (Civeira 2004). Penetrance of the disease is almost 100%, so in the family of affected parent there is 50% likelihood of inheriting FH in children regardless of gender (Civeira 2004).

The spectrum of mutations found in different population is very heterogeneous. To date, 1946 different mutations were identified worldwide according to the Human Genome Mutation Database accessed on April 24, 2015 (Stenson et al. 2009). The opposite is true for founder populations, where small number of *LDLR* mutations account for the vast majority of FH cases. This is very clearly seen in the screening of FH individuals in the North Karelian region of Finland with over 80% of FH patients heterozygous for the same *LDLR* mutation p.Pro309Lysfs*49 (Koivisto et al. 1992; Vuorio et al. 2001; Vuorio et al. 1997). Also the spectrum of mutations differs for founder and nonfounder populations.

The distinction between a small number of common alleles and a large number of rare alleles is important for the strategy of DNA testing and the diagnosis of FH (Austin et al. 2004).

LDLR gene and its structure

Goldstein and Brown (1973) have discovered and described the LDL receptor as a molecule responsible for autosomal dominant form of hypercholesterolemia (Goldstein and Brown 1973). The causal gene was identified in 1985 by *in situ* hybridization (Lindgren et al. 1985). LDL receptor gene (*LDLR*) is located on chromosome 19 (19p13), spans 45kb and contains 18 exons and 17 introns (Sudhof et al. 1985). This region is transcribed into 5.3 kb mRNA, which is translated into 860 amino acids of LDL receptor (Fig. 1) (Fahed and Nemer 2011).

LDLR gene consists of several functional domains, i.e. ligand binding domain (encoded by exons 2-6), which mediates the interaction between the receptor and lipoproteins containing apolipoprotein B and apolipoprotein E (Esser et al. 1988), the epidermal growth factor (EGF) precursor homology domain (encoded by exons 7-14), the O-linked sugar domain (encoded by exon 15) (Kozarsky et al. 1988), transmembrane domain (Schneider 2002), and finally cytoplasmic tail of LDL receptor is a short 55 amino acid part (encoded by rest of exon 17 and exon 18) which take place in internalization of receptors into clathrin-coated pits (Schneider 2002).

LDL receptor protein

Mature LDL receptor is highly conserved membrane glycoprotein consisting of 839 amino acids divided into five domains. Structure from N-terminal end is ligand-

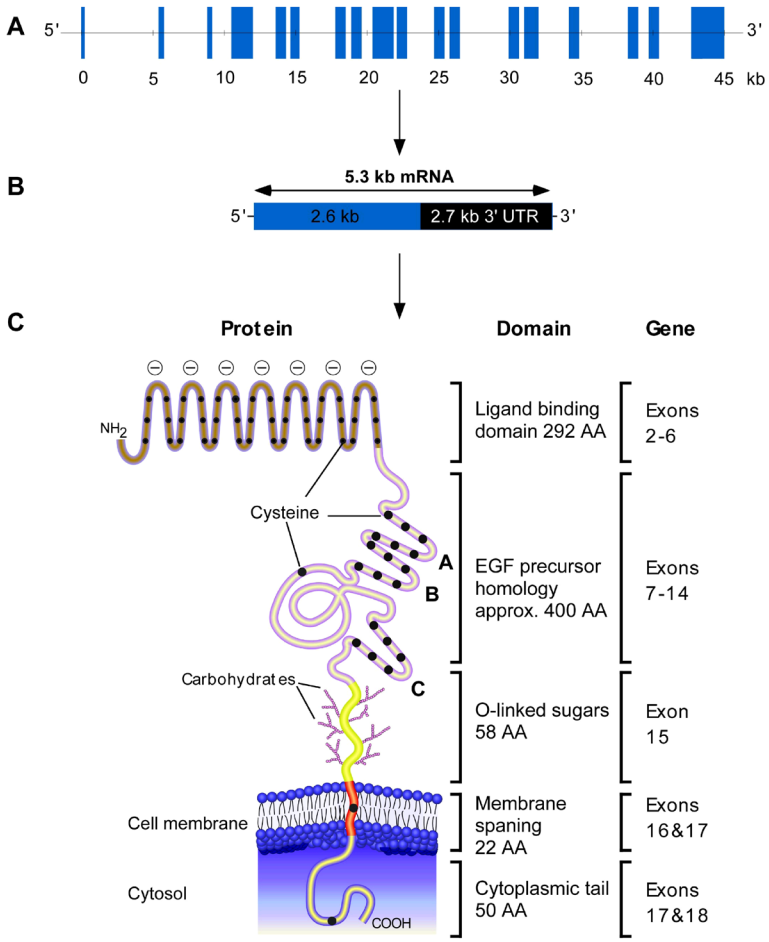

Fig. 1. Structure of LDL receptor. A) schematic representation of the human *LDLR* gene, exons are marked blue, size in kb is listed below the figure; B) mRNA a 3'UTR untranslated region; C) LDL receptor domains. Adapted from Al-Allaf et al. (2010).

binding site, domain with high homology to the epidermal growth factor, O-carbohydrate domain, transmembrane domain and cytoplasmic tail (Russell et al. 1984). From the original protein of 860 amino acids, first 21 amino acids (AA) consists of signal sequence (encoded by exon 1), which directs the ribosome synthesizing LDL receptor to the ER membrane (Sudhof et al. 1985).

