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The c.-133A > G polymorphism in *NPC1L1* gene influences the efficacy of ezetimibe monotherapy on apolipoprotein A1 in hyperlipidemic patients

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Niemann-Pick C1-like 1 protein (*NPC1L1*) plays a critical role in intestinal cholesterol absorption. Previous studies found that the *NPC1L1* c.-133A > G SNP, but not other *NPC1L1* SNPs, was associated with response to statin treatment and statin-ezetimibe combinations. To date effect of *NPC1L1* c.-133A > G SNP on ezetimibe monotherapy has not been studied. Our objective was to examine whether SNP c.-133A > G at the *NPC1L1* gene has effects on lipid levels and on the efficacy of 3, 6 and 12 months of 10 mg daily ezetimibe monotherapy in hyperlipidemic patients with statin induced adverse effects. One hundred and one type IIa and IIb hyperlipidemic patients (72 females, 29 males; age: 61.23 ± 9.87 ys; BMI: 28.18 ± 4.29 kg/m^2) were enrolled. The genotype frequencies were conformed to Hardy-Weinberg equilibrium. We could not find significant differences in initial lipid levels between AA and AG + GG patients. While plasma levels of apolipoprotein A1 (ApoA1) did not significantly decrease after ezetimibe treatment (1.96; 3.39 and 2.74%) in AA patients, a significant elevation in ApoA1 levels has been found after treatment in AG + GG patients (9.15; 8.54 and 13.58%). The effect of *NPC1L1* c.-133A > G on the ApoA1 levels was found significant ($p < 0.05$). Efficacy of treatment with ezetimibe on other plasma lipid parameters after 3, 6 or 12 months did not differ significantly. *NPC1L1* -133A > G SNP influences the ApoA1 response to ezetimibe monotherapy, therefore, may alter the effect of ezetimibe on the structure and function of the high-density lipoprotein particles.

1. Introduction

Coronary heart disease (CHD) remains the leading cause of death in the developed countries (WHO 2011; Riccioni and Sblendorio 2012). Dyslipidemia, manifested by elevated low-density lipoprotein cholesterol (LDL-C) and decreased high-density lipoprotein cholesterol (HDL-C) levels, is central to the development and progression of atherosclerosis (Riccioni and Sblendorio 2012; Hazen 2010). The contribution of dyslipidemia to cardiovascular risk was illustrated in several studies (Hansson 2005; Emerging Risk Factors Collaboration et al. 2009; Ballantyne and Hoogeveen 2003). Therefore, dyslipidemia has become a primary target of intervention in strategies for the prevention of cardiovascular events (Sazonov et al. 2010; Brown et al. 1993a; Brown 1993b). Factors that influence the efficacy of lipid lowering agents including genetic variations may explain the significant inter-individual differences in response to these drugs.

The cholesterol homeostasis is controlled mainly by hepatic cholesterol-synthesis, cholesterol absorption in the proximal region of the jejunum and biliary excretion (Cohen 2008). Niemann Pick C1-like 1 (*NPC1L1*) protein is a polytopic transmembrane protein localized in the brush border membrane of jejunal epithelial cells and plays a crucial role in the absorption of cholesterol and plant sterols (Altmann et al. 2004; Davis et al. 2004; Betters and Yu 2010). The significance of *NPC1L1* has especially increased since it turned out that ezetimibe interfered with cholesterol absorption by blocking this protein (Garcia-Calvo et al. 2005). On average, ezetimibe causes a 15–20 % reduction in cholesterol level and thereby it is the only clinically successful lipid lowering molecule since the introduction of statins (Knopp et al. 2003; Pandor et al. 2009; Athyros et al. 2008). The gene of *NPC1L1* is encoded in the 7th chromosome and its size is 29 kb. *NPC1L1* knockout mice have been demonstrated to have lower cholesterol absorption than wild-type, and in these animals there was no additional reduction in cholesterol absorption after ezetimibe treatment (Altmann et al. 2004).

