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ENDOTHELIUM-DEPENDENT VASODILATION AND SCREENING FOR MOLECULAR DEFECTS OF THE LDL-R GENE IN BULGARIANS WITH UNTREATED AYMPTOMATIC SEVERE HYPERCHOLESTEROLEMIA

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SUMMARY

Background: Severe hypercholesterolemia and family history of early vascular diseases are important determinants of the development of the endothelium dependent vasodilation in familial hypercholesterolemia. Mutations of the LDL-R-gene (low-density lipoprotein receptor gene) are characterized by a high genetic heterogenicity. Nearly 80% of the population specific heterogenicity are due to point mutations along the LDL-R gene. The different expression of the defect gene in LDL-R mutation carriers as well as the presence of elevated LDL levels in non-mutation carriers makes diagnosis difficult. So far, there is no optimal diagnostic algorithm in familial hypercholesterolemia. **Objective** To study the endothelium-dependent vasodilation (flow-mediated vasodilation) in carriers and noncarriers of LDL-R defective gene and utilize them to screen for defects in the LDL receptor (spot mutations and polymorphisms) in severe hypercholesterolemia (HC) in molecular biological analysis. Study was conducted in patients with diagnosed and presumed familial hypercholesterolemia according to Simon-Broome criteria. **Materials and methods:** 464 first relatives of families with familial hypercholesterolemia were tested, of them 120 meet Simon-Broome inclusion criteria. Total cholesterol, triglycerides, cholesterol of high density lipoproteins, cholesterol of low density lipoproteins are determined, using the enzyme- colorimetric method. Molecular analysis included: DNA isolation, amplification of a target DNA fragment by polymerase chain reaction, Single Strand Conformation Polymorphysm (SSCP) and direct DNA sequencing. The Hewlett Packard SONOS 5500 with a 7.5 MHz triplex transducer and the MedicaSoft.IMT lab software packet were used to determine the flow-mediated vasodilation. **Results:** According to the presence or absence of genetic mutations, patients were assigned into two groups - carriers of LDL-R gene point mutations - 22 (18.33%) patients - of them 16 (13.33%) were carriers of an unknown mutation (not registered in the available data bases-1073G>A) and non-carriers - 98 patients - 81.67%. All 18 exons of the LDL-R gene were tested. Median age of non-carriers is 43.41±0.43 years, that of carriers –45.40±0.27 years ($p<0.001$). There is no statistically significant difference in the patients' distribution according to sex ($\chi^2=0.06$; $p>0.05$). There is not statistical significant difference on all biomarkers of the atherogenic risk between two group ($p>0.05$), as for flow-mediated vasodilation ($p>0.05$). **Conclusion:** Endothelium-dependent vasodilation (flow-mediated vasodilation) are not an indicator of the presence of point mutations and polymorphisms of the LDL-R gene.

Key words: hypercholesterolemia, low-density lipoprotein, LDL-R, flow-mediated vasodilation, apolipoproteins,

Familial hypercholesterolemia (HH) is one of the solved genetic reasons for coronary artery disease.[1,2] It is a potential mortality and it's caused from defectly endocytosis of the LDL-cholesterol from LDL-R.[3-8] Mutations of the LDL-R-gene (low-density lipoprotein receptor gene) are characterized by a high genetic heterogenicity. Nearly 80% of the population specific heterogenicity are due to point mutations along the LDL-R gene. This fact requires genetic scanning of all exons. The different expression of the defect gene in LDL-R mutation carriers as well as the presence of elevated LDL levels in non-mutation carriers makes diagnosis difficult.[8] So far, there is no optimal diagnostic algorithm in clinical diagnosis familial hypercholesterolemia.[9] Research efforts are focused on finding solutions that would facilitate everyday clinical practice. The datas of the research in the literature, as previous our researches show that routin lipid profile has a small amount of information to distinguish the difference of the non-carriers versus carriers on the defect of the LDL-R.[9-14] Severe hypercholesterolemia and family history of early vascular diseases are important determinants of the development of the flow-mediated vasodilation (%FMD) in familial hypercholesterolemia.[15,16] There are small datas in the literature for the importance of the non-invasive vessel researches, expecially flow-mediated vasodilation, as direct steps to the screening for the molecular biological analysis

in patients with clinical diagnosis familial hypercholesterolemia.[15,16] In Bulgaria, studies of severe familial hypercholesterolemia are carried out mainly in patients with manifested coronary artery disease, data for patients with asymptomatic hypercholesterolemia are scarce.[9-14]

AIM

To study the endothelium-dependent vasodilation (flow-mediated vasodilation) in carriers and noncarriers of LDL-R defective gene and utilize them to screen for defects in the LDL receptor (spot mutations and polymorphisms) in severe hypercholesterolemia (HC) in molecular biological analysis.