Function of LDL receptor

The LDL receptor (low density lipoprotein receptor) is a glycoprotein which binds LDL particles, the primary carriers of plasma cholesterol. Apolipoprotein B-100 is a surface protein of LDL particles which is recognized by ligand-binding site of LDL receptor. After binding of LDL particle to the LDL receptor, clathrin-coated vesicles are formed, which are then transported into endosome through the interaction with LDL receptor adaptor protein 1 (LDLRAP1). After dissociation of the ligand-receptor complex, the LDL receptor is recycled back to the cell surface and free cholesterol is used within the cell (Fig. 2). This process handles up to 70% of circulating cholesterol in plasma (Costet et al. 2008; Fahed and Nemer 2011).

Regulation of LDL receptor production

When the cell lacks exogenous cholesterol access, it leads to endogenous production of cholesterol in the cell nucleus. Nuclear regulation of LDL receptor production includes two metabolic pathways shown in Fig. 2. First pathway, the bond of steroid response element binding protein (SREBP) to steroid response element on DNA stimulates the transcription of LDLR as a result of low intracellular cholesterol levels (Costet et al. 2008). This metabolism is activated during the therapy with HMG-Co A (β hydroxyl-β-metylglutaryl-coenzyme A) reductase inhibitors (statins). Second role in LDLR regulation plays sterol mediated nuclear receptor LXR, which induces transcription of Inducible Degrader of the LDLR (IDOL). IDOL triggers ubiquitination of LDL receptors, which are marked for degradation (Zelcer et al. 2009). This metabolism ensures the uptake of LDL cholesterol from circulation. Any defect in the metabolic pathway leading to a false uptake or high plasma concentrations of LDL cholesterol leads to clinical manifestation of familial hypercholesterolemia (Fahed and Nemer 2011).

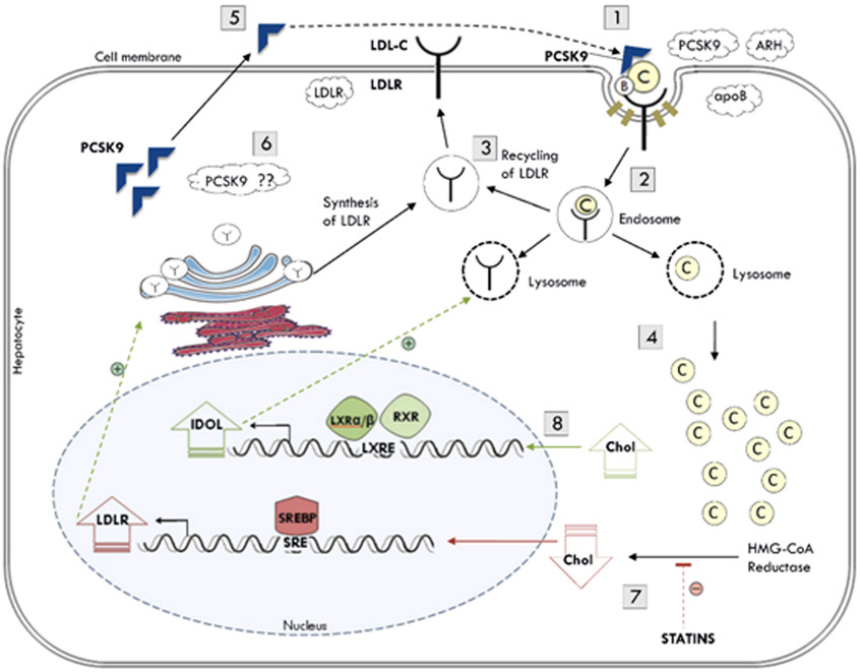

Fig. 2. LDL cholesterol metabolism adapted from Fahed and Nemer (2011): LDL receptor on the surface of hepatocytes binds ApoB-100 of LDL particles, what produces complex of these molecules (1). Formation of clathrin-coated pits is endocytosed into the cell by interactions which include LDLRAP1 (*LDLRAP1* – gene encoding LDLRAP1) (2). Inside of hepatocyte the complex is dissociated, LDL receptor is recycled back to plasma membrane (3) and free cholesterol is processed inside the cell (4). PCSK9 functions as post-transcriptional inhibitor of LDL receptors. PCSK9 is secreted inside the cell and inhibits LDLR by interactions with cell surface (5). The presence of intracellular process by which PCSK9 inhibits LDL receptors is still unknown (6). Clouds in the figure show genes, which are associated with high levels of LDL cholesterol.

Mutations in *LDLR*

Mutations in *LDLR* gene account for approximately 75% of all FH patients with an identified mutation (Marduel et al. 2010). To date, 1946 allelic mutations in *LDLR* gene were identified. These mutations comprise mostly of missense, nonsense or splicing substitutions (60.1%), small deletions and insertions (22.7%) and large rearrangements (17.2%) (Stenson et al. 2009). The greatest number of changes is in exon 4, which is the largest exon of *LDLR* (Leigh et al. 2008).

Spectrum of mutations in European countries is very variable. From Greece where 6 mutations are causal in 60% of all FH patients to Norway with the most heterogeneous group of 148 mutations associated with FH phenotype (Traeger-Synodinos et al. 1998; Dedoussis et al. 2004; Futema et al. 2013). In several European countries such as Denmark, Czech Republic, Poland, Germany, three most common, for each country different, mutations are responsible for the majority of FH cases (Table 1) (Nauck et al. 2001; Chmara et al. 2010; Benn et al. 2012; Tichy et al. 2012).