In humans Cohen et al. sequenced the gene of *NPC1L1* and demonstrated that in patients carrying rare *NPC1L1* variants significantly reduced cholesterol absorption could be found compared to non-carriers (Cohen et al. 2006). Several studies investigated the possible genetic basis for this inter-individual variation, and to date over 140 single nucleotide polymorphisms (SNPs) and 5 insertions/deletions have been identified in the *NPC1L1* gene (Hegele et al. 2005; Simon et al. 2005; Lupatelli et al. 2013). Certain single nucleotide polymorphisms, including the c.-133A > G SNP of the *NPC1L1* gene have been shown to influence the response to statins (hydroxy-methylglutaryl-CoA reductase inhibitors) or statin-ezetimibe combinations (Hegele et al. 2005; Simon et al. 2005).

So far most trials investigated the efficacy of ezetimibe in combination with statins. In a study of 375 healthy individuals

Table 1: Clinical and anthropometric data of the study population.

Age (years)	61.23 ± 9.87						
Gender (female/male)	69/32						
BMI (kg/m2)	28.18 ± 4.29						
Waist circumference (cm)	98.54 ± 11.74						
	Before treatment	After treatment					
		3 months	change (%)	6 months	change (%)	12 months	change (%)
Total cholesterol (mmol/l)	7.46 ± 1.42	6.79 ± 1.46***	−9.67	6.72 ± 1.32***	−7.9	6.48 ± 1.39***	−11.70
LDL-C (mmol/l)	4.65 ± 1.52	4.16 ± 1.30***	−10.15	4.07 ± 1.14***	−10.38	3.91 ± 1.09***	−8.52
HDL-C (mmol/l)	1.45 ± 0.43	1.43 ± 0.41	−0.39	1.41 ± 0.42	−0.91	1.39 ± 0.45	1.45
Triglyceride (mmol/l)	2.45 (1.62−4.38)	2.20 (1.70−3.70)	−9.93	2.29(1.55−3.84)**	−13.45	2.10(1.60−3.70)*	−19.47
ApoB (g/l)	1.30 ± 0.35	1.23 ± 0.33	−2.65	1.17 ± 0.30**	−7.61	1.15 ± 0.28**	−6.62
ApoA (g/l)	1.66 ± 0.37	1.68 ± 0.36	3.07	1.62 ± 0.31	2.18	1.66 ± 0.37	5.01
hsCRP (mg/l)	2.78 (1.69−5.95)	2.46(1.25−5.25)**	−17.68	2.54(0.87−4.87)**	−14.96	2.94(1.50−5.80)	−3.28
Creatine kinase (U/l)	131.5(87.00 −235.50)	123.00(88.00 −225.00)	−1.10	138.00(98.00 −220.00)	2.07	120.00(92.00 −224.00)	−4.17

*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. BMI = body mass index; LDL-C = low-density lipoprotein-cholesterol; HDL-C = high-density lipoprotein-cholesterol; ApoB = apolipoprotein B; ApoA = apolipoprotein A; hsCRP = high-sensitivity C-reactive protein

and hypercholesterolemic patients 10 mg ezetimibe was added onto existing statin therapy. No association was found between baseline cholesterol level and the examined *NPC1L1* single-nucleotid polymorphisms. However, they could demonstrate associations between cholesterol-response to ezetimibe treatment and these SNPs (Simon et al. 2005).

To date, the effect of single nucleotide polymorphisms in the *NPC1L1* gene on the response to ezetimibe monotherapy has not been studied. We supposed that ezetimibe monotherapy could improve the lipid parameters in hyperlipidemic patients and the efficacy of ezetimibe might be different in patients with different *NPC1L1* genotypes. Our aim was to examine the effect of SNP c.-133A > G at the *NPC1L1* gene on lipid levels and on the efficacy of 3, 6 and 12 months of 10 mg daily ezetimibe monotherapy in hyperlipidemic patients.

