European Journal of Human Genetics (2001) 9, 836–842
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www.nature.com/ejhg

ARTICLE

Familial defective apolipoprotein B-100 in a group of hypercholesterolaemic patients in Poland. Identification of a new mutation $Thr_{3492}Ile$ in the apolipoprotein B gene

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The prevalence of the familial defective apolipoprotein B-100 (FDB) $Arg_{3500}Gln$ mutation in 525 unrelated hypercholesterolaemic Polish subjects was evaluated. DNA samples were screened for FDB mutation using SSCP method. Presence of mutation was confirmed using a mismatch MspI PCR strategy. Plasma lipid levels and clinical characteristics of 13 patients identified as carriers of the mutation and of their 23 affected relatives were analysed and compared with non-affected ones. In the affected individuals a variable expression of lipid concentrations and of atherosclerosis symptoms were observed. The prevalence of FDB $Arg_{3500}Gln$ mutation in hypercholesterolaemic Polish subjects (3.7%) seems to be similar to the frequency reported in other Caucasian hypercholesterolaemic populations. The estimated prevalence of the mutation in general Polish population is relatively high being 1/250. The same haplotype at the apoB locus in the carriers of this mutation in Poland as in other populations from Western Europe suggests its common origin. In one hypercholesterolaemic subject a non-hitherto described mutation was identified. It consisted in C→T transition in apoB codon 3492 leading to threonine to isoleucine substitution in 3492 position of apoB gene ($Thr_{3492}Ile$). *European Journal of Human Genetics* (2001) **9,** 836–842.

Keywords: Familial defective apolipoprotein B-100 (FDB); prevalence in Poland; new mutation

Introduction

Familial defective apolipoprotein B-100 (FDB) is an autosomal dominantly inherited genetic disease first described by Innerarity *et al*[1] and characterised by hypercholesterolaemia and premature atherosclerosis.

Apo B-100 is the only protein of low density lipoproteins (LDL) and its binding to the LDL receptor is essential for the normal removal of LDL from plasma. The disorder is characterised by clinical and lipoprotein abnormalities very similar to familial hypercholesterolaemia caused by the LDL receptor deficiency.[2,3] Recently numerous authors report that the symptoms in FDB are less severe than in disease caused by LDL receptor mutations.[4–7]

The most common mutation in FDB is an adenine for guanine substitution in complementary DNA of exon 26 of apo B gene. It results in an arginine for glutamine change in the 3500 amino acid position of the protein.[8] The mutation

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Received 27 July 2001; revised 16 August 2001; accepted 19 August 2001

strongly reduces protein binding by the receptor. The occurrence of other kinds of mutations was also reported – most of them very rare. They are either localised in the same 3500 site – ($Arg_{3500}Trp$)[9]– or in its close proximity ($Arg_{3531}Cys$).[10]

FDB has been identified basing on DNA analysis in Caucasian populations of North America and in numerous European countries with a frequency ranging from 1:210 to 1:1250. It was however not found in Finland, Spain, Russia and Japan.[11–14]

The vast majority of the individuals carrying the apo B $Arg_{3500}Gln$ mutation has an identical apo B haplotype which strongly suggests that the mutation occurred on a single ancestral gene.[11,15] Only a few independent mutations ie, accompanied by other haplotypes were described.[14]

Five families carrying the apo B Arg3500Gln mutation were recently described in Poland.[16]

In the present study the frequency of the FDB $Arg_{3500}Gln$ mutation in 525 unrelated hypercholesterolemic Polish subjects was evaluated.

Subjects

Five hundred and twenty-five unrelated patients (309 men and 216 women) with moderate and severe hypercholesterolaemia (LDL cholesterol $\geqslant$160 mg/dl), aged 20–82 years (mean age 57.5$\pm$9.7 years) recruited from the outpatient lipid clinic of the National Institute of Cardiology in Warsaw, Poland, were screened by single-strand conformation polymorphism (SSCP) analysis for the presence of FDB mutation. 351 (206 men and 145 women) had primary type IIA hyperlipoproteinaemia (HLP IIA) (triglycerides $<$200 mg/dl) and 174 of them (103 men and 71 women) had type IIB hyperlipoproteinaemia (HLP IIB) (triglycerides $\geqslant$200 mg/dl but $<$400 mg/dl). Sixty-five patients (42 men and 23 women) from HLP IIA group could be diagnosed as familial hypercholesterolaemia (FH_{clin}) basing on the clinical criteria according to Study Group European Atherosclerosis Society[17] ie, LDL cholesterol level above 190 mg/dl and the presence of tendon xanthomas in the patient or in a first-degree relative.