In case of Czech Republic the p.Gly592Glu; p.Asp266Glu; and p.Cys209Tyr are responsible for up to 39.8% of FH in cases. From these mutations the most common one is p.Gly592Glu, responsible for more than 19% of Czech FH patients (Tichy et al. 2012). This mutation is also the most common in Poland, where it accounts for more than 22% of FH cases (Chmara et al. 2010). In Slovakia, this mutation also appears to be the most common accounting for 5.8% of FH patients (unpublished data).

Effect of mutations on protein structure

Mutations in *LDLR* gene are divided into five classes according to the effect on the protein formation (Hobbs et al. 1990):

1st class (null receptor) is caused by point mutations causing premature termination in protein coding region, large deletions, nonsense and frame-shift mutations or mutations in promoter region that block the transcription, resulting in lack of LDLR synthesis (Hobbs et al. 1987; Goldstein et al. 2001).

2nd class (slow or absent processing of the precursors). Mutations are primarily located in the ligand-binding domain and EGF homology domain. These alleles represent more than half of all mutations in the LDL receptor. From these mutations arise receptor precursors that are not capable of passing through the membrane, or through endoplasmic reticulum, Golgi apparatus to the cell membrane. While some mutations slow down the processing of LDL receptors, most of them are able to transport the LDLR into the endoplasmic reticulum, but fail to transport to the cell surface (Austin et al. 2004).

3rd class: defective ligand-binding. These receptors are able to locate in hepatocyte membrane, but lack the ability of effective ApoB binding due to structural changes near or in the ligand-binding domain. Mutations are primarily located in ligand-binding domain or EGF precursor homology domain (Hobbs et al. 1990; Goldstein et al. 2001; Austin et al. 2004).

4th class: internalization defective. Missing internalization into clathrin-coated pits. The inability of these receptors to localize into clathrin-coated vesicles results from mutation in carboxyl-terminal part of receptor. Most of these mutant truncated proteins are secreted to the cell surface (Schneider 2002). Mutations in form of large deletions occur in the cytoplasmic or transmembrane domain, inhibiting the aggregation of LDL receptors on cell surface, so the complex of receptor and LDL particle cannot be internalized (Austin et al. 2004).

5th class: recycling-defective. The classification of these mutations in a single class is based on the observation that deletion of the EGF precursor domain permits the truncated protein to bind and internalize the ligand. However, in the acid environment of endosomes the release of LDL particle is blocked and the mutant receptor is degraded and the recyclation on hepatocyte surface is missing (Schneider 2002). This mutation class includes mutations in the EGF precursor homology domain (Hobbs et al. 1990; Goldstein et al. 2001; Austin et al. 2004).

In 2010, a study of 436 FH patients has shown that patients with *LDLR* mutations classified as null alleles showed a more severe clinical phenotype and worse carotid atherosclerosis than those with receptor defective mutations, independently of age, gender, lipid and nonlipid risk factors (Junyent et al. 2010).

Functional characterization of new variants in *LDLR*

In almost every publication about mutation spectrum in a specific population or ethnic group with FH, a few new variants are found which have not been previously described. For example in Czech population from 2239 patients were identified 55 new variants that have not been described in other FH population (Tichy et al. 2012). In the British study of 289 patients were identified 90 individuals with 54 different mutations and another 12 previously unpublished variants (Futema et al. 2013).

In case of identifying new variant in coding sequence, the causality of the variant cannot be confirmed until functional studies are performed (Lombardi et al. 1997; Naoumova et al. 2004). If the functional studies are not available, the pathogenicity of the mutation might be predicted by other approaches such as cosegregation of the genotype and phenotype in the family pedigree, exclusion of the presence of the variant in healthy population, verification of the mutation in literature or in the mutation databases and *in silico* analyses of the variant. The *in silico* analyses are computer programs, which on the statistic basis predict the pathogenicity of the variant. But few studies have shown that *in silico* analyses do not provide 100% sensitivity and specificity of the data (Silva et al. 2012). Therefore for the final determination of causality, functional characterization needs to be performed (Romano et al. 2011).

For functional characterization of *LDLR* variants in exons, two slightly different approaches are published (Etxebarria et al. 2012; Silva et al. 2012). In the past, classification of variant pathogenicity was most extensively used in cultured fibroblasts of homozygous FH patients (Hobbs et al. 1992). Whereas homozygous FH is very rare, to mimic this situation *in vitro*, transfection of cell lines with plasmid carrying the homozygous variant was used for identification of exact effect of the variant on LDL receptor activity (Ranheim et al. 2006; Silva et al. 2012).

Second approach uses the isolation of T-lymphocytes directly from blood sample of the patient (Romano et al. 2010; Etxebarria et al. 2012). By this way residual affinity of LDL receptors is measured directly in the patient with mutant and normal LDL receptors. Consequently, including both methods, cells are cultured and labeled with fluorescent dye. Subsequently, by flow cytometry and confocal microscopy the expression of LDLR, the binding of LDL particles to the receptor and LDL cholesterol uptake is measured. For the identification of intron variants, mRNA is isolated from lymphocytes of the patient, reversely transcribed into cDNA and sequenced. This method will reveal the missing exons or introns in the translated LDLR protein (Romano et al. 2010; Etxebarria et al. 2012).

