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Familial dyslipidemia, clinical, therapeutic, and genetic considerations in pediatric patients

PhD THESIS SUMMARY

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Abbreviations and symbols list

ABCG5	ATP-binding cassette subfamily G, member 5
ABCG8	ATP-binding cassette subfamily G, member 8
AD	autosomal dominant
ANGPTL3	angiopoietin-like protein 3
Apo E	apolipoprotein E
ApoA-1	apolipoprotein A1
ApoA-V	apolipoprotein A-V
ApoB	apolipoprotein B
Apo-CII	apolipoprotein CII
AR	autosomal recessive
ATP	adenosine triphosphate
CESD	cholesteryl ester storage disease
CCL2	monocytic chemoattractant protein 1
CRISPR-Cas9	clustered regularly interspaced short palindromic repeats
DGGE	denaturing gradient gel electrophoresis
DHA	docosahexaenoic acid
EPA	eicosapentaenoic acid
GPIHBP1	glycosylphosphatidylinositol anchored high density lipoprotein binding protein 1
HDL	high density lipoproteins
HeFH	heterozygote familial hypercholesterolemia
FH	familial hypercholesterolemia
HoFH	homozygote familial hypercholesterolemia
AIP	atherogenic index of plasma
ICAM1	intercellular adhesion molecule 1
IDL	intermediate density lipoproteins
IL-8	interleukin 8
INSMC	Institutul Național pentru Sănătatea Mamei și Copilului „Alessandrescu-Rusescu”, Bucharest
LAL	lysosomal acid lipase

LIPA	lysosomal acid lipase protein
LIPC	lipase C, hepatic type
LIPG	lipase G, endothelial type
LDL	low density lipoproteins
LDLR	receptor for LDL-cholesterol
LDLRAP	low density lipoprotein receptor adaptor protein 1
LPL	lipoprotein lipase
MLPA	Multiplex Ligation-Dependent Probe Amplification
PCSK9	protein convertase subtilisin/kexin type 9
PGM	Personal Genome Machine
SSCP	Single-Strand Conformation Polymorphism
STAP1	Signal Transducing Adaptor Family Member 1
TG	triglyceride
VCAM1	vascular cell adhesion molecule 1
VLDL	very low-density lipoproteins

Introduction

In the medical world, the interest in dyslipidemia is growing since it is one of the risk factors for early cardiovascular disease. Many times, dyslipidemia starts at a young age, in childhood. Accompanied by an unhealthy lifestyle, wrongful food choices (processed food and fast-food), the tendency towards a sedentary lifestyle, could have severe consequences such as coronary disease at a young age (before 50 years).

The presence of modifiable and non-modifiable risk-factors in childhood is associated with cardiovascular events in adulthood. [1]. This is why approaching cardiovascular risk-factors, including dyslipidemia in childhood is essential for improving prognosis, in a preventive approach.

In Romania, there is no screening program for dyslipidemia in pediatric patients.

This doctoral thesis wishes to be a wake-up call regarding dyslipidemia, as a disease per-se as well as secondary to other diseases or induced by medication.

This thesis started from several working hypotheses. The first working hypothesis was that patients with dyslipidemia have diseases or follow treatments that influence their lipid profile values. The objectives included analyzing the disease that led to a rise in LDL-cholesterol values; describing the diseases that could determine changes in the lipid profile.

To demonstrate this hypothesis we conducted a retrospective, descriptive, transversal study which was extended on a 9-year period (2011-2020) and includes the evaluation of over 3000 lipid panel results from National Institute for Mother and Child Health "Alessandrescu-Rusescu", Bucharest. Of the 2413 patients included in the study, 18,23% had high LDL-cholesterol levels (over 130 mg/dL). These patients had various diagnosis. A significant number of patients (25,91%) were diagnosed with overweight, obesity and other hyperalimentation. Of the patients included in the study, 9,09% were diagnosed with dyslipidemia and 6,36% specifically with hypercholesterolemia.

The second working hypothesis was that patients with familial hypercholesterolemia can be identified by screening and their diagnosis can be confirmed through genetic testing. Patients with genetically confirmed familial hypercholesterolemia do not respond as well to hygiene-

dietetic treatment measures compared with the ones with negative genetic testing results. Objectives included identifying patients suspected to have familial hypercholesterolemia; identifying and describing genetic mutations identified in patients with positive genetic testing; determining the response to hygiene-dietetic measured for patients with familial hypercholesterolemia.

In order to demonstrate this hypothesis, we conducted a prospective, longitudinal study focused on familial hypercholesterolemia, including innovative components for this subject in our country. As far as we know it is the only study in Romania that includes genetic testing and the description of mutations identified for familial hypercholesterolemia for pediatric patients. We selected 20 patients that underwent genetic testing for familial hypercholesterolemia. Eight of them had mutations involved in the ethiopathogeny of familial hypercholesterolemia. These mutations were located in the genes coding for the receptor for low density lipoproteins (LDLR), apolipoprotein B (APOB) and protein convertase subtilisin/kexin type 9 (PCSK9).