Seventy available relatives and 12 spouses of identified probands were subsequently investigated.

The study was approved by the Ethics Committee of National Institute of Cardiology. All the participants gave their informed consent for the investigation.

Methods

Blood samples were obtained after an overnight fast. Lipid levels were determined either before starting hypolipaemic therapy or after the period of at least 6 weeks without treatment. Total serum cholesterol (TC) and triglycerides (TG) were determined by enzymatic assays (Boehringer Mannheim). High density lipoprotein cholesterol (HDL-C) was determined after precipitation of apo B-100 containing lipoproteins. LDL cholesterol (LDL-C) concentration was calculated according to Friedewald formula. Lipoprotein(a) [Lp(a)] concentration was measured by electroimmunodiffusion (Sebia).

Genomic DNA was obtained from the white blood cells by phenol/chloroform extractions and ethanol precipitation according to Maniatis.[18]

A 320 bp fragment of the apo B gene was amplified with oligonucleotides UOL and LOL as described by Schuster *et al*[19] using GeneAmp PCR System 2400 (Perkin-Elmer Corp., Norwalk, Connecticut, USA). PCR product was screened for the presence of FDB mutation by single-strand conformation polymorphism (SSCP) analysis according to Chaves *et al*[20] with minor modifications. A mixture consisting of 5 μl of PCR product and 5 μl of formamide was heated for 5 min at 95°C and then plunged into ice prior to loading onto the 12% acrylamide (50 : 1 acrylamide : bisacrylamide) gel with 5% glycerol. Electrophoresis was carried out at 200 V for 5 h at 4°C. After electrophoresis the gels were stained with silver. As positive controls, DNA from Dutch and German patients heterozygous for the FDB $Arg_{3500}Gln$ mutation were used.

The presence of $Arg_{3500}Gln$ mutation was confirmed using a mismatch MspI PCR modified strategy as described by Motti[21] with modifications of Defesche[3] (and personal communication) and our own minor changes (instead of carrying out a hot start prior to addition of the Taq polymerase, AmpliTaq Gold DNA polymerase (Perkin-Elmer Corp.) was used and therefore the number of PCR cycles was increased to 35). A 477 bp fragment (final PCR product was 517 bp because of the 40 base long CG clamp at the 5′ end of the 5′-primer) of exon 26 of the apo B gene was amplified using oligonucleotides Apo-B-5 and Apo-B-3. After an initial denaturation step at 95°C for 5 min, reaction was carried out for 35 cycles of amplification as follows: denaturation at 95°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 2 min. A final extension at 72°C for 7 min was also performed. Twenty-five μl of reaction mixture was digested by the restriction endonuclease *Msp*I (Amersham Life Science), using the conditions recommended by manufacturer. Digestion products were separated by electrophoresis at room temperature on 2% agarose gels, stained with ethidium bromide and viewed over ultra violet light.

Sequencing was performed in DNA samples from the individual with atypical mutation (P132) and from a normal control. Exon 26 was amplified by polymerase chain reaction using oligonucleotide primers UOL and LOL and reaction conditions as described before. The products were purified using a Qiagen polymerase chain reaction kit and sequenced manually using the Thermo Sequenase Cycle Sequencing Kit (Amersham Life Science) under conditions recommended by the manufacturer.

Three biallelic markers were analysed to establish haplotypes at the apo B locus. The RFLPs of *Xba*I,[22] MspI[8] in exon 26 and *Eco*RI[15] in exon 29 were determined. The MspI RFLP was also confirmed by the technique used for the detection of

FDB $Arg_{3500}Gln$ mutation as described earlier. Construction of the haplotypes was based on the assumption that there had been no recombination event within the apo B gene.