Familial hypercholesterolemia due to *APOB* mutations

Familial hypercholesterolemia due to the mutations in *APOB* gene has similar phenotype as mutations in *LDLR* gene, however the clinical picture could be less severe (Myant 1993; Vohnout et al. 2003). In some cases it is very difficult to distinguish between heterozygous *APOB* patient and heterozygous *LDLR* patient on the clinical basis. Frequency of FH due to *APOB* mutation in general population is 0.08-0.041%. Patients with defect in apolipoprotein B represent approximately 2 to 5% of all FH cases (Fahed and Nemer 2011). However the proportion of FH cases differs among European countries ranging from 0 to 11%. In Slovakia 9.7% of FH cases (Gasparovic et al. 2007) have an *APOB* mutation, what is similar to data from Czech Republic and Poland (Bednarska-Makaruk et al. 2001; Freiberger et al. 2004)

Gene for apolipoprotein B

Gene for the synthesis of *APOB* is located on short arm of 2nd chromosome and spans 43kb of this chromosome (2p24-23). This gene is transcribed particularly in hepatocytes and enterocytes. Transcript of 14kb mRNA has a half-life of 16 hours and consists of 29 exons and 28 introns. The largest exon 26 (7572bp) accounts for more than half the size of ApoB protein (Ludwig et al. 1987; Scott 1989).

Structure and function of apolipoprotein B (ApoB)

Apolipoprotein B (ApoB) is quantitatively dominating and non-replaceable apolipoprotein of lipoproteins rich in cholesterol and triglycerides such as chylomicrons, VLDL, IDL and LDL particles. ApoB is essential for the formation and secretion of these lipoproteins (Olofsson et al. 1987).

Two main isoforms are known in human: ApoB-48 and ApoB-100. ApoB-48 has 48% identity with apolipoprotein B-100 from N-terminal end (Innerarity et al. 1987). The full size of ApoB-100 is 4536 amino acids with a weight of 517 to 550 kDa, making it one of the largest known monomers. The structure of ApoB-100 contains five domains: βα1, β1, α2, β2 a α3, named according to the structure of α-helix and β-pleated sheet (Segrest et al. 1994; Rutledge et al. 2010).

Apolipoprotein B-100 is synthetized in the liver and is essential for initial lipidation of VLDL particles (Aguie et al. 1995). It is also the cornerstone of IDL and LDL particles. These related molecules are used for the transport of fat and cholesterol in the circulation. Apolipoprotein B-100 is the only remaining component of the LDL particles. LDL particles are removed from circulation by binding the LDL receptors; subsequently this complex is internalized and degraded in liver (Benn 2009).

When apolipoprotein B is mutated, LDL particles cannot bind to its ligand, what results in higher LDL

concentrations in plasma. There is only a limited number of mutations in this gene that cause the FH phenotype (Fahed and Nemer 2011).

Mutations in *APOB* gene

The first mutation in *APOB* gene was identified by Innerarity and coworkers in 1987 p.Arg3500Gln, now known as p.Arg3527Gln (Innerarity et al. 1987). It is a nucleotide substitution of G to A at position 10708, resulting in amino acid change of arginine for glutamine at protein position 3527 (p.Arg3527Gln). The frequency of this mutation in general population is 1:500 to 1:700 (Tybjaerg-Hansen et al. 1998). This mutation has not been identified in genetically isolated Finnish population, however the highest frequency was published in Switzerland study (Hamalainen et al. 1990; Miserez et al. 1994). The knowledge of geographic distribution of the mutation and ways of spreading humans among Europe and Asia established the hypothesis that this mutation originates from Central Europe 7000 years ago. The fact that the same mutation is found in the majority of FH patients, suggests that it comes from a mutual European ancestor (Rauh et al. 1991; Vrablik et al. 2001).

Another known mutation p.Arg3527Trp is a substitution of arginine for tryptophan, which has the same biochemical and clinical features as the previous mutation. Higher incidence of this mutation and its association with unique haplotype in Asia, indicates a mutual Asian ancestor (Tai et al. 1998; Vrablik et al. 2001). Gaffney et al. (1995) identified only two hyperlipidemic patients with this mutation of Caucasian ethnicity.

In 2014, Alves and collaborators published a study where they analyzed whole *APOB* gene in 65 patients without a mutation in *LDLR*, *PCSK9* or exon 26 and 29 of *APOB* gene which are routinely screened. They identified 10 variants in this group and functional studies shown a 40% reduction in internalization in lymphocytes and HepG2 cells in two variants p.Arg164Thr (exon 22) and p.Gln4494del (exon 29). Similar results were demonstrated in the most common mutation p.Arg3527Gln. Remaining variants were considered as neutral polymorphisms. Their results show that in negative patients, screening of additional parts in the known FH genes would be appropriate (Alves et al. 2014).

Familial hypercholesterolemia due to mutations in *PCSK9* gene

Proprotein convertase subtilisin kexin 9 (*PCSK9*) was discovered in 1987 as the third genetic variation involved in autosomal dominant form of FH. PCSK9 is the ninth member of subtilisin kexin-like proconvertase family. PCSK9 functions as posttranslational inhibitor of LDL receptors due to their increased internalization and degradation in lysosomes (Park et al. 2004). FH phenotype due to *PCSK9* mutations is very similar to heterozygous *LDLR* mutations. The prevalence of *PCSK9* heterozygotes is 1:2500 (Hegele 2009).