2. Investigations and results

2.1. Study population

Patients were recruited at the First Department of Internal Medicine, University of Debrecen. One hundred and one (72 females, 29 males), Fredrickson type IIa and IIb hyperlipidemic with previously diagnosed statin induced adverse effects were enrolled. The mean age of studied patients was 61.23 ± 9.87 years. In 42 cases statin-induced myopathy (myalgia with or without creatine-kinase (CK) elevation), in 28 cases statin-induced liver enzyme elevation (hepatopathy), in 15 cases statin-induced severe gastrointestinal symptoms were proved. Sixteen patients had more than one statin-induced symptom: two patients had myopathy, hepatopathy and gastrointestinal symptoms, seven had myopathy and hepatopathy, five had myopathy and gastrointestinal symptoms, and two patients had hepatopathy and gastrointestinal symptoms. Patients with alcoholism, drug dependence, malignancy, pregnancy or lactation, as well as patients on anticoagulant or lipid-lowering therapy were excluded. After 6 weeks on the National Cholesterol Education Program step 1 diet, patients received 10 mg/day ezetimibe (Ezetrol) for 12 months. Informed consent was obtained from all patients after explaining the nature and the purpose of the study. The Ethics Committee of the University of Debrecen, the National Institute of Pharmacy approved the study. The study is registered by the European Clinical Trials Database (EudraCT number 2009–017732–40).

2.2. Results

Baseline characteristics and initial laboratory parameters of the subjects are presented in Table 1.

The tolerability of ezetimibe was excellent. No patient had to discontinue the drug due to side effects, altogether one person complained about transient ezetimibe-induced headache. During the one year of ezetimibe therapy there were no serious adverse events.

After 3 months of ezetimibe treatment the total cholesterol, LDL-C and hsCRP levels significantly decreased, while the other studied parameters (triglyceride, HDL-C, ApoB, ApoA1 and CK levels) did not change significantly in the whole study population. Six months of treatment significantly decreased the total cholesterol, LDL-cholesterol, triglyceride, ApoB and hsCRP levels compared to the initial results. After 12 months of treatment we found significantly decreased total cholesterol, LDL-cholesterol, triglyceride, ApoB levels (Table 1, Table 2).

In the studied population the *NPC1L1* c.-133A > G genotype distribution was as follows: 57.42 % were AA, 34.65% were AG and 7.92% were GG genotype. Because of the low ratio of GG patients we divided the study population into two groups: AA (G non-carriers) and AG + GG (G carriers). There were no significant differences in initial lipid levels between AA and AG + GG patients. We could not find significant differences in age, body mass index (BMI) and waist circumference between the two groups (Table 3.).

Interestingly, plasma levels of ApoA1 did not change significantly after 3, 6 and 12 months of ezetimibe treatment (1.96; 3.39 and 2.74%, respectively) in AA patients. However, significant elevation in ApoA1 levels has been found after treatment in AG + GG patients (9.15; 8.54 and 13.58%, respectively). The effect of *NPC1L1* -133A > G on the efficacy of ezetimibe treatment on ApoA1 level was significant ($p < 0.05$). HDL-cholesterol levels remained unchanged in both groups, and there was no significant difference between the two groups (Fig., Table 3).

Comparing the two groups we could not find any significant difference in the efficacy of treatment with ezetimibe on other plasma lipid parameters after 3, 6 or 12 months. After 3 and 12 months ezetimibe treatment LDL-cholesterol and total cholesterol levels significantly decreased both in the AA and in the AG + GG groups. There was no significant difference between the two groups. In the AG + GG group the rate of LDL-cholesterol level decrease was greater than in the AA group,

Table 2: Changes (%)of lipoprotein and apolipoprotein levels compared to the initial levels after 3,6 and 9 months of ezetimibe treatment in various NPC1L1 genotype groups and in the whole patient population