Genotype of apolipoprotein E was identified using the method of Hixson and Vernier.[23]

Statistical analysis

Prevalence estimates of the mutation were expressed as percentages with 95% confidence intervals. Data for quantitative variables were expressed as mean values $\pm$SD. Lp(a) concentrations were expressed also as median values. Differences in means between FDB and non-FDB subjects were tested by the Mann-Whitney two-sample test. The lipid results were also adjusted by sex, age and family-connection using analysis of covariance. Statistical significance of the differences in the frequencies of the apo E alleles and of other qualitative variables was evaluated using χ^2 or Fisher exact test. *P*-values lower than 0.05 were considered as statistically significant.

Results

SSCP screening of the whole group of 525 hypercholesterolaemic patients resulted in an initial identification of FDB mutation in 14 individuals. All the patients belonged to the HLP IIA group. The mutation was not found in any of 174 patients with HLP IIB. In one patient (P132) the SSCP pattern differed slightly from the patterns characteristic for both positive and negative results (Figure 1). In all but this one person with the atypical SSCP pattern the presence of the $Arg_{3500}Gln$ mutation has been confirmed by the mismatch MspI PCR method. All of them were heterozygous for the mutation.

The calculated prevalence of the $Arg_{3500}Gln$ mutation was 2.5% in the whole hypercholesterolaemic group and 3.7% in the IIA type HLP group. In 65 patients with the clinical characteristics of familial hypercholesterolaemia (FH_{clin}) the frequency can be evaluated as 10.8% (Table 1).

In the patient P132 with atypical SSCP pattern and non confirmed $Arg_{3500}Gln$ mutation further investigations by DNA sequencing revealed another kind of mutation in the same region of the apo B gene. A C→T transition in codon 3492 (ACT→ATT) which is producing a change from threonine to isoleucine in the encoded amino acid sequence was found ($Thr_{3492}Ile$) (Figure 2). Such mutation was not described before. The patient was heterozygous for the mutation.

Table 2 presents the clinical and biochemical characteristics of 14 probands with both mutations. Out of 13 subjects with the $Arg_{3500}Gln$ FDB mutation, eight had coronary artery disease (CAD) and two peripheral artery disease (PAD) symptoms and in four of them tendon xanthomas were found. The patient with $Thr_{3492}Ile$ mutation was highly hypercholesterolaemic, had first symptoms of angiographically confirmed CAD at the age of 39 years as well as angiographically confirmed PAD at the age of 38 years and had tendon xanthomatosis. All

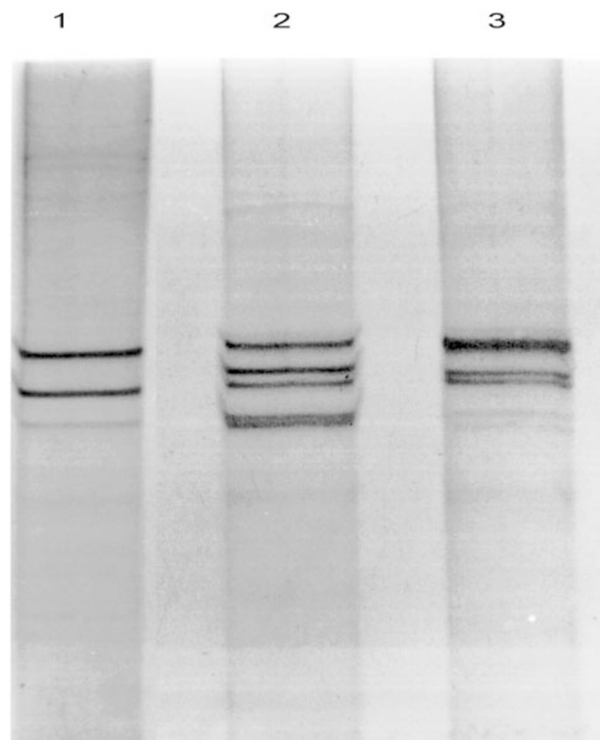

Figure 1 DNA screening for FDB mutation by SSCP analysis of PCR products. Lane 1, unaffected individual; Lane 2, subject heterozygous for $Arg_{3500}Gln$ mutation; Lane 3, atypical SSCP pattern in P132 proband heterozygous for the new $Trp_{3492}Ile$ mutation.

investigated relatives ie, his two sons and brother were carriers of the mutation, but their total and LDL cholesterol were not elevated. The brother of the proband had the first symptoms of angiographically confirmed CAD at the age of 50 years. Both sons (still young – 20 and 22 years) were asymptomatic. The father of the proband died at the age of 73 years because of the third myocardial infarction (his first MI was at the age of 40 years).