***PCSK9* gene**

Human gene *PCSK9* is located on first chromosome (1p32.3) and contains 12 exons spanning 3617 bp. *PCSK9* gene encodes 692 amino acids long glycoprotein (Benjannet et al. 2004).

Structure and function of PCSK9

PCSK9 is synthesized as an inactive proenzyme containing 692 amino acids. The structure of PCSK9 is similar as other members of the subtilisin convertase family - signal sequence (1-30 AA), following prodomain (31-152 AA) and catalytic domain (Seidah et al. 2003). After the catalytic domain there is a C-terminal region of 279 amino acids rich in cysteine and histidine (CHRD domain). The protein is synthesized as 72 kDa precursor that underlies autocatalytic cleavage between prodomain and catalytic domain (Basak 2005). The prodomain (14 kDa) is in the process of passing through the ER cleaved from the mature protein (63 kDa). The prosegment works as an inhibitor and intramolecular chaperone of catalytic domain, which is necessary for the proper folding of PCSK9 and its release from the ER (Seidah et al. 2003). Benjannet et al. (2014) discovered that for autocatalytic cleavage the presence of calcium is required.

Culture cell studies indicate that PCSK9 directly interacts with LDL receptors within the cell in endoplasmic reticulum and also on the cytoplasmic membrane, where it mediates the degradation. In cells that do not express the LDL receptor, the PCSK9 molecules remain located in the ER. However, in cells expressing LDLR, PCSK9 molecules are moved to other parts of post-endoplasmic cell organelles (endosomes in cell lines and into Golgi apparatus in hepatocytes), where they are colocalized with LDL receptors (Nassoury et al. 2007).

At neutral pH, PCSK9 binds the extracellular EGF homology domain of LDL receptor. The complex of PCSK9, LDL particle and LDLR enters the endosomal pathway as a result of decreasing pH, and then the C-terminal end of PCSK9 binds to ligand-binding domain of LDLR, what reinforces the strength of the bond

(Yamamoto et al. 2011). The affinity of PCSK9 to LDLR in the endosome is increased and thus leads to failure of PCSK9 release from the complex, which prevents recycling of the receptor. This process aligns PCSK9/LDLR complex in the lysosomes, where the complex is degraded (Tibolla et al. 2011).

Mutations in *PCSK9* gene

In 2015, The Human Gene Mutation Database records 68 mutations in *PCSK9*, 40 of them result in familial hypercholesterolemia (Stenson et al. 2009). These mutations are divided into two classes depending on their phenotypic effect: gain of function and loss of function variants.

Mutations in *PCSK9* are uncommon cause of FH compared to mutations in the *LDLR* gene. The French ADH Research Network published the frequency of *PCSK9* mutations in 1358 probands. They have identified *PCSK9* mutations in only 0.7% of FH patients compared to 73.9% with a *LDLR* mutation and 6.6% with a mutation in *APOB* (Marduel et al. 2010).

Effect of mutations on protein structure and function

According to the structure of the protein, the effect of natural occurring loss of function or gain of function mutations on PCSK9 function can be predicted.

In the case of gain of function variants, PCSK9 constantly marks LDL receptors for degradation, so they are not capable to effectively process LDL-C from circulation. An example is the gain of function mutation p.Asp374Tyr that increases the interaction between PCSK9 and LDLR 5-30 times compared to wild type PCSK9. Aspartic acid at protein position 374 (D374) produces no contact with other amino acids in the PCSK9 structure, but it creates hydrogen bond with histidine 306 of EGF homology domain of LDLR, thereby increases the affinity to LDL receptor (Pandit et al. 2008). The class of gain of function mutations is associated with increased plasma levels of LDL-C and the development of CVD (Wu and Li 2014).

Loss of function mutations result in loss of function of PCSK9 which is not capable of mediating the degradation of LDL receptor, that leads to continuous catabolism of LDL cholesterol. Three nonsense mutations p.Leu82*, p.Tyr142* a p.Cys679* result in a shortened version of PCSK9 which disrupt the proper folding and secretion of the protein (Fasano et al. 2007). These loss of function mutations are associated with decreased levels of plasma LDL cholesterol and decreased cardiovascular risk (Kwon et al. 2008; Lambert et al. 2009).

Familial recessive hypercholesterolemia

Khachadurian and collaborators already in 1973 described a different form of hypercholesterolemia which is clinically similar to classic FH but represents an autosomal recessive pattern of inheritance (Khachadurian and Uthman 1973). The gene responsible for autosomal recessive hypercholesterolemia-ARH (*LDLRAP1*) was mapped a few years later by Garcia et al. (2001). Patients with recessive hypercholesterolemia have decreased clearance of LDL receptors from hepatocyte surface into the cell. In contrast with homozygous *LDLR* patients, they have normal or slightly reduced function of LDLR (Zuliani et al. 1999). The clinical phenotype of ARH is at the interface between the heterozygous and homozygous *LDLR* patients. Total cholesterol (TC) in plasma is in range of 13 to 17 mmol/l and concentrations of LDL-C between 9.6 to 14.3 mmol/l. If patients are not effectively treated, they evolve large xanthomas and coronary artery disease that occurs in third decade of life. Most of them die of myocardial infarction (MI) or stroke (Arca et al. 2002).