	AA		AG + GG		all	
	Δ (%)	*SD*	*Δ (%)*	*SD*	*Δ (%)*	*SD*
Cholesterol						
3 months	−9.12	14.8 Δ	−10.27	13.4 Δ	−9.67	14.1
6 months	−7.21	20.9	−8.80	18.3	−7.9	19.7
12 months	−12.95	15.5 Δ	−10.06	17.5 Δ	−11.7	16.3
Triglyceride						
3 months	−8.15	36.8	−10.63	30.7	−9.93	33.8
6 months	−11.11	64.5	−15.84	30.12	−13.45	52.0
12 months	−19.47	60.2	−19.80	49.7	−19.47	55.6
LDL-C						
3 months	−9.29	18.7 Δ	−11.02	16.6 Δ	−10.15	17.6
6 months	−10.78	29.3	−9.96	21.5	−10.38	25.6
12 months	−5.94	32.8 Δ	−11.00	21.0 Δ	−8.52	27.2
HDL-C						
3 months	−4.45	12.6	3.94	21.4	−0.39	17.8
6 months	−5.09	24.8	3.58	23.2	−0.91	24.3
12 months	−1.67	22.9	4.57	30.6	1.45	26.9
ApoB						
3 months	−1.16	24.1	−4.44	14.6	−2.65	20.2
6 months	−9.12	15.8	−5.88	17.5	−7.61	16.5
12 months	−6.83	29.1	−6.38	16.6	−6.62	23.7
ApoA1						
3 months	−1.96	10.4	9.15	21.1 Δ x	3.07	16.9
6 months	−3.39	15.1	8.54	22.6 Δ x	2.18	19.7
12 months	−2.74	19.2	13.58	25.7 Δ x	5.01	23.7

LDL-C = low-density lipoprotein-cholesterol; HDL-C = high-density lipoprotein-cholesterol; ApoB = apolipoprotein B; ApoA1 = apolipoprotein A1 Δ: $p < 0.05$ x: $p < 0.05$ between AA and AG + GG

Table 3: Initial laboratory parameters and anthropometric data of the patients with various NPC1L1 genotypes

NPC1L1 genotype	AA	AG + GG	p
n	57	44	
Age (years)	62.02 ± 10.80	60.20 ± 8.54	ns
Gender (female/male)	38/19	31/13	ns
BMI (kg/m2)	28.67 ± 4.79	27.59 ± 3.60	ns
Waist circumference (cm)	99.29 ± 13.12	97.71 ± 10.11	ns
Total cholesterol (mmol/l)	7.37 ± 1.33	7.59 ± 1.95	ns
LDL-C (mmol/l)	4.62 ± 1.29	4.69 ± 1.78	ns
HDL-C (mmol/l)	1.51 ± 0.50	1.37 ± 0.31	ns
Triglyceride (mmol/l)	2.50(1.50−3.50)	2.30(1.80−4.87)	ns
ApoB (g/l)	1.30 ± 034	1.30 ± 0.38	ns
ApoA1 (g/l)	1.72 ± 0.40	1.56 ± 0.29	ns
hs CRP (mg/l)	2.64(1.67−7.69)	3.02(1.71−4.56)	ns
Creatinine kinase (U/l)	120.00(85.00−245.00)	137.00(94.00−227.00)	ns

n = number of patients, BMI = body mass index, LDL-C = low-density lipoprotein-cholesterol, HDL-C = high-density lipoprotein-cholesterol, ApoB = apolipoprotein B, ApoA1 = apolipoprotein A1, CRP = C-reactive protein.

however, the difference was not significant. The ApoB level decreased significantly only in the AA group after 6 months and 12 months therapy, but there was no significant difference between the two groups (Table 3).

3. Discussion

We demonstrated firstly that *NPC1L1* c.-133A > G SNP influences the ApoA1 response to ezetimibe monotherapy and we could confirm that ezetimibe monotherapy was able to improve the lipid parameters in hyperlipidemic patients with statin-induced adverse effects.