Haplotype analysis of 10 families with FDB $Arg_{3500}Gln$ mutation demonstrated that the mutant allele segregated with the haplotype *Xba*I$^-$/*Msp*I$^+$/*Eco*RI$^-$. The lack of DNA from family members of two probands (P82 and P537) and non-informative family pedigrees of another proband (P526) precluded unambiguous resolution of their haplotypes. Analysis of probands genotype (Table 2) showed however that one of the two alleles could also represent the *Xba*I$^-$/*Msp*I$^+$/*Eco*RI$^-$ haplotype.

A comparison of the results of lipid and lipoprotein determinations and of clinical characteristics in 36 FDB subjects (13 probands and their affected relatives) with 44 non-FDB relatives is shown in Table 3. The carriers of the $Arg_{3500}Gln$ mutation had markedly higher mean TC and LDL-C levels. The median Lp(a) level was similar in both groups, but in

Table 1 The prevalence of FDB in hypercholesterolaemic patients

		Frequency of FDB mutation (screening – SSCP analysis)			*Frequency of $Arg_{3500}Gln$ mutation (confirmation by mismatch MspI PCR)*		
	Number of subjects	*Absolute (n)*	*Relative (%)*	*95% confidence interval (%)*	*Absolute (n)*	*Relative (%)*	*95% confidence interval (%)*
All hypercholesterolaemic group:	525	14	2.7	(1.3 – 4.0)	13[a]	2.5	(1.1 – 3.8)
Type IIA hyperlipoproteinaemia	351	14	4.0	(1.0 – 6.0)	13[a]	3.7	(1.7 – 5.8)
• FH_{clin}	65	7	10.8	(3.2 – 18.3)	7	10.8	(3.2 – 18.3)
• non-FH	286	7	2.4	(0.66 – 4.2)	6	2.1	(0.43 – 3.8)
Type IIB hyperlipoproteinaemia	174	0	0		0	0	

[a]One patient (P132) screened for FDB by SSCP shows a pattern slightly differing both from other FDB individuals and nonaffected subjects. The apo B-100 $Arg_{3500}Gln$ mutation was not confirmed using the mismatch MspI PCR. This subject was a carrier of another hitherto nondescribed ($Thr_{3492}Ile$) mutation in the same region of apo B-100 gene identified by sequencing of this DNA fragment.