The patients selected for genetic testing received recommendations for hygieno-dietetic measures and were evaluated for their response to these measures. The ones that had negative genetic testing had higher decreases of total cholesterol and LDL-cholesterol levels when compared with the ones with positive genetic testing.

This thesis includes two case presentations. The first case, a girl, was diagnosed with hereditary dyslipidemia type V and was a challenging case for the medical team taking into consideration the extreme values of her lipid panel as well as the difficulties in her management due to her young age (she was 4 months old). The second case presented is of a young boy diagnosed with heterozygous compound familial hypercholesterolemia, who, due to his high LDL-cholesterol levels required off-label treatment with statins, before the age they are approved for by the European Medicines Agency.

Treatment of dyslipidemia starts and is based on hygieno-dietetic measures and lifestyle changes. In certain situations, medical treatment is required. Medical treatment options for patients with dyslipidemia include statins, PCSK9-inhibitors, and monoclonal antibodies. Recent data, demonstrate that initiating treatment with statins in young individuals under 18

years of age diagnosed with familial hypercholesterolemia reduces the risk for cardiovascular disease in adulthood [1].

Approaching dyslipidemia in pediatric patients, in a preventive manner, can improve their quality of life by preventing cardiovascular events for which they have high risk. A screening program for familial hypercholesterolemia could help avoid 46 heart attacks, 50 cases of angina, 8 cerebral strokes and 16 deaths for every 1000 persons tested over a 20 years timeframe [2].

The studies included in this thesis have certain limitations. One of these is the high cost of genetic testing in our country. This is why a limited number of patients could have the benefice of molecular diagnosis. Further, larger studies are required, on a larger geographic area in order to describe appropriately the mutations responsible for the ethyopathogeny of familial hypercholesterolemia in Romania.

This doctoral thesis brings additional information regarding dyslipidemia secondary to certain treatments or other diseases. It also brings new information for the molecular diagnosis of patients with familial hypercholesterolemia and specifically the mutations identified in pediatric patients in our country, information that could contribute to starting a national screening program for dyslipidemia in pediatric patients and a registry for patients with familial hypercholesterolemia.

I. General part

1. Dyslipidemia

Dyslipidemia is defined by high plasmatic values for total cholesterol and/or triglycerides (TG) or low plasmatic values of high-density lipoproteins (HDL-cholesterol). The effect of dyslipidemia on long term is atherosclerosis [3].

The association of risk factor in childhood and cardiovascular disease in adulthood was recently demonstrated [4, 5]. Certain risk factors including hyperlipemia, hypertriglyceridemia, hypertension and obesity in young age can accelerate the atherosclerosis process [6].

There are four classes of dyslipidemia in pediatric patients: dyslipidemia due to lifestyle, dyslipidemia as a secondary effect of medical treatments, genetic dyslipidemia and secondary to other diseases [7, 8].

2. Familial hypercholesterolemia

Familial hypercholesterolemia (FH) is a frequent genetic disorder (estimated prevalence in the USA is 1:220 persons). The EUROASPIRE-IV study that investigated FH prevalence using Dutch Lipid Clinic Network criteria in 24 countries estimates the prevalence of FH between 8,3%-20% in patients aged less than 50 years, with large variations from one country to anothers (3,4% in Finland vs. 20,8% in Bosnia Herzegovina) [9]. When it is not diagnosed and treated FH leads to heart attacks in 50% of men before the age of 50 years and 30% of women before the age of 60 years [10].

Most patients are asymptomatic in young age and are usually identified at the time of the cardiovascular event, when it was observed that the coronary arteries are affected more than peripheral or cerebral arteries [11]. Organizations such European Atherosclerosis Society and American Academy of Pediatrics recommend universal screening for children in order to identify individuals at risk for early cardiovascular disease [1, 12–14].

Screening for FH is accessible for family physicians and pediatricians because it includes questions regarding family history (family history of cardiovascular events such as heart attack or stroke at young age) and determining plasmatic level of total cholesterol and LDL-cholesterol

[15, 16]. Plasmatic values of LDL-cholesterol suggestive for FH vary between authors from 140 mg/dl to 190 mg/dL [15, 17–19].

2.1. Familial hypercholesterolemia physiopathology

FH is determined by mutations in genes coding key proteins involved in endocytosis and recycling pathways of the LDL-cholesterol receptor (LDLR). These mutations led to high LDL-cholesterol plasmatic levels [20]. High LDL-cholesterol levels lead to the retention of cholesterol in the arterial wall and the apparition of foam cells in arterial intima. Such early lesions determine obstructive atherosclerosis and later, angina pectoris and/or the rupture of the atheroma plaque followed by cardiovascular events such as heart attack [20].

2.2. Diagnostic protocols and algorithms for familial hypercholesterolemia

Multiple approaches regarding the ideal algorithm for identifying patients with FH have been proposed. Simon Broome Criteria and Dutch Lipid Clinic Network criteria that are used currently in adult patients for the diagnosis of FH are not applicable in children.

There is no consensus regarding the age for screening. There is also no consensus regarding starting treatment with statins which some authors recommend from the age of 8 years , others from the age of 10 years, or even starting treatment only when other cardiovascular risk factors are identified [18, 21].