ApoA1 secreted by the liver and intestine is the main structure protein of HDL in plasma. Lipid-poor ApoA1 acquires cholesterol and phospholipids, in particular from hepatocytes and enterocytes (Brunham et al. 2006; Timmins et al. 2005). The initial lipidation of apoA-I is primarily mediated by ABCA-1 and results in the formation of nascent HDL (Fig.). Nascent HDL acquires additional phospholipids and free cholesterol from extrahepatic tissues, therefore, exerts an antiatherogenic effect *via* reverse cholesterol transport. Knocking out ApoA1 in human apoB-transgenic female mice, LDL receptor-deficient and LDL receptor/apoE-deficient mice resulted in an accelerated atherosclerosis development (Moore et al. 2003, 2005; Voyiazi-akis et al. 1998). Furthermore, ApoA1 is capable of binding and removing lipid hydroperoxides of LDL *in vitro* and *in vivo* (Navab et al. 2000). Therefore, direct or indirect elevation of ApoA1 level may improve the atheroprotective function of HDL particle.

To date, the effect of ezetimibe monotherapy on the ApoA1 containing HDL-cholesterol is unclear. In former studies different

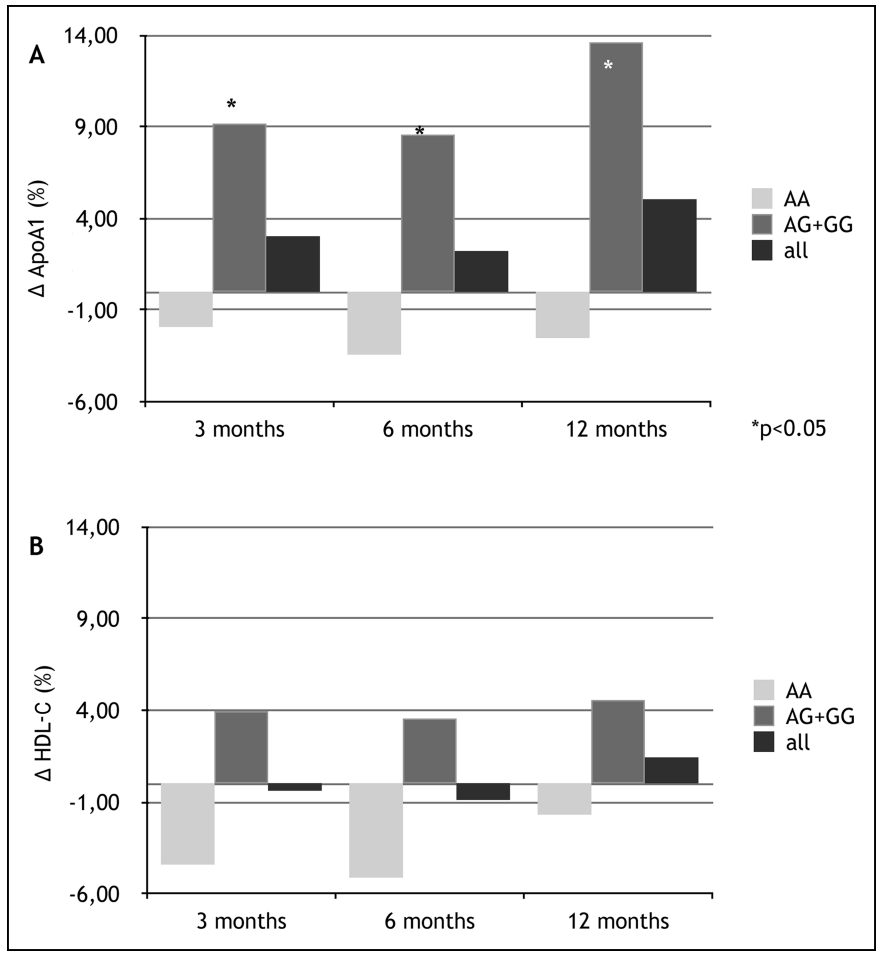

Fig: Changes (%) of Apolipoprotein A1 (A) and high-density lipoprotein cholesterol (HDL-C) levels after 3,6 and 9 months of ezetimibe treatment in various NPC1L1 genotype groups and in the whole patient population.