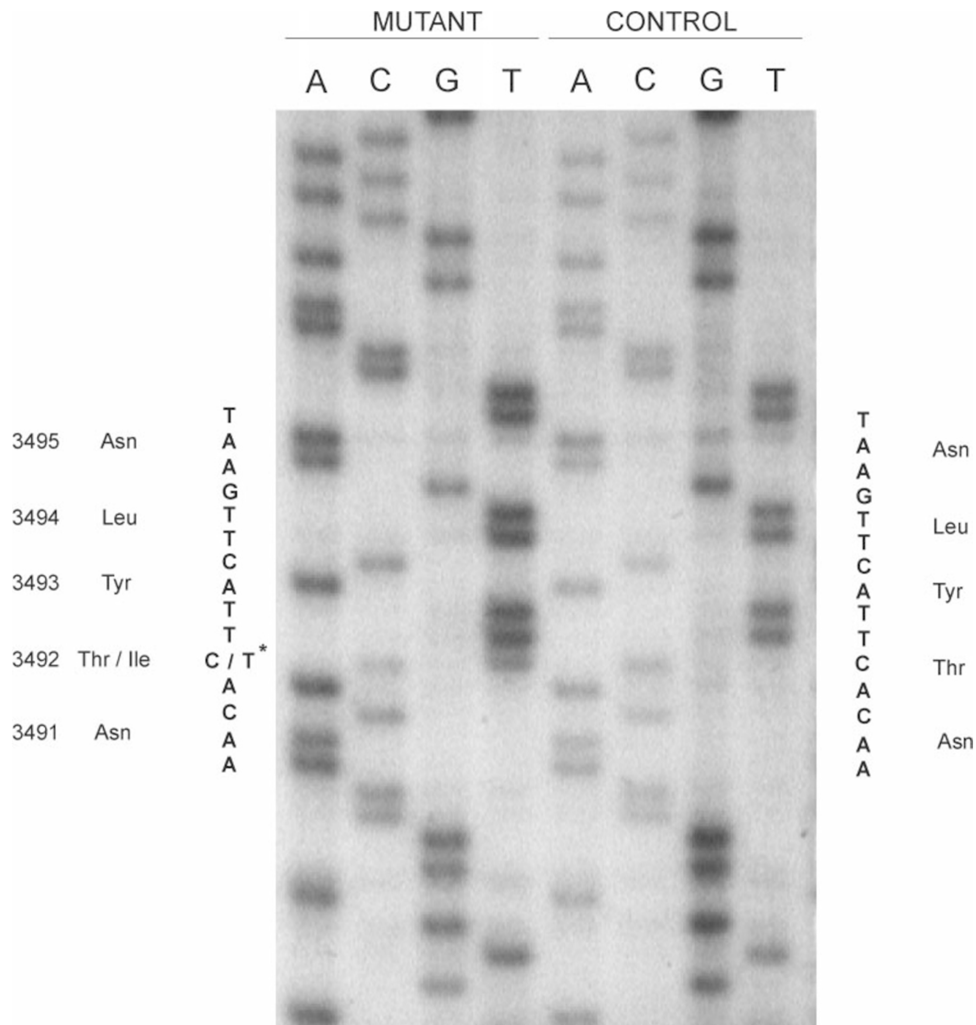

Figure 2 Autoradiograph of the 6% polyacrylamide gel, showing the nucleotide sequences of amplified double-stranded DNA fragment of exon 26 of the apo B gene from the patient P132 with atypical SSCP pattern (mutant) and from normal control.

affected subjects Lp(a) level above 30 mg/dl occurred more frequently than in nonaffected ones (31.4% *vs* 16.3%, respectively). 22.6% of adult FDB subjects (above 20 years of age) had tendon xanthomas. Cardiovascular disease as defined by angina or history of MI was present in 38.7% of the adult FDB subjects as compared with 10.3% in their non-FDB relatives.

Table 2 Clinical and biochemical characteristics of probands with $Arg_{3500}Gln$ mutation causing FDB and proband with new mutation $Thr_{3492}Ile$

Proband No	*Sex*	*Age at DNA analysis (years)*	*Age at lipid measurement (years)*	*Mutation*	*Haplotype marker* XbaI	MspI	EcoRI	*TC (mg/dl)*	*LDL-C (mg/dl)*	*HDL-C (mg/dl)*	*TG (mg/dl)*	*Lp(a) (mg/dl)*	*ApoE genotype*	*Clinical findings (age, years)*
P82	F	70	60	$Arg_{3500}Gln$	+/−	+/+	+/−	365	283	62	98	12	3/3	MI (59), HT
P161	M	46	43	$Arg_{3500}Gln$	−/−	+/+	+/−	301	211	79	56	48	3/3	MI (38), CABG
P165	M	64	60	$Arg_{3500}Gln$	+/−	+/+	+/−	398	327	48	113	13	3/3	MI (54), CABG, PAD
P186	M	59	56	$Arg_{3500}Gln$	+/−	+/+	+/−	295	225	42	140	10	4/3	CAD (50), CABG
P275	M	45	39	$Arg_{3500}Gln$	+/−	+/+	+/−	317	244	37	178	124	3/3	CAD (37), MI (40), CABG, XT
P398	M	57	47	$Arg_{3500}Gln$	+/−	+/−	+/−	285	205	45	175	132	3/3	MI (47), CABG
P432	M	43	44	$Arg_{3500}Gln$	−/−	+/+	−/−	298	230	45	114	12	3/3	CAD (38), MI (39), PCTA
P526	F	31	21	$Arg_{3500}Gln$	+/−	+/−	+/−	316	236	57	113	37	3/3	XT
P537	F	61	55	$Arg_{3500}Gln$	−/−	+/+	+/−	371	283	61	134	NA	2/3	XT
P668	F	60	59	$Arg_{3500}Gln$	+/−	+/+	+/−	347	278	56	147	102	4/3	CAD (41), MI (49), PAD, HT
P687	F	65	65	$Arg_{3500}Gln$	−/−	+/+	+/−	306	235	35	180	<5	3/3	Asymptomatic
P694	F	57	55	$Arg_{3500}Gln$	−/−	+/+	−/−	295	197	81	93	26	4/3	HT, Diabetes
P696	F	47	45	$Arg_{3500}Gln$	+/−	+/+	+/−	359	281	56	108	24	4/3	XT
P132	M	49	39	$Thr_{3492}Ile$	+/−	+/+	+/+	281	203	41	186	5	2/3	CAD (39), PAD (38), XT