2.3. Differential diagnosis

Differential diagnosis is important for differentiating FH from other disease with similar manifestations such as cerebrotendinous xanthomatosis, cholesteryl ester storage disease and sitosterolemia [22].

2.4. Genetic diagnosis

FH is transmitted in an autosomal dominant manner. There have been identified certain mutations responsible for reduced catabolism of LDL-cholesterol particles. Until now, five major mutations have been reported: APOB, APOE, LDLR, PCSK9 and STAP1 [23]. Worldwide, there have been documented over 1200 LDLR mutations that have an impact on the functional domains of the receptor for LDL-cholesterol. Heterozygous mutations for LDLR are identified in approximately 90% of familial hypercholesterolemia cases while APOB mutations

are identified in approximately 5% of cases and PCSK9 mutations in approximately 1% of cases. There prevalence of the mutations has geographic variation [20].

Identifying the genetic mutation causing FH confirms diagnosis. Genetic diagnosis is essential for cascade screening that allows for confirmation or invalidation of FH in aother family members [24].

2.5. Cost-efficacity of national screening programs

The benefit for health and cost-efficacity of national screening program are negatively correlated with the age at diagnosis [25]. A study from Spain estimates that the incremental cost-efficacity ratio is 26.792€ per avoided coronary event and 111.567€ per avoided death [2].

2.6. Familial hypercholesterolemia treatment

Familial hypercholesterolemia requires life-long treatment for the patients [17].

Treatment of FH in mild to medium forms is based on hygieno-dietetic measures. Multiple studies have confirmed that risk factors identified in adults with atherosclerosis contribute to atherogenesis in children. Among these risk factors are: high levels of LDL-cholesterol (characteristic for FH), obesity, hypertension, second-hand smoking, diabetes and others [26, 27].

2.6.1. Non-pharmacologic management

Treatment options for FH depend on the patient's age and LDL-cholesterol levels. Patients with FH require counseling regarding lifestyle changes. Interventions on lifestyle include a low-fat diet, physical activity, quitting smoking, and continues even if medical treatment is initiated [18].

2.6.2. Pharmacologic management

According to European guidelines treatment with statins is recommended in pediattric age patients starting the age of 10 years, along with lifestyle changes [28]. In Romania, according to the National Medication and Medical Devices Agency, rosuvastatin is approved for use in pediatric patients from the age of 6 years.

If statin treatment is proven to be inefficient, increasing the dose, changing the treatment with another medication from the same class, adding another medication to the treatment with the same effect but different action mechanism are all viable choices [29].

Other treatment option available for pediatric patients are cholesterol absorption inhibitors (ezetimibe), ion changing resins (cholestyramine)[29]. Lately, progress has been made regarding other medication used to lower cholesterol levels. PCSK-9 inhibitors (evolocumab, alirocumab) can help lower LDL-cholesterol values by 50-60% when associated with statins treatment [19, 30, 31].

Optimizing FH treatment for children and adolescents entails a multidisciplinary team that should include the family physician, the pediatrician and the continuation of care towards adult-care services [32].

II. Personal contribution

3. Working hypothesis and general objectives

This research with the title Familial dyslipidemia, clinical, therapeutic, and genetic considerations in pediatric patients is based on the theory that individuals with a particularly genetic heritage, exposed to certain environmental factors have higher chances of developing certain disease and respond differently to treatment.

Initial research starts with two clinical cases that are the fundament for the authors interest for this pathology. Both cases are rare, severe and were challenging for the medical team in terms of management. By presenting these cases, certain particularities of patients with primary dyslipidemia are highlighted.

The first study, a descriptive transversal study, aims to approach all types of dyslipidemia identified in a pediatric hospital. Objectives include describing the diagnosis of patients with dyslipidemia and evaluating atherogenic risk for them by using certain indexes (Atherogenic Index of Plasma, Castelli I and II indexes).

The second study, a prospective interventional study, is centered on familial hypercholesterolemia. Objectives of this study include describing certain aspects of genetic diagnosis for patients suspected of having familial hypercholesterolemia, approaching some clinical and therapeutic elements essential for the management of these patients.

4. Case presentations

4.1. Clinical case I

We present the case of a 4 month old female infant admitted at INSMC ”Alessandrescu-Rusescu” Bucharest. The patient was sent to the hospital by her family physician.

Results of her laboratory tests, especially her lipid profile were surprising: total lipids 22.390 mg/dL (normal value 400-800 mg/dL), triglycerides 18.260 mg/dL (normal value 35-160 mg/dL), LDL-cholesterol 900 mg/dL, HDL-cholesterol 20 mg/dL, cholesterol/triglyceride ratio 0,04. Hepatic function could not be evaluated (transaminases level could not be measured).

Lipid electrophoresis results: α-lipoproteins 19,9%, preβ-lipoproteins 39,3%, β-lipoproteins 39.2%, chylomicrons 1.6%. It is obvious there is an increase in preβ-lipoproteins and chylomicrons.

In this stage the following diagnosis was made hereditary dyslipidemia type V with hypercholesterolemia and hypertriglyceridemia.