LDLRAP1 gene

In 2001, Garcia et al. mapped the *LDLRAP1* gene (previously known as *ARH*) encoding the LDL receptor adaptor protein 1 on chromosome 1p35-36.1. This gene contains 9 exons and 8 introns within 25kb.

Structure and function of LDLRAP1

LDLRAP1 is an adaptor protein that interacts with the cytoplasmic tail of LDL receptors, phospholipids and clathrin components of endocytosis mechanism (Garuti et al. 2005).

This protein encoded by *LDLRAP1* gene is a cytosolic protein consisting of 308 amino acids that comprises 107 AA-long motif called phosphotyrosine-binding domain (PBD) (Arca et al. 2002). PBD is a part of several adapter proteins which play role in intracellular signaling and transport. PBD domain interacts with motifs of LDLR cytoplasmic tail which plays role in the endocytosis process of LDL receptors (Schneider 2002). This domain interacts with phosphoinositols of cell membrane (Mishra et al. 2002) and simultaneously the specific sequence of C-terminal end of LDLRAP1 binds clathrin and its adaptor AP-2 (He et al. 2002). These interactions allow LDLRAP1 to function as an adapter for clathrin-mediated endocytosis of LDL receptors in the liver (Dvir et al. 2012).

Mutations in *LDLRAP1*

Mutations in *LDLRAP1* gene cause the failure of internalization of LDL receptors inside the hepatocyte (Garcia et al. 2001). In 2015, 17 unique allelic mutations in *LDLRAP1* gene have been listed in The Human Genome Mutation Database. Samples from two families originated from Sardinia were used for gene mapping. Children born in these families had a homozygous mutation in *LDLRAP1* gene. The first mutation that was present in two affected siblings from northeastern Sardinia was the insertion of one nucleotide (adenine). The insertion caused alternative frame-shift at codon 144, resulting in premature stop codon at protein position 145 (c.432_433insA in exon 4; p.Ala145Lysfs*26). The second mutation identified in another family from central Sardinia was the substitution of G for A nucleotide that caused the substitution of tryptophan for stop codon at position 22 (c.65G>A in exon 1; p.Trp22*) (Garcia et al. 2001; Arca et al. 2002).

Clinical characterization of FH

Familial hypercholesterolemia (FH) is an autosomal dominant disorder that leads to elevated LDL and total cholesterol levels in plasma. As it was mentioned previously the disease is caused by mutations in currently four known genes including *APOB*, *LDLR*, *PCSK9* and *LDLRAP1* gene. Majority of the patients have a heterozygous *LDLR* mutation with a moderate phenotype; rare mutations of both alleles of *LDLR* gene are causing a more severe phenotype. Lifetime elevated total and LDL cholesterol levels result in premature occurrence of coronary heart disease (CHD), myocardial infarction (MI) and angina pectoris (AP) (Austin et al. 2004). LDL cholesterol deposits in several tissues of FH patients, especially in skin and tendons (xanthomas) and in arterial plaques (atheromas). Homozygotes and heterozygotes both may have tendon xanthomas, especially in the Achilles tendons and in the extensor tendons of the hand. Cholesterol deposits may also occur in the iris (arcus lipoides cornae) or around the eyes (xanthelasmas or palpebral xanthomas) (Goldstein et al. 2001).

Since heterozygous *LDLR* patients have only one functional gene, therefore only half of the functional LDL receptors, their hepatocytes are able to process only about half of LDL cholesterol (LDL-C) compared to unaffected individual. The risk of CHD development in untreated patients is 20 times higher (Hopkins 2010). As heterozygous patients remain untreated with total cholesterol (TC) concentrations in the range between 8 and 15 mmol/l, typically the cardiovascular disease occurs between 55 to 60 years of age (Nordestgaard et al. 2013). Coronary heart disease develops before age of 50 years in 50% of men with FH and in women with FH, the CHD occurs within the age of 60 in approximately 30% of cases (Hopkins et al. 2011).

The incidence of homozygous form of FH is very rare (1 case in a million). This is a very severe type of disease with almost total or total loss of function of the LDL receptor (LDLR). The homozygous form of FH can be caused due to mutations in *LDLR*, *APOB* genes but also due to gain of function *PCSK9* mutation. TC levels are in the range of 14 to 24 mmol/l. Since all LDL receptors are dysfunctional (in case of homozygous or double heterozygous *LDLR* mutations), the deposition of cholesterol in most arteries is accelerated, thus leads to premature atherosclerosis. In patients with homozygous form, atherosclerosis involving the aorta and coronary arteries is rapidly progressive, leading to MI, AP or sudden death before age of 30 years (Goldstein et al. 2001).