M, male; F, female; CAD, coronary artery disease; MI, myocardial infarction; CABG, coronary artery bypass graft; PAD, peripheral artery disease; HT, hypertension; XT, tendon xanthomas; NA, no data available.

Table 3 Comparison of FDB ($Arg_{3500}Gln$ mutation) subjects and their non-FDB relatives

	FDB (n=36)	*Adjusted mean*[a]	*Range*	*non-FDB (n=44)*	*Adjusted mean*[a]	*Range*	P	P[b]
Sex (M/F)	18/18			18/26			ns	
Age, mean ± SD (years)	44.3 ± 18.6		(7–77)	32.7 ± 20.2		(6–74)	0.0109	
TC, mean ± SD (mg/dl)	288 ± 57.1	280.7	(149–398)	200 ± 51.0	207.3	(116–330)	<0.0001	<0.0001
LDL-C, mean ± SD (mg/dl)	212 ± 53.2	204.1	(86–327)	118 ± 45.3	124.9	(45–213)	<0.0001	<0.0001
HDL-C, mean ± SD (mg/dl)	52 ± 12.0	53.4	(34–81)	56 ± 13.0	55.0	(30–89)	ns	ns
TG, mean ± SD (mg/dl)	136 ± 92.4	131.9	(55–552)	120 ± 74.7	123.8	(35–450)	ns	ns
Lp(a), mean ± SD (mg/dl)	34.4 ± 43.7	32.4	(<5–140)	19.4 ± 29.9	21.4	(<5–140)	0.077	ns
Lp(a), median (mg/dl)	12			9			ns	
percentyl (25–75) (mg/dl)	6.5–41			<5–16				
Lp(a) >30 mg/dl (%)	31.4			16.3			ns	
Apo E allele								
ε2 (%)	4.3			8.1			ns	
ε3 (%)	78.6			75.6			ns	
ε4 (%)	17.1			16.3			ns	
CAD (⩾20 years) (%)	38.7			10.3			0.0164	
Age of CAD onset, mean ± SD (years)	47.6 ± 8.7		(37–62 years)	52.3 ± 6.1		(47–59 years)	ns	
MI (⩾20 years) (%)	25.8		(38–51 years)	3.4		(59 years)	0.0265	
XT (⩾20 years) (%)	22.6			0			0.0109	

[a]Means adjusted for age, sex and family connection; [b]p after adjustment of means for age, sex and family connection; CAD, coronary artery disease; MI, myocardial infarction; XT, tendon xanthomas.

Comparison of lipid and lipoprotein levels in affected and non-affected family members showed some degree of overlapping of the results. Four young FDB mutation carriers had LDL-C in normal or in the upper range of normal levels. Two diabetic FDB patients had also hypertriglyceridaemia. Some non-FDB relatives and spouses showed lipid abnormalities: mostly mild or moderate hypercholesterolaemia, and some of them mild hypertriglyceridaemia.

Lipid and lipoprotein levels did not differ significantly between FDB patients carriers of various apolipoprotein E allele (data not shown).