In the context of these extreme lipid profile values and reduced therapeutic option (small infant, fed with human milk exclusively), hygieno-dietetic measures were initiated. On short term, in the first month the patient had two episodes of acute edematous pancreatitis. Thereafter, with hygieno-dietetic measures, her prognosis was good.

4.2. Clinical case discussions and conclusions I

Hyperlipoproteinemia type V is a rare phenotype of dyslipidemia characterized by the presents of chylomicrons in fasting plasma and extreme hypertriglyceridemia [33]. The prevalence of this type of dyslipidemia is 0,13-0,15% in adult population [34].

In this case, the measures usually used for hypertriglyceridemia were very hard to implement, the only available measure being adjusting the milk quantity and starting early diversification.

This case, investigated in the clinic before this thesis started, was marked by management difficulties. At that time, treatment options were few. It is the case that initiated the authors' interest in hereditary dyslipidemia.

4.3. Clinical case II

We present the case of a 4 years old male patient, diagnosed with familial hypercholesterolemia, that had the following lipid profile values at diagnosis: total cholesterol 942 mg/dL (normal value $\leq$ 200mg/dL [35]), LDL-cholesterol 792 mg/dL (normal value $\leq$ 130 mg/dL), HDL-cholesterol 20 mg/dL (normal value $\geq$ 40 mg/dL), total lipids 2286 mg/dL. Both parents and the child were genetically tested (Holland). Due to genetic testing he was diagnosed with compound heterozygous familial hypercholesterolemia, inheriting one mutation from his mother and one mutation from his father (mutation/genotype 1 LDLR:C201X, exon 4; mutation/genotype 2 LDLR:G571E, exon 12). It was recommended to start treatment with statins (rosuvastatin) and ezetimibe.

He first came to INSMC ”Alessandrescu-Rusescu” Bucharest when he was 5 years old. At that time, he had already started a low-fat diet and managed to lower his lipid profile values to total cholesterol 453 mg/dL and LDL-cholesterol 401 mg/dL. In agreement with his parents off-label treatment with rosuvastatin was started. Treatment was started with a dose of 2.5 mg/day, after 4 months the dose was increased to 5 mg/day and then to 10 mg/day.

The best outcome after 2 years and 6 months of treatment was lowering total cholesterol levels with 36.4% and LDL-cholesterol with 42.39%, after the first time his rosuvastatin dosage was doubled (5mg/kg/zi). After this, his lipid panel values were stationary, reaching a plateau and then increased, which made the medical team decide to introduce ezetimibe in his treatment scheme. The patient tolerated medical treatment well without any of the side-effects mentioned in the product leaflet.

This case was published: *Statins treatment and oro-dental aspects in a case of hereditary hypercholesterolemia in a child under 6 years* in Acta Endocrinol (Buchar) (2019 Jul-Sep;15(3):378-383. doi: 10.4183/aeb.2019.378. PMID: 32010359; PMCID: PMC6992394), ISI, Impact factor 0,55 [36] .

4.4. Clinical case discussions and conclusions II

Homozygous familial hypercholesterolemia (HoFH) is rare, estimated prevalence is 1:160.000-1:300.000 individuals and it is characterize by extreme values of LDL-cholesterol, over 500 mg/dL [37]. Patients HoHF have atherosclerosis by the age of 20 years and do not survive over 30 years [38].

In our patients’ case after starting treatment with statins LDL-cholesterol decreased with 42,39%. If take into consideration his LDL-cholesterol from diagnosis, through diet and medication his LDL-cholesterol level decreased by 70,83%. Despite all this, his LDL-cholesterol level remained over the 135 mg/dL recommended target.

In conclusion, the results obtained by this patient are supported by data in literature at an international level and support the hypothesis that treatment with statins, in the short term, does not seem to have major side-effects. Further studies are necessary to evaluate long-term treatment safety of statins in patients with FH because these patients will probably need life-long treatment.

5. Retrospective transversal study

5.1. Working hypothesis and specific objectives

The working hypothesis for the retrospective transversal study was that patients with dyslipidemia have certain diseases or follow certain treatments which could influence their lipid panel values.

Specific objectives include:

- ✓ Describing lipid profile characteristics for patients evaluated in a pediatric hospital;
- ✓ Identifying patients with dyslipidemia that were evaluated in a pediatric hospital;
- ✓ Describing characteristics of patients with dyslipidemia that were evaluated in a pediatric hospital;
- ✓ Analyzing diseases that led to an increase in LDL-cholesterol levels;
- ✓ Describing the disease that could modify the lipid profile;
- ✓ Evaluating atherogenic risk in patients with high LDL-cholesterol values;
- ✓ Selection of patients suspected to have familial hypercholesterolemia.

5.2. Materials and methods

We conducted a transversal, retrospective, descriptive study. We evaluated total cholesterol, LDL-cholesterol, HDL-cholesterol, triglyceride, and lipid levels for consecutive patients in the timeframe 2011-2020.

Threshold value for LDL-cholesterol was set at 130 mg/dL, according to the Pediatrics Romanian Protocols [39]. Acceptable, borderline, and abnormal values for total cholesterol total, LDL-cholesterol and triglyceride were define according to the recommendations of the Expert Pannel National Cholesterol Education Program (NCEP) [40].