Genotype-phenotype correlation

FH is a hereditary disease with high variability in phenotypic expression. In unrelated patients with the same type of *LDLR* mutation, large individual differences in plasma cholesterol, atherosclerosis severity and onset of cardiovascular disease appear (Bertolini et al. 2004). These differences are likely to be modulated by other genetic and environmental factors. The environmental factors contributing to the differences are probably the same factors that predispose the general population to CHD, such as smoking, low HDL cholesterol levels, male sex and increasing age (Soutar and Naoumova 2007). Despite many efforts that have been made for identification of other genetic variants that could affect the clinical phenotype of FH patients with *LDLR* mutation, most of the studies were inconclusive because of small number of patients. Even though it is likely that such variants do exist, it could be that many of these will be rare alleles that can have marked effect in one family but may not be detectable in groups of FH patients in SNP association studies (Soutar and Naoumova 2007). To the differences in plasma lipid levels in patients with the same *LDLR* mutation can contribute also epigenetic modifications of key lipoprotein metabolism genes. The data from a Canadian study show that DNA methylation levels of *ABCG1*, *LIPC* and *PLTP* genes was significantly associated with HDL-C, LDL-C and triglyceride levels in a sex-specific manner. They suggest that these epigenetic

changes contribute to the interindividual variability at least in the FH phenotype (Guay et al. 2014).

The penetrance of *APOB* mutations has been shown to be less than 100% and patients with *APOB* mutations usually have a less-severe phenotype than FH patients due to *LDLR* mutations (Myant 1993; Vrablik et al. 2001). The clinical phenotype of the p.Arg3527Gln *APOB* mutation carriers is variable, despite the presence of the same mutation (Vrablik et al. 2001; Soutar and Naoumova 2007).

Mutations in *LDLR* and *APOB* gene show a gene dosage effect (Singh and Bittner 2015). Levels of LDL-C are dependent on the genotype of the patient, from less severe phenotype as follows: heterozygote *APOB* <heterozygote *LDLR* <double heterozygote (*LDLR*/*APOB*/gain of function *PCSK9* mutation) <homozygote *APOB*/gain of function *PCSK9* mutation <homozygote *LDLRAP1*/*LDLR*-defective mutations <compound heterozygote *LDLR*-defective and *LDLR*-negative mutations <homozygote *LDLR*-negative mutations (Cuchel et al. 2014).

Diagnostic criteria

Currently, there are several diagnostic criteria for identifying patients with FH. The most commonly used are three types of clinical scoring systems – diagnostic criteria of MedPed USA program, which assess FH only based on lipid levels corresponding to age of the individual and his family relationship to the proband (Table 2) (Williams et al. 1993). Diagnosis based only on total cholesterol levels may be ambiguous, especially in children. The incidence of false positive and false negative results is in the range from 8 to 15% (Leonard et al. 1977; Kwiterovich 1993; Naoumova et al. 2004).

Other frequently used criteria were established by members of Scientific Committee for Simon Broom register (Table 3) and The Dutch Lipid Network criteria also known as WHO criteria (Table 4) (Betteridge et al. 1991; WHO 1999). Both take into account not only biochemical parameters, but also family history, therefore have a higher informative value.

Table 1
MedPed criteria for FH diagnosis (Williams et al. 1993)

Total cholesterol (LDL cholesterol) in mg/dl and mmol/l (98% specificity, 87% sensitivity)				
Age	**Degree of relatedness to closest FH relative**			**General population**
	First	**Second**	**Third**	
<18	220 (150) mg/dl 5,7 (3,9) mmol/l	230 (165) mg/dl 5,9 (4,3) mmol/l	240 (170) mg/dl 6,2 (4,4) mmol/l	270 (200) mg/dl 7,0 (5,2) mmol/l
20	240 (170) mg/dl 6,2 (4,4) mmol/l	250 (180) mg/dl 6,5 (4,7) mmol/l	260 (185) mg/dl 6,7 (4,8) mmol/l	290 (220) mg/dl 7,5 (5,7) mmol/l
30	280 (200) mg/dl 7,3 (5,2) mmol/l	280 (200) mg/dl 7,3 (5,2) mmol/l	290 (210) mg/dl 7,5 (5,4) mmol/l	340 (240) mg/dl 8,8 (6,2) mmol/l
40+	300 (215) mg/dl 7,8 (5,6) mmol/l	300 (215) mg/dl 7,8 (5,6) mmol/l	310 (225) mg/dl 8,0 (5,8) mmol/l	360 (260) mg/dl 9,3 (6,7) mmol/l

Table 2
Simon Broom diagnostic criteria (Betteridge et al. 1991)

Diagnose of definite familial hypercholesterolemia	**Diagnose of possible familial hypercholesterolemia**
Total cholesterol > 7.5 mmol/l in adult	Total cholesterol > 7.5 mmol/l in adult
Total cholesterol > 6.7 mmol/l in child	Total cholesterol > 6.7 mmol/l in child
LDL cholesterol > 4.9 mmol/l in adult	LDL cholesterol > 4.9 mmol/l in adult
LDL cholesterol > 4.0 mmol/l in child	LDL cholesterol > 4.0 mmol/l in child
Plus one of the following clinical signs	
Tendon xanthomas in first- or second-degree relative	Family history of total cholesterol > 7.5 mmol/l in adult or > 6.7 mmol/l in child in first- or second-degree relative
DNA-based evidence of *APOB*, *LDLR*, *PCSK9* mutation	Family history of myocardial infarction aged <50 in second-degree relative or <60 in first-degree relative

Table 3
The Dutch Lipid Network criteria (WHO 1999)