Discussion

In this study Polish unrelated individuals with moderate and severe hypercholesterolaemia were screened for the presence of the FDB $Arg_{3500}Gln$ mutation. The mutation was detected

only in type IIA hypercholesterolaemic patients. Its frequency was 3.7%. The prevalence of FDB Arg_{3500}Gln mutation in the investigated group was similar to the frequency (1–6%) observed in hypercholesterolaemic subjects from other Caucasian populations of Western Europe, North America and Australia.[14,24]

In the present study all FDB heterozygotes were identified among individuals classified as having either clinical symptoms of FH (10.8%) or type IIA HLP without FH symptoms (2.1%). Our sample of hypercholesterolaemic patients containing a relatively high number of probably FH subjects is not representative of the general population. A crude estimate of the frequency of the Arg_{3500}Gln mutation in Polish population can be made based on the frequency of hypercholesterolaemia (>250 mg/dl, without hypertriglyceridaemia) in Poland evaluated as approximately 19% (data from Pol-MONICA Warsaw Program[25] – 1519 individuals, age 35–64, screened in 1993 as the part of international WHO-MONICA Project). Assuming the frequency of FH in general population of 1/500 (0.2%),[26] an estimate of the frequency of the Arg_{3500}Gln mutation in Polish population was $10.8\% \times 0.2\%$ plus $2.1\% \times (19\% - 0.2\%) \approx 1/250$.

In our study relatively high frequency (10.8%) of the mutation was found among clinically diagnosed FH patients. In most other studies based on the same clinical criteria FDB frequency in this group was reported to be 2–3%.[14,24] A higher frequency (12%) was stated only in Switzerland.[5] Recently in Poland FDB mutation was found in five of 30 families (17%) with clinical signs of familial hypercholesterolaemia.[16] It suggests that FDB mutation may be a rather frequent cause of clinically recognised familial hypercholesterolaemia in Poland.

Comparison of the lipid parameters in FDB subjects and their non-FDB relatives (in which environmental and other genetic factors were similar) revealed that cholesterol concentration was not fully informative for FDB diagnosis as TC and LDL-C concentrations in subjects with and without mutation were overlapping to some extent. Such observations were also noted in other studies.[4,6,27] DNA analysis in the relatives of FDB mutation carriers should therefore be strongly recommended as a method of an early identification of other carriers.

In one of the examined hypercholesterolaemic patients a new mutation was detected. It was localised in codon 3492 of the apo B gene causing threonine to isoleucine substitution. The patient-carrier of this mutation was hypercholesterolaemic and had symptoms of premature atherosclerosis in coronary and peripheral arteries, tendon xanthomatosis and family history of premature atherosclerosis. All investigated relatives of the proband were carriers of the mutation and all were normocholesterolaemic. However as it was observed for the Arg_{3500}Gln mutation, the TC and LDL-C concentrations might be not fully informative for FDB diagnosis. The Thr_{3492}Ile mutation is situated in close proximity of the typical Arg_{3500}Gln one. To determine the influence of this mutation on the apo B-100 ligand function, the study of binding affinity of proband's LDL to LDL receptor is necessary.

A high prevalence of another kind of apo B mutation – the Arg_{3531}Cys mutation was recently stated in Denmark[7] and in France.[28] The frequency of this kind of mutation in Poland has not yet been established.

In summary, our study showed that the prevalence of the Arg_{3500}Gln mutation in Polish hypercholesterolaemic patients was 3.7% which was similar to the frequency detected in hypercholesterolaemic groups in several other European countries. The estimated prevalence of the mutation in the general Polish population could be relatively high – 1/250. The same haplotype at the apo B locus in the carriers of this mutation in Poland suggests its common origin with other populations from Western Europe. A not hitherto reported kind of mutation in apo B gene – a Thr_{3492}Ile mutation was found in one hypercholesterolaemic subject with early CAD symptoms. The determination of the influence of the mutation on LDL binding to LDL receptor is necessary.

Acknowledgments
This work was supported by grant No 4 P05D 038 09 from the Komitet Badań Naukowych, Poland. DNA from a Dutch patient heterozygous for the FDB Arg_{3500}Gln mutation and Apo-B-5 and Apo-B-3 primers and the description of the mismatch MspI PCR method were kindly supplied by JC Defesche, PhD, Amsterdam, The Netherlands. DNA from a German patient heterozygous for the FDB Arg_{3500}Gln mutation was the gift from Prof H Schuster, Berlin, Germany.

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