Depending on LDL-cholesterol level patients were divided into two groups: first group (A) with LDL-cholesterol level lower than 130 mg/dl and the second group (B) with LDL-cholesterol ≥ 130 mg/dL.

To measure atherogenic risk in patients with high LDL-cholesterol the following indexes were calculated: Castelli I, Castelli II and the Atherogenic Index of Plasma (AIP).

Statistic was performed using Microsoft Excel, Epi Info™ and MedCalc® Statistical Software. Parametrical or non-parametrical tests used considered the significance level threshold at 0.05.

The study was approved by the ethics committee of the hospital and was conducted in accordance with the Declaration of Helsinki.

5.3. Results

5.3.1. Study cohort

For this study more than 3000 lipid panel results were evaluated dating from the timeframe 2011-2020. After eliminating duplicates (multiple evaluations for the same patient) 2413 patients were included.

Table 5.2. Mean lipid panel values by age-groups

	0–2 year (n = 613)	**3–5 year (n = 354)**	**6–8 year (n = 444)**	**9–11 year (n = 455)**	**12–14 year (n = 338)**	**15–18 year (n = 209)**
Total cholesterol (mg/dL)	146,4 (±51,9)	165,9 (±50,5)	167,3 (±44,9)	167,3 (±40,2)	156,9 (±40,1)	154.5 (±39.3)
LDL-cholesterol (mg/dL)	88,2 (±37,1)	107,0 (±43,6)	108,4 (±38,5)	111,0 (±37,2)	102,6 (±36,0)	101.8 (±37.0)
HDL-cholesterol (mg/dL)	41,3 (±15,4)	53,6 (±29,0)	55,5 (±14,1)	53,1 (±14,0)	50,0 (±14,5)	49.1 (±13.6)
Triglyceride (mg/dL)	135,4 (±101,2)	72,6 (±36,9)	81,9 (±55,6)	87,9 (±46,3)	95,5 (±54.7)	86.1 (±49.4)

5.3.2. High LDL-cholesterol cohort

From the study cohort we identified 440 patients (18,23%) with LDL-cholesterol over 130 mg/dl. The mean age of patients with high LDL-cholesterol is 7,90 years (±4.46). Almost half (47,50%) of them were female. Most of them (69,09%) were from urban areas and 44,77% from Bucharest.

Table 5.5. Diagnosis of patients with high LDL-cholesterol (over 130 mg/dL)

Diagnostic	**Percentage of patients diagnosed (n=440)**
Endocrine, nutritional**,** and metabolic disease	52,75%
• Overweight, obesity and other hyperalimentation	25,91%
• Metabolic disorders (including familial hypercholesterolemia)	11,37%
• Malnutrition	11,14%
• Disorders of the thyroid gland (hypothyroidism)	4,10%
• Diabet**es** mellitus	0,23%
Other	28,65%
Diseases of the respiratory system	3,41%
Mental, Behavioral and Neurodevelopmental disorders	3,21%
Diseases of the digestive system	2,96%
Diseases of the nervous system	2,51%
Congenital malformations, deformations, and chromosomal abnormalities	2,29%
Disease of the musculoskeletal system and connective tissue	1,59%
Diseases of the genitourinary system	0,92%
Diseases of the blood and blood-forming organs and certain disorders involving the immune mechanism	0,69%
Diseases of the eye and adnexa	0,68%
Diseases of the circulatory system	0,23%
Neoplasms	0,23%

Table 5.6. Lipid panel mean values by pathology

Diagnostic	Total cholesterol (mg/dL)	LDL-cholesterol (mg/dL)	HDL-cholesterol (mg/dL)	Triglyceride (mg/dL)
Endocrine, nutritional, and metabolic diseases	216,19 (± 48,12)	161,84 (± 41,26)	51,73 (± 15,08)	110,26 (± 63,50)
Other	216,70 (± 66,24)	155,19 (± 30,01)	57,20 (±42,74)	100,35 (± 59,51)
Diseases of the respiratory system	206,53 (± 38,47)	151,33 (± 21,00)	50,71 (± 21,11)	128,28 (± 64,90)
Mental, behavioral, and neurodevelopmental disorders	229,46 (± 46,19)	176,35 (± 38,81)	53,78 (± 12,39)	101,35 (± 58,02)
Diseases of the digestive system	224,08 (± 62,36)	166,84 (± 47,57)	52,30 (± 20,23)	80,23 (± 65,10)
Diseases of the nervous system	222,40 (± 38,12)	151,90 (± 25,06)	49,00 (± 14,14)	135,70 (± 69,84)
Congenital malformations, deformations, and chromosomal abnormalities	207,75 (± 49,52)	158,90 (± 35,50)	41,30 (± 16,85)	123,77 (± 76,08)
Diseases of the musculoskeletal system and connective tissue	211,85 (± 56,63)	158,85 (± 42,27)	50,00 (± 16,75)	75,14 (± 20,31)
Diseases of the genitourinary system	432,00 (± 114,89)	325,00 (± 111,24)	57,75 (± 6,80)	253,75 (± 287.98)
Diseases of the blood and blood-forming organs and certain disorders involving the immune mechanism	236,00 (± 22,06)	183,66 (± 39,39)	42,00 (± 2,64)	147,33 (± 38,00)
Diseases of the eye and adnexa	205,66 (± 33,82)	172,00 (± 24,87)	48,00 (± 6,00)	99,00 (± 24,02)

