Title: Genetics and pharmacogenetics of familial hypercholesterolemia in Serbia

Research rational

Familial hypercholesterolemia (FH) is an autosomal dominant disorder that affects the metabolism of low-density lipoprotein cholesterol (LDL-C) through variants in the gene for LDL receptor (*LDLR*), and less commonly in those for apolipoprotein B (*APOB*), proprotein convertase subtilisin-kexin type 9 (*PCSK9*), and others.

FH is a common yet underdiagnosed autosomal dominant disorder with a prevalence approximately 1 in 200 individuals, meaning that about 35,000 people are expected to have FH in Serbia. FH is characterized by lifelong elevation of LDL-C and if untreated leads to early-onset atherosclerosis and increased risk of cardiovascular events. Affected men and women who are untreated have a 30% to 50% risk of a fatal or nonfatal cardiac event by ages 50 and 60 years, respectively.

The most common causes of FH are pathogenic variants of the LDL-R gene, which are responsible for 85% to 90% of genetically confirmed FH. Pathogenic variants of the APOB gene, resulting in decreased binding of LDL to the LDL-R, or gain-of-function mutations in the gene for PCSK9, resulting in increased destruction of LDL-R, are responsible for 5% to 15% and 1% of cases of FH, respectively. Autosomal recessive FH, caused by homozygous mutations in the LDL-R adaptor protein-1, is associated with a mild homozygous FH (HoFH) phenotype.

FH is significantly underdiagnosed and undertreated, particularly in children. Timely pharmacological interventions can significantly reduce morbidity and mortality risks. Recently published results from Serbia show that the majority of FH patients are treated with the lowest statin doses, and only 17.9% of the studied population were using high intensity statin, per the accepted European guidelines.

There are modest data on FH in Serbia (with a population of 6.7 mil. it is estimated that a maximum of 20% of patients with FH have been recognized in Serbia (about 3000-7000 patients)), while there are no genetically confirmed FH in Serbia.

The main objective of the research is detection of causative gene mutation on clinically diagnosed patients with FH. Specificity of local causative gene mutation compared to clinical characteristics can lead to facilitation of faster diagnosis of these patients. Determination of most common genetic variants in Serbian FH patients will enable development of population-specific diagnostic panel for FH (PCR- based or NGS-based) and could accelerate initiation of national screening program for FH in the near future that could increase diagnosis, patient refferal and treatment optimization as unmet medical need of health care system.

The second aim is to determine local statin intolerance and gene polymorphisms that is affecting efficacy/safety of statins to be used as easy detectable personalized medicine, thus timely pharmacological intervention can significantly improve patients' wellbeing.

Generation of local data can facilitate improvement of local Register of FH patients that could be helpful in the decision-making process of appropriateness of innovative therapy choice and ensure adequate treatment to prevent the development of ASCVD in these patients.

Study design

Multi-centric epidemiological research aiming to identify causative gene in patients with clinically diagnosed FH. Patients were identified based on clinical and family history data, physical examination, and LDL-C levels. Target population is 100 clinically diagnosed patients with FH. Blood samples and Informed Consent Forms are maintained in University Clinical Centre, Lipidology Centre within the Endocrinology department.

Genetic testing will be performed in Institute of Molecular Genetics and Genetic Engineering. Independent Ethical Committee (IEC) will be obtained before research start up.

To identify the mutational profile involved in the pathogenesis of FH, only confirmed disease-causing and disease-associated genes will be analyzed. This is a pilot research that will provide first data on genetic basis of FH in Serbia. Analysis of disease-causing and disease-associated genes and the determination of the most frequent variants could be used for design of cost-effective population specific diagnostic panel for the Serbian population. If the analysis shows in this sample size that certain variant is present with frequency >30%, or if several variants are present with frequency >80%, then PCR based diagnostic panel can be developed in order to achieve cost- effective and user-friendly diagnostics tool. If it were not the case, it would be recommended that NGS analysis should be applied for molecular diagnosis of FH.