HDL-C raising effect of ezetimibe has been shown. Some researchers found that ezetimibe could increase the HDL-C level significantly (Dujovne et al. 2002), while in a study of healthy men there were no difference in HDL levels between the placebo group and the ezetimibe monotherapy group (Berneis et al. 2010). *NPC1L1* single nucleotide variants are known to influence the sterol absorption and therefore can affect the formation of HDL-C (Ginsberg et al. 1998). Nevertheless, the effect of *NPC1L1* polymorphisms on the HDL-C response to ezetimibe monotherapy has not been studied yet. In previous studies it was found that the *NPC1L1* c.-133A > G SNP was one of the SNPs that may influence the response to some statins (Polisecki et al. 2008). Carriers of the *NPC1L1* c.-133 A > G common allele was shown to have higher LDL-C levels (Willer et al. 2008). Others demonstrated that patients carrying *NPC1L1* -133 GG had a lesser LDL-cholesterol lowering response to statin-ezetimibe combinations (Simon et al. 2005; Polisecki et al. 2008).

In our study we could not find a significant difference in the response to ezetimibe on HDL-C between the patients carrying *NPC1L1* -133 AA and in AG + GG patients. Contrarily, in AA patients plasma levels of ApoA1 did not decrease significantly after 3, 6 and 12 months of ezetimibe treatment. While, a significant elevation in ApoA1 levels has been found after treatment in AG + GG patients. Based on these results *NPC1L1* c.-133A > G SNP influences the ApoA1 response to ezetimibe monotherapy, therefore, may alter the effect of ezetimibe on the structure and function of the HDL particles.

In a previous study genotype distribution of the -133 A > G *NPC1L1* polymorphism was similar to our patients. In this study among the autosomal dominant hypercholesterolemic group, 48% of patients were AA allele carriers, 46 % and 6% were AG and GG allele carriers, respectively. While in the control group the genotype distribution was as follows: 58% were AA, 35% were AG and 7% were GG genotype (Martin et al. 2010). In our study ezetimibe monotherapy significantly reduced the total (-11.70 %), LDL-C (-8.52 %) and the triglyceride levels (-3.70 %), while the HDL-C levels (1.45 %) did not increase significantly. A previous meta-analysis found that ezetimibe monotherapy decreased LDL-C level by -18.58 %, and reduces total cholesterol and triglyceride concentration by -13.46 % and -8.06% compared to placebo, respectively. HDL-cholesterol level was also found to significantly increase by 3% (Pandor et al. 2009). In our study the reduced cholesterol altering effect of ezetimibe can be explained by our study population. Other studies have investigated the effects of ezetimibe in patients with diverse lipoprotein abnormalities (e.g. type 2 diabetes and mixed hyperlipidemia), while we enrolled a specific statin-intolerant hyperlipidemic population.

Firstly, Bays et al. (2001) established that the maximum cholesterol-lowering effect of ezetimibe evolved the first 2 weeks, and then it remained the same. So far, most trials have followed the ezetimibe efficacy only for 12 weeks. Though in short-term (12 weeks) trials the safety profile of ezetimibe monotherapy seems to be like placebo's, there has been limited information about the long-term safety of ezetimibe (Pandor et al. 2009). In previous trials only isolated case reports of myopathy have been described with ezetimibe (Dujovne et al. 2002). In our study a one-year ezetimibe treatment was examined, and it was well tolerated.

We found significantly reduced hsCRP level after 3 and 6 months of ezetimibe monotherapy, but we could not find significant difference after 12 months in all patients. The studied *NPC1L1* polymorphism did not alter the effect of ezetimibe on CRP level significantly. In former trials ezetimibe monotherapy decreased hsCRP level compared with placebo, but the differences had generally not been found to be statistically significant as well (Pearson et al. 2007).