Diseases of the circulatory system	200,00 (NA*)	138,70 (NA*)	60,00 (NA*)	91,00 (NA*)
Neoplasms	205,00 (NA*)	148,00 (NA*)	52,00 (NA*)	185,00 (NA*)

Table 5.4. Mean values of Castelli I and II and of the Atherogenic Index of Plasma in the high-LDL-cholesterol group and normal LDL-cholesterol group

	Whole group (n=2413)	**High LDL-cholesterol (n=440)**	**Norma LDL-cholesterol (n=1973)**	**p**
Atherogenic Index of Plasma	-0,11 (± 0,33)	-0,07 (± 0,32)	-0,12 (± 0,34)	0,03
Castelli I Index	3,46 (± 3,09)	4,73 (± 6,23)	3,17 (± 1,55)	0,00
Castelli II index	2,26 (± 1,44)	3,51 (± 2,48)	1,98 (± 0,83)	0,00

*Atherogenic Index of Plasma = log(TG/HDL) ; Castelli I Index = total cholesterol /HDL-cholesterol; Castelli II Index = LDL-cholesterol/HDL-cholesterol

5.4. Discussion

Prevalence of lipid panel abnormalities in pediatric population is estimated at 8-20% [41]. In this study, 18,23% of the patients had high LDL-cholesterol (≥130 mg/dL), a high percentage when compared with the prevalence of dyslipidemia in pediatric patients reported by other studies.

In this study, 31,82% of the patients aged between 0 and 9 years had high TG levels (≥100 mg/dL), and 21,6% of the ones aged between 0 and 2 years. We can safely assume that most of the children aged 0-2 years are breast-fed or receive some kind of milk formula. In this age group, the TG plasmatic levels is often high (up to 150 - 200 mg/dL) [42–44]. This high value could be explained by the feeding particularities and timing. Infants are fed at short intervals and the probability for the blood test to have been drawn in a fasting state is low.

More than a quarter of the patients (25,91%) were diagnosed with obesity. In another recent study, that took part in the west part of Romania [45], the prevalence of overweight and obese children was estimated under 30%.

In this study 4,1% of the patients with high LDL-cholesterol were diagnosed with congenital hypothyroidism. In other studies, among patients with hypothyroidism, 30% had high total cholesterol and LDL-cholesterol while 90% had dyslipidemia [46, 47].

Results from this study were published in the Journal Medicina with the title *Dyslipidemia in Pediatric Patients: A Cross-Sectional Study,* ISI, Impact factor 2,6 [48].

5.5. Conclusions

This retrospective descriptive study supports through its results the utility and necessity for implementing a national screening program. Such a measure could reduce the cardiovascular disease burden at young ages (before 50 years) that falls on the patient because it affects his quality of life and reduces his work capacity as well as on the health system through the costs this potentially severe pathologies generate.

In our country there is no national screening program for dyslipidemia in pediatric patients. Taking this into consideration, primary dyslipidemia is underdiagnosed.

Results from this study support the working hypothesis and the expected results were confirmed, towards the objectives set.

6. Prospective study

6.1. Working hypothesis and specific objectives

This study starts from the hypothesis that patients with familial hypercholesterolemia can be identified through screening and the diagnosis can be confirmed through genetic testing.

Specific objectives included:

- ✓ identifying patients suspected to have familial hypercholesterolemia;
- ✓ identifying and describing genetic mutations identified in patients with positive genetic testing;
- ✓ evaluation of atherogenic risk for the patients included in the study;
- ✓ initiating hygieno-dietetic treatment in patients suspected to have familial hypercholesterolemia;
- ✓ determining the response to hygieno-dietetic for patients diagnosed with familial hypercholesterolemia.

6.2. Materials and methods

We conducted a prospective longitudinal study regarding the genetic testing of patients with clinical diagnosis for familial hypercholesterolemia. The study was conducted at INSMC ”Alessandrescu-Rusescu” in Bucharest. Patients were selected from the retrospective transversal study described before.

6.2.1. Patients’ selection

Inclusion criteria were age under 18 years, evaluation of the lipid profile in the timeframe 2011-2020, LDL-cholesterol value over 130 mg/dL in at least on evaluation, no other disease that could explain the high cholesterol levels, never underwent genetic testing, agreement to enter the study.

6.2.2. Clinical and paraclinical evaluation

Selected patients were evaluated from a clinical and paraclinical point of view. Clinical evaluation included measuring the weight, height, and arterial pressure. Paraclinical evaluation included collecting blood vials for evaluation of lipid profile and genetic testing. Due to financial considerations (extremely high cost of genetic testing) only 20 genetic tests were available. Genetic testing took place at Regional Genetic Testing Center Dolj.

For each patient, the body-mass-index (BMI) was calculated. For the atherogenic risk we calculated Castelli I, Castelli II Indexes, the Atherogenic Index of Plasma (AIP) and ApoB/ApoA ratio.