Inclusion criteria

Age >18 years with no upper age limit.

Patients with clinically diagnosed FH using the Dutch Lipid Clinic Network (DLCN) score based on clinical examination, LDL-C levels and family and personal history.

Fasting levels of LDL-C in plasma >5mmol/L.

Patient medical records on physical examination could be presented with tendon xanthomas on the dorsal aspect of the metacarpophalangeal joints or at the calcaneal tendon, or xanthelasmas on the eyelid.

All patients suspected of FH will be included.

Exclusion criteria

Informed Consent Form not obtained.

Research objectives

Primary objectives

- FH phenotype determination and detection of most common variants that causes the disease in Serbia population
- Evaluation of pharmacogenetic variants relevant to efficacy/toxicity of statins

Secondary objectives

- Correlation among clinical and genetic diagnosis and its similarities in case presentations
- Design population specific diagnostic panel for FH

Primary endpoints

1. The number of FH suspected patients analyzed on genomic level
2. The number of genetically confirmed FH in Serbia
3. Detection of the most common variants causing FH in Serbia

4. The number of FH patients with pharmacogenetic variants influencing statin efficacy and safety

5. The estimated number of patients who would benefit from the avoiding primary therapy approach (statins) and be treated with advanced therapeutics from the start

Secondary endpoints:

1. Assessment of the possibility to design population specific FH diagnostic kit for time- and money-consuming diagnosis of FH in Serbia

Methodology

Blood samples from clinically diagnosed FH patients in Lipidology Centre, Clinic for Endocrinology, University Clinical Centre of Serbia, Belgrade, Serbia from who signed inform consent was obtained, will be transferred to Institute of Molecular Genetics and Genetic Engineering.

Patients who are carriers of variants in confirmed disease-causing and disease-associated genes (classified as pathogenic, likely pathogenic or VUS, according to ACMG) will be considered as FH positive. To exclude potential benign variants in FH genes characteristic for Serbian population, available data on NGS analysis (already performed on healthy Serbian population) will be used for comparison of FH and non-FH patients. Only nucleotide variants in confirmed FH disease-causing and disease-associated genes will be considered as diagnostic markers. This study is not planned to establish predictive markers of FH. The goal of the project is to provide initial data on genetic profile of FH Serbian patients, genotype-phenotype correlation and to recommend most efficient method for diagnosis and screening of FH in Serbia.

Genomic DNA will be isolated from the whole blood sample according to the established laboratory protocols using QIAamp DNA Blood Mini Kit (Qigen, Germany). Samples for NGS will be prepared following the manufacturer's protocol using Illumina's TruSight One Sequencing Panel (Illumina, San Diego, CA) containing exons and exon-intron boundaries of 4813 clinically relevant genes, among which are all genes associated with FH (LDLR, LDLRAP1, PCSK9, APOE genes). Also, all relevant genes for pharmacogenomics of statins (gene families ABC, SLC, CYP, UGT1) are included in TruSight One Sequencing Panel. So, in one NGS reaction, all data for this study will be obtained. Samples will be sequenced on NextSeq 550 sequencer with NextSeq 500/550 High Output v2.5 kit (300 cycles) (both Illumina, USA) following the manufacturer's protocol including recommendations for quality control parameters. Variants reported in the disease-specific database [Leigh S, et al.] and HGMD Professional database (www.biobase-international. com/product/hgmd) will be classified as potentially disease-causing. In silico analysis of novel variants will be performed with PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/), SIFT (http://sift.jcvi.org/www/SIFT_enst_submit.html), CADD (http:// cadd.gs.washington.edu/) and MutationTaster (www.mutationtaster.org) bioinformatic tools. The novel variants will be annotated as pathogenic, likely pathogenic as well as the variants of uncertain significance (VUS), according to ACMG classification and ClinVar. Single nucleotide variants and small duplications/ deletions with potentially disease-causing effect will be confirmed by targeted Sanger DNA sequencing.