PATIENTS AND METHODS

Between June 2003 and September 2006 a total of 460 patients with primary HC were examined in the Preventive Cardiology Surgery of the Clinic of Cardiology, St George University Hospital, Plovdiv. Of these, only 120 (age over 16 years) met the Simon-Broome Register criteria and were included in the study (Table 1).[17] We performed the following cardiologic, laboratory and instrumental studies: cardiologic screening including clinical history, hospital based investigations of blood pressure (ERKAMETAR 3000, Germany), ECG (Hellige EK-56), echocardiography (Hewlett-Packard SONOS 5500), 24-hour Holter ECG (Innomed Medical INC), stress echocardiography (Hellige EK-56) and complete blood and urine analysis. Laboratory tests were performed at the Central Clinical Laboratory of St George University Hospital, Plovdiv. The biochemical parameters of blood glucose, total cholesterol, triglycerides (TG), high density lipoprotein cholesterol, urea, creatinine, and uric acid were measured using a biochemical analyzer Konelab 60i (Thermo Electron Co, USA). Creatinine clearance was calculated using the Cockcroft-Gault formula [140 – Age x Mass (kg) x 0.85 if female/72 x serum creatinine]. Plasma concentrations of fibrinogen were determined by the Clause method. Determination of LDL serum cholesterol was performed using a direct analysis and reagents from Thermo Electron Co KonelabTM (Finland).

MOLECULAR BIOLOGICAL ANALYSIS.

The present study included 120 DNA samples of FH patients of both genders.[18-20] Samples of high-molecular DNA isolated from nuclear blood cells were used as material for the genetic analysis. The blood samples were withdrawn 30 min to 1 hour after meals in plastic tubes with EDTA anticoagulant. They were stored at +4°C for 48 hours. The technique included several stages: 1. Isolation of DNA; 2. Amplification of a specific target of DNA fragment using polymerase chain reaction; 3. A single strand conformation polymorphism SSCP analysis; 4. Direct sequencing.

Determination of flow mediated vasodilation of brachial artery was performed based on Celermajer's recommendation (1992) and on %FMD manual book (2002).[21,22] The diameter of the brachial artery was measured with a 7.5 MHz transducer of Hewlett Packard 5 500, using automated computer software MedicaSoft. IMT. lab. A marker is placed at the starting point (the leading margin of the intima-lumen surface of the proximal wall) and at the end point (10 mm from the starting point). The diameter is measured automatically to the distal intima- lumen wall at the same distance. The percentage variation in the diameter of brachial artery was determined following 5-min compression with the cuff of the blood pressure monitor up to 50 mmHg above the measured systolic blood pressure. The non-dominant arm was used. The examination was performed at a room temperature of 22-24°C. The subjects had fasted for 8-12 hours, and were advised to abstain from coffee, vitamin C and vasoactive drugs.

Ten minutes after determination of %FMD, Nitroglycerine (NTG) mediated vasodilation was achieved by administration of 0.4 mg NTG. After 4 minutes, non-endothelium dependent vasodilation was determined.

Variation analysis, Student's t-criterion and analysis of covariance (ANCOVA) were used in the statistical analysis. Results were expressed as the mean ± standard deviation (SD). $p<0.05$ was used as a level of significance of the null hypothesis. Statistical analysis was carried out using the SPSS v.11.0 statistical software (SPSS Inc. Chicago, III).

Prior to the study procedures a written informed consent was obtained from patients and controls. The procedures used in this study were approved by the Ethics Committee at Medical University of Plovdiv.

Table 1. Inclusion and exclusion criteria

Inclusion criteria	Exclusion criteria
1. Total cholesterol level above 7.5 mmol/l in people over 16 years of age.	1. Diabetes mellitus, impaired glucose tolerance or fasting blood glucose > 5.6 mmol/l.
2. Tendon xanthomata in first or second degree relatives.	2. Cigarette smoking.
3. A myocardial infarction before age 60 in first degree relatives and before age 50 in second degree relatives.	3. Clinical and laboratory evidence of:
4. Total cholesterol above 7.5 mmol/l in first or second degree relatives.	3.1. Coronary artery disease (CAD) in all forms
A diagnosis of definite FH requires meeting criteria 1 and 2.	3.2. Cerebrovascular disease
	3.3. Arterial hypertension
	3.4. COPD, bronchial asthma

A diagnosis of possible FH requires meeting criteria 1 and 3 or 1 and 4.	3.5. Chronic arterial insufficiency of the extremities (peripheral arteries) – ABI < 0.9 3.6. Chronic renal and hepatic dysfunction 3.7. Systemic disorders of connective tissues – collagenosis, rheumatoid arthritis, SLE 3.8. Neoplasms 3.9. Acute inflammation or chronic inflammatory process requiring active treatment. 4. Long-term use of NSAID (over the last six months and during the period of investigation), corticosteroids, hormonal medications, psychotropic drugs, lipid modifying drugs (fibrates, statins, antioxidants). 4.1. Chronic use of alcohol and drug abuse.