At the initial visit the patients received counseling for lifestyle changes and maintaining these changes. After a year from the initial visit the patients were invited for reevaluation.

For every patient included in this study informed consent was obtained. The study was approved by the ethics committee of the hospital and was conducted in accordance with the Declaration of Helsinki.

6.2.3. Genetic testing method

For genetic testing TruSigh Cardio enrichment kit was used. The genetic testing was evaluated, optimized, and evaluated at Reginal Medical Genetic Center Dolj.

6.2.4. Dietetic recommendations

Dietetic recommendations were elaborated from several bibliographic sources [8, 49–51]. It was recommended to approach these changes inclusive, as a family, in order to avoid the child feeling punished.

6.2.5. Statistical processing

The study group was divided in two: group A – negative genetic testing for FH and group B – positive genetic testing for FH. The result for genetic testing was considered positive if a mutation was pathogenic or VUS (variant of unknown significance). Statistic was performed using Microsoft Excel, Epi Info™, and MedCalc®. Parametrical or non-parametrical tests used considered the significance level threshold at 0.05.

6.3. Results

Tabel 6.1. Mutations identified in patients with familial hypercholesterolemia

Case no.	FH	Gena	Variant		Variant type
#4	Type 2 OMIM #144010 AD*	APOB	NM_000384.3:c.10580G>A rs5742904 (p.Arg3527Gln)	Heterozygote	Probable pathogenic
#3	Type 2 OMIM #144010 AD	APOB	NM_000384.3:c.12443_12444delinsAA rs1558559244 (p.Ala4148Glu)	Heterozygote	VUS*
#2	Type 1 OMIM #143890 AR*/AD	LDLR	NM_000527.5:c.1618G>A rs769370816 (p.Ala540Thr)	Heterozygote	Pathogenic
#6	Tip 1 OMIM #143890 AD/AR	LDLR	NM_000527.5:c.1775G>A rs137929307 (p.Gly592Glu)	Heterozygote	Pathogenic
#7	Tip 1 OMIM #143890 AD/AR	LDLR	NM_000527.5:c.502G>A rs200727689 (p.Asp168Asn)	Heterozygote	Pathogenic
#8	Tip 1 OMIM #143890 AD/AR	LDLR	NM_000527.5:c.81C>G rs2228671 (p.Cys27Trp)	Heterozygote	Probable pathogenic
#1	Tip 3 OMIM #603776 AD	PCSK 9	NM_174936.4:c.836C>T rs1049662014 (p.Pro279Leu)	Heterozygote	VUS
#5	Tip 3 OMIM #603776 AD	PCSK 9	NM_174936.4:c.836C>T rs1049662014 (p.Pro279Leu)	Heterozygote	VUS

*AD : autosomal dominant; AR autosomal recessive; VUS variant of uncertain significance

In this study 20 patients were genetically tested for familial hypercholesterolemia. The mutations identified are presented in table 6.1.

Ten patients came for reevaluation, 4 from the genetic negative group and 6 from the genetic positive group. In the genetic negative group, after initiation of hygieno-dietetic treatment and maintaining it for 1 year, the total cholesterol level decreased by 13,34% while for the patients with positive genetic testing the decrease was of only 7,93%. In the genetic negative group, after initiation of hygieno-dietetic treatment, LDL-cholesterol decreased by 18,5% while for the patients with positive genetic testing the decrease of LDL-cholesterol was insignificant.

Tabel 6.3. Mean lipid panel values for the entire study group and two subgroups (positive genetic testing and negative genetic testing).

	Entire group (n=20)	**Negative genetic testing (n=12)**	**Positive genetic testing (n=8)**	**p**
Colesterol total	224,35 mg/dL (± 30,75)	213,16 mg/dL (± 21,31)	241,12 mg/dL (± 36,29)	0,07
LDL-cholesterol	154,25 mg/dL (± 26,79)	147,00 mg/dL (± 18,98)	165,12 mg/dL (± 34,01)	0,24
HDL-cholesterol	59,55 mg/dL (± 17,69)	54,41 mg/dL (± 12,51)	67,25 mg/dL (± 22,14)	0,16
ApoA	149,05 mg/dl (±25,53)	139,30 mg/dL (± 21,72)	161,25 mg/dL (± 32,64)	0,14
ApoB	117,77 mg/dl (±17,54)	114,40 mg/dL (± 10,80)	122,00 mg/dL (± 23,68)	0,39

Table 6.4. Mean values for Castelli I and II Indexes and the Atherogenic Index of Plasma in the study group and the two subgroups

	Study group (n=20)	**Negative genetic testing (n=12)**	**Positive genetic testing (n=8)**	**p**
Atherogenic Index of Plasma	-0,24 (± 0,27)	-0,18 (± 0,28)	-0,32 (± 0,32)	0,30
Castelli I Index	4,02 (± 1,09)	4,11 (± 1,05)	3,89 (± 1,20)	0,66
Castelli II Index	2,82 (± 0,98)	2,87 (± 0,92)	2,74 (± 1,13)	0,78
ApoB/ApoA ratio	0,82 (± 0,20)	0,84 (± 0,17)	0,79 (± 0,25)	0,63

* Atherogenic Index of Plasma = log(TG/HDL) ; Castelli I Index = total cholesterol /HDL-cholesterol; Castelli II Index = LDL-cholesterol/HDL-cholesterol

6.4. Discussions

FH is characterized by high LDL-cholesterol since birth, which, in time, leads to an increased risk for atherosclerotic cardiovascular disease. Despite all information accumulated about FH it remains underdiagnosed and undertreated, being marked by high mortality and morbidity due to atherosclerotic cardiovascular disease [52].