Descriptive statistics (mean, 95% confidence interval [95%CI], standard deviation and ratio) will be used to characterize the analyzed population. Statistical analysis will be performed using IBM SPSS.

Safety reconciliation should be performed one year after FPFV and annually thereafter, ending at within 3 months of the LPLV.

Timelines

Start date: May 2024

Completion date: Dec 2024 for Interim analysis

Planned publication/abstract: International Journal HI mid 2025

Reference literature

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Team:

Principal Investigators:

1. Dr Sonja Pavlovic

Dr Sonja Pavlovic is a head of the Center for Genetic Diagnosis of Rare Diseases at the Institute of Molecular Genetics and Genetic Engineering, University of Belgrade, Serbia. She received her PhD in Molecular Biology from the University of Belgrade in 2001. She was named Full Research Professor, the highest scientific title, in 2011. She is a member of the Academy of Medical Sciences in Serbia. Her research interests include molecular genetics of rare diseases, hematological malignancies, and pharmacogenomics. She has published more than 200 peer-reviewed journal articles and book chapters, cited for more than 2900 times. Dr Sonja Pavlovic has introduced numerous molecular genetic diagnostic tests in medical practice in Serbia, including NGS. Teaching activities of dr Sonja Pavlovic include seminars

and courses at the Faculty of Biology and Faculty of Medicine, University of Belgrade. She has been a supervisor of more than 50 master and doctoral theses.

2. Dr Katarina Lalic

Dr Katarina Lalic is a head of Department for lipid disorders and cardiovascular complications in diabetes, Clinic for Endocrinology, Diabetes and Metabolic Diseases, University Clinical Center of Serbia. She is full Professor on Faculty of Medicine, University of Belgrade. The principal interest of Prof Dr Lalic scientific work in diabetology/endocrinology has been concentrated on the relations between insulin secretion and insulin sensitivity impairments in Type 2 diabetes and their relevance for the pathogenesis its late complications, especially coronary artery disease. Also, in the field of lipidology she was involved in the research regarding treatment of patients with lipid metabolism disorders including LDL apheresis, being the only center in Serbia who applied this therapy. Within her department they actively screen, follow and treat patients with familiar hypercholesterolemia. Professor has been an investigator in several studies regarding the new therapeutic approaches in diabetes and lipid treatment.

Research Associates

1. Dr Vladimir Gasic, MD, PhD is an Assistant Research Professor in the Laboratory for molecular biomedicine, at the Institute of Molecular Genetics and Genetic Engineering, University of Belgrade, Serbia. He received his PhD in Molecular Biology from the University of Belgrade in 2019. His research interests are related to molecular basis of rare diseases, pharmacogenomics and pharmacotranscriptomics and, especially, molecular endocrinology and the role of microRNA and long non-coding RNA in diseases. He has published more than 30 peer-reviewed journal articles. He has a great experience in NGS analysis.
2. Dr Jovana Komazec, PhD, is an Assistant Research Professor in the Laboratory for rare diseases research and therapeutic development, at the Institute of Molecular Genetics and Genetic Engineering, University of Belgrade, Serbia. She received his PhD in Molecular Biology from the University of Belgrade in 2020. The scientific research area - genetic basis of MODY diabetes (Maturity-onset diabetes of the Young, MODY), functional characterization of disease-causing gene variants. She has a great experience in NGS analysis.
3. Dr Sandra Singh Lukač is working in Clinic for Endocrinology, Diabetes and Metabolic Diseases, University Clinical Center of Serbia and she is Assistant Professor for Internal Medicine at Faculty of Medicine, University of Belgrade. She obtained Academic specialization in neurology evaluating potential role of statins in multiple sclerosis. She is a member of the Academy of Medical Sciences in Serbia and European Association for the Study of Diabetes (EASD).