RESULTS

1. Characteristics of the studied population. There was a statistically significant difference ($p<0.001$) in the age distribution of patients, carriers and non-carriers of molecular defects. This conclusion was confirmed by the calculated mean age in both patient groups. The mean age of non-carriers was 45.41 ± 0.43 years, the mean age of carriers - 48.40 ± 0.67 years. The two patient groups were compared with respect to the their sex distribution using the Pearson's criterion. There were no statistical differences in the sex distribution in the study sample ($\chi 2 = 0.06$; $p>0.05$) and therefore it was excluded from further analysis.

2. Molecular biological analysis - according to whether there were or were not molecular defects, patients were assigned to two groups: carriers (22 patients, 18.33%) and non-carriers (98 patients, 81.67%). At first we screened the apo B gene for R3500Q mutation in the patients as it is well known that even a single mutation can lead to a phenotype variation. The most common family defect in Apo-B is caused by a substitution mutation at nucleotide position 10 780, which results in replacement of Arg in the defected polypeptide chain by Gln at position 3500. No mutation of this type, with reported incidence of only 3.1% in Bulgarian population, was found in the studied contingent, in patients with TC above 7.0 mmol/l. This results are most probably due to the small sample size. The second stage included testing for spot mutations in the LDLR gene. According to literature data, their frequency is higher (87.6%). We screened all 18 exons of the LDLR gene (apart from the recommended analysis of exons 6, 4 and 9).

3. Biomarkers of the atherogenic risk in the examined group. Higher values of total cholesterol (non-carriers -8.55±0.14 mmol/l, carriers - 9.54±0.47 mmol/l - $p>0.05$ and LDL-cholesterol (6.77±1.29 mmol/l, versus 7.42±0.04 mmol/l - $p>0.05$) were established in carriers, but the difference was not statistically insignificant. Lower HDL- cholesterol level in carriers, compared to non carriers was found (non-carriers - 1.01±0.24 mmol/l, carriers - 0.95±0.02 mmol/l, $p>0.05$), the difference being not statistically significant. Similar results were established for triglycerides (non-carriers - 0.97±0.09 mmol/l, versus carriers – 0.94±0.26 mmol/l ($p>0.05$).

4. Endothelium-dependent and independent vasodilatation in the examined groups. Higher values of %FMD (Table 2) were established in non-carriers, but the difference was not statistically significant ($p>0.05$). There isn't statistical significant difference between carried and non-carried in respect of the nitroglycerin-mediated endothelium-independent vasodilation (Table 2).

Table 2. Endothelial dependent and independent vasodilatation in the examined groups

Parameters		N	mean ± SEM	SD	u	P
Flow-mediated vasodilation (%FMD)						
	Non carriers	98	4.47±0.58	1.23	0.7	> 0.05
	Carriers	22	4.24±0.55	0.98	1.1	
Nitroglycerin -mediated vasodilation (%)						
	Non carriers	98	16.41 ± 0.67	0.06	- 1.83	> 0.05
	Carriers	22	16.13 ± 1.05	0.10		

DISCUSSION

By literature datas cholesterol-dependent endothelial dysfunction is connected to LDL-oxidation, but not with LDL–concentration. [1,15,16] %FMD significantly and directly correlated with coronary risk and it increases, when % FMD lowers. HH increases the answer to vasoconstrictor's agonists and leads to disturbance in the endothelium-

dependent vasodilation. [15,16] The all patients with HH (with or without spot mutations and polymorphisms of the LDL-R) we have discovered a disturbed %FMD (<7%), which we connect with the fact, that HH leads to lowering of the endothelial NO, respectively lowered endothelium-dependent vasodilation. The mean values of the %FMD in the two examined groups don't distinguish significantly: in the group of the non-carriers - 4.47±0.58%, and this of the carriers are 4.24±0.55%. The lack of the difference between the two investigation groups, with or without molecular defect, it's probably because of the fact, that it shows functional abnormality of vascular wall, tht's shows the real – in this moment the situation of the vessel, which can to sirvaved a different in the hours with all of the power and difect of this example of the imitation. [15,16] The lack of the significant difference in the phenotype manifestation, assessment through %FMD between group of the affecting and non-affecting patients with FH, is probable responsible for the influence of the factors of the surrounding.

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