From a clinical point of view and looking at the lipid profile of patients with positive and negative genetic testing for FH the differences were not statistically significant. However, there has been a higher decrease of LDL-cholesterol levels after hygieno-dietetic measures in patients with negative genetic testing, therefore a better outcome.

This study is among the first ones in Romania that aims to contribute to the early diagnosis of FH and reduce significantly the morbidity and mortality that these patients have because of atherosclerotic cardiovascular disease. In this study Next Generation Sequencing (NGS) was used to identify genetic mutations in patients with clinical clues for FH. As far as we know there is no other Romanian study describing genetic mutations associated with FH in pediatric patients.

In this study the detection rate was 40%, 8 patients with mutations identified, 5 with pathogenic/probable pathogenic mutations and 3 with variants of uncertain significance. The pathogenic/probable pathogenic variants were mostly on LDLR gene. In this study 2 patients had APOB mutations, one classified as VUS and one probable pathogenic. In this study there were no new mutations identified, all the mutations being described before.

The lack of genetic studies for FH in our country does not allow us to correlate the results geographically. We consider that additional studies are required, including more patients, extending on a larger area, in order to identify mutation responsible for FH phenotype in Romania.

This study has certain limitations. The number of patients included is very small due to the high costs of genetic testing. Also, this study was conducted in a single center and can not be generalized for the country or region.

Results from this study were published in the journal Diagnostics, with the title *Genetic Testing for Familial Hypercholesterolemia in a Pediatric Group: A Romanian Showcase*, ISI, impact factor 3,6. [53].

6.5. Conclusions

In this study 8 out of 20 patients were genetically tested positive for FH. Most of them were on LDLR gene but there were also mutations in APOB and PCSK9 genes. Several variants have been reported in other studies (LDLR c.1618G>A, LDLR c.1775G>A).

Evaluating the lipid profile is the first step towards clinical diagnosis for FH and it is universally available, at low costs. Genetic testing remains, however the gold standard for FH diagnosis [54].

Additional studies, on larger patient groups are needed, aiming the genetic spectrum of FH in Romania, and to identify indexes appropriated for evaluating atherosclerotic risk in these patients.

Results from this study support the working hypothesis and the expected results were confirmed, towards the objectives set.

7. Conclusions and personal contribution

This doctoral thesis, with the title ”Familial dyslipidemia, clinical, therapeutic and genetic considerations in pediatric patients”, aims to be an alarm sign for a massively underdiagnosed disease not only in our country but worldwide. Risk factors for cardiovascular disease (obesity, sedentarism etc.) can be present since childhood and through prolonged exposure can expedite the apparition of cardiovascular events.

This doctoral thesis has achieved its goals. Studies conducted in thesis were published in prestigious journals, with a cumulative impact factor of 6,75, representing an important source of information regarding dyslipidemia for the international academic community.

The study of identified genetic mutations for children with clinical suspicion for FH is as far as we know the first study of its kind in Romania in pediatric patients and it lays the foundation for a better knowledge of genetic characteristic of familial hypercholesterolemia in Romania. Therefore, these results contribute to the augmentation of knowledge on familial hypercholesterolemia and genotype-phenotype correlation.

Based on the data included in this doctoral thesis the following conclusions can pe drawn:

1. Dyslipidemia, no matter the cause (genetic, secondary, induced by medication) increases the risk for cardiovascular disease. There are multiple conditions that are accompanied by dyslipidemia, frog genetic diseases (Down Syndrome, Prader-Willi syndrome) to congenital hypothyroidism. These patients require supplementary care and a multidisciplinary approach.

2. Genetic dyslipidemias are frequent. Familial hypercholesterolemia (FH), although rare in homozygous form, is quite frequent in heterozygous form.
3. Patients with FH do not usually have, in heterozygous form, clinical sighs that would draw attention in clinical examination. None of the patients included in this thesis, diagnosed through genetic testing with heterozygous FH had any distinctive specific clinical signs.
4. Confirming diagnosis through genetic testing is important in the management of patients with FH because, sometimes, multiple cholesterol-lowering agents are required.
5. Management of patients with dyslipidemia is difficult especially at young age (infants) and during their lifetime because of the complications (heart attack, stroke, acute pancreatitis) requiring a multidisciplinary team.
6. Prevention measures such as universal screening and counseling the family of the patients with dyslipidemia and especially FH cand make a difference in their quality of life and reduce the costs that the complication of FH generate for the healthcare system.

Perspective directions include using the data obtained through these studies as solid arguments towards implementing a national screening program for dyslipidemia in children.

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(Chapter 4, Subchapter 4.3. and Subchapter 4.4., pages 43-49)

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