Some limitations of the study can be noted. The power of the study may be reduced because of the relatively small sample size. Presumably, in a larger study population -133 A > G *NPC1L1* polymorphism could significantly influence the efficacy of ezetimibe on further lipid parameters, especially HDL-C. Data on the efficacy of cholesterol absorption and distribution of HDL subfractions would improve our knowledge about ezetimibe effect on lipoproteins and could demonstrate the supposed functional consequences of the c.-133A > G *NPC1L1* polymorphism as well.

In conclusion *NPC1L1* -133A > G SNP influences the ApoA1 response to 10 mg daily ezetimibe monotherapy, therefore, may alter the effect of ezetimibe on the structure and function of the HDL particles. Further studies are required to prove that SNP -133A > G ezetimibe treatment induce structural and functional changes in the HDL particles, therefore alters its anti-atherogenic properties.

4. Experimental

4.1. Laboratory measurements

At baseline and after 3, 6 and 12 months of treatment with ezetimibe, after 12 h of fasting, a 10-ml venous blood sample was taken between 07.30 and 08.00 a.m. Lipid parameters were determined from fresh serum.

Serum cholesterol and triglyceride levels were measured using enzymatic, colorimetric tests (GPO-PAP, Modular P-800 Analyzer; Roche/Hitachi, Basel, Switzerland), whereas HDL-C was assessed by homogenous, enzymatic, colorimetric assay (Roche HDL-C plus 3rd generation). LDL-C was measured by homogenous, enzymatic, colorimetric assay (Roche LDL-C plus 2nd generation, Basel, Switzerland). Apolipoprotein examination was performed by immunoturbidimetric assay Tina-Quant APO A (Version 2; Roche), Tina-Quant APO B (Version 2; Roche). Accuracy ((mean/target)x100) was in the range 97.3–106 %, precision (run to run CVa) of lipid measurements was in the range 1.39–5.15% on Cobas6000 analyzer (Roche).

Creatine-kinase activities were determinetd by UV kinetic assay (CK liquid), in accordance with the IFCC recommendations on Roche/Hitachi Modular P800 analyser and C-reactive protein (CRP) was analysed on the same equipment by immunoturbidimetric assay (CRPLX).

4.2. NPC1L1 genotype analysis

Genomic DNA was isolated from EDTA (ethylenediaminetetraacetic acid) or citrate-anticoagulated blood, using the QIAamp Blood Mini Kit (Qiagen GmbH, Hilden, Germany).

The presence of the c.-133A > G polymorphism (rs17655652) was tested by BlpI (New England Biolabs, Ipswich, MA) enzyme digestion of PCR-amplified products. A 365-bp fragment of the *NPC1L1* gene was amplified using primers NPC1L1rs17655652F (5'- GAC CCT AGC ACC TGC GTG ATG A -3') and NPC1L1rs17655652R (5'- GTA ACG CTC GCC TGG TAC ACG G -3'). In homozygous wild type samples, digestion with BlpI yielded a 272-bp and a 93-bp restriction fragment while this BlpI site is lost in the case of the mutated allele.

4.3. Statistical methods

Statistica for Windows 6 and IBM Statistical Package for the Social Sciences (SPSS) Statistics Version 19 computer softwares were used for statistical analysis. Normality of distribution was tested by the Kolmogorov-Smirnov test. In case of normal distribution the differences between anthropometry and laboratory parameters in control and patient groups were analyzed with one-way analysis of variance (ANOVA), followed by post hoc comparisons using the Newman-Keuls test. In cases of non-normal distributions the differences between groups were compared with Kruskal-Wallis and Mann-Whitney U tests. Data were expressed as means ± standard deviation (S.D.) in case of normal distribution, and median (lower/upper quartile) in case of non-normal distribution. A value of $P < 0.05$ was considered to be statistically significant.

The genotype-dependence of changes was analyzed by Welch's robust test. The frequencies of complications in persons with various genotypes and alleles were compared using the chi-square and Fisher's exact test.

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