Family history	First-degree relative with premature CVD (men: <55 years; women <60 years) or LDL-C above 95th percentile	1
	First-degree relative with tendon xanthomas and/or children aged <18 years with LDL-C above 95th percentile	2
Clinical history	Patient with premature CAD (men:<55years; women <60years)	2
	Patient with premature cerebral or peripheral vascular disease (men:<55years; women <60years)	1
Physical examination	Tendous xanthomas	6
	Arcus lipoides cornae (ALC) prior to age 45 years	4
LDL cholesterol levels (mmol/l)	LDL-C ≥ 8.5 mmol/l	8
	LDL-C 6.5 – 8.4 mmol/l	5
	LDL-C 5.0 – 6.4 mmol/l	3
	LDL-C 4.0 – 4.9 mmol/l	1
DNA analysis	Pathogenic mutation in *APOB*, *LDLR*, *PCSK9* gene	8

Definitive diagnosis > 8 points, probable diagnosis 6-8 points, possible diagnosis 3-5 points

Table 4
Spectrum of most common mutations in LDLRin European countries (partially addapted by Tichy et al. 2012)

Country	Diagnostic criteria	Number of probands	Number of detected mutations in LDLR	Most common mutations (frequency %)	Total % of the most frequent mutations out of all mutations	Reference
Great Britain	Simon Broom (SB)	272	54	p.Glu101Lys (6.6) p.Trp87Gly (3.3) c.313+1G>A (3.3) p.Asp227Glyfs*12 (3.3) p.Asp227Glu (3.3) p.Glu374fs*8 (3.3) p.Pro685Leu (3.3)	26.4	Futema et al. 2013
Slovakia	SB (not exactly)	226	51	p.Gly592Glu (10.5) c.68-2A>T (5.3)	15.8	unpublished data
Czech Republic	MedPed	2239	127	p.Gly592Glu (19.3) p.Asp266Glu (16.4) p.Arg416Trp (4.1)	39.8	Tichy et al. 2012
Poland	WHO	378	71	p.Gly592Glu (22.5) Exon4_8dup (9.5) p.Asp221Gly (5.3)	37.3	Chmara et al. 2010
Germany	SB (not exactly)	100	37	p.Asp266Glu (3.7) p.Glu288* (3.1) c.313+1G>A/C(3.1)	9.9	Nauck et al. 2001
Austria	MedPed	263*	46	p.Asp266Glu(11.1) p.Asp221Gly (5.6) p.Asp178Glu (4.6) p.Val506delfs*14 (4.6)	25.9	Widhalm et al. 2007

Treatment of FH

There are currently several pharmacological treatment options for FH. With knowledge of HMG-CoA reductase as a central enzyme in endogenous pathway of cholesterol production, inhibitors of this enzyme called statins came on market. Treatment guidelines for adults recommend treating FH patients with high-dose statin to achieve at least 50% reduction of LDL-C levels. According to international guidelines the target LDL-C levels should be in the majority of FH patients below 2.6 mmol/l and in the patients with CAD or other risk factors below 1.8 mmol/l. However, if the therapy fails to meet target cholesterol levels, a combination of statin with ezetimibe, nicotic acid or bile acid sequestrants is recommended (Watts et al. 2014). For children and adolescents with FH exist a different treatment guidelines (Feldman et al. 2014).

In more severe homozygous and heterozygous FH resistant to statin treatment due to myopathy, LDL apheresis is an option, but this possibility is not available in many countries. For the treatment of homozygous FH, two molecules are approved - lomitapide and mipomersen (Cuchel et al. 2014). Lomitapide is an oral molecule that inhibits microsomal triglyceride transfer protein (MTTP) which is needed for the synthesis of chylomicrons and VLDL particles in the intestine and liver (Hussain et al. 2012). Inhibition of MTTP leads to decreased secretion of lipoproteins into circulation. Mipomersen is an antisense oligonucleotide that binds mRNA of apolipoprotein B what prevents the synthesis of ApoB on ribosome. In the absence of ApoB, atherogenic lipoproteins including LDL particles are not able to be synthesized (Crooke and Geary 2013).

Currently, PCSK9 monoclonal antibodies show very promising entrance in FH treatment. Although these are not yet commercially available, the third stage clinical trials indicate their potential in reduction of cholesterol and CAD occurrence (Schwartz et al. 2014). PCSK9 mediates the LDL receptor's degradation in lysosomes. Inhibition of PCSK9 function leads to continuous processing of cholesterol leading to a reduction in LDL-C levels.

The final and last option for very severe hypercholesterolemia that does not respond to drug treatment is the possibility of liver transplantation but such cases are extremely rare (Pejic 2014).

Conclusions

Familial hypercholesterolemia is one of the most common inherited metabolic disorders that can cause death or disability in young age. FH is very heterogeneous group of patients with very variable phenotype in LDL-C levels and severity of phenotype. It is still not possible to elucidate the genetic background of each FH patient. The majority FH patients are due to an *LDLR*, *APOB*, *PCSK9* or *LDLRAP1* mutation, but in 10-40% we cannot detect the genetic background of the disease. But it is very clear that this group of patients demand early diagnosis confirmed by DNA analysis, and early treatment in patients and their families help to reduce morbidity and mortality associated with FH. In Slovakia, the DNA analysis of the p.Arg3527Gln *APOB* mutation and mutations in *LDLR* and *PCSK9* genes is available in the DIABGENE laboratory (diabgene@savba.sk).

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