VARIANT WITHIN *CELSR2/PSRC1/SORT1*, BUT NOT WITHIN *CILP2/PBX4*, *PCSK9* AND *APOB* GENES, HAS A POTENTIAL TO INFLUENCE STATIN TREATMENT EFFICACY

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Summary

Statins have become a cornerstone of cardiovascular prevention. However, their lipid lowering efficacy and, thus, also impact on event risk reduction, differ substantially between individuals. Major part of this interindividual difference can be explained by genetic factors. Using the GWA approach candidate genes that may modify the response to statin treatment have been detected. Variants rs646776 (*CELSR2/PSRC1/SORT1*), rs16996148 (*CILP2/PBX4*), rs11206510 (*PCSK9*) and rs693 (*APOB*) were analyzed in 370 (146 males) dyslipidemic patients treated with statins (46.6% simvastatin, 41.5% atorvastatin, 11.9% lovastatin, 10 or 20mg/day) and 470 normolipidemic controls (188 males). Lipid levels were available prior and after 8-12 weeks of therapy. There was a significant decrease both in total (7.36±1.28→5.43±1.01 mmol/L) and LDL-cholesterol (4.72±1.35→3.19±0.98 mmol/L) after treatment. Genotype frequencies of the three SNPs differed between patients and controls (rs646776, rs16996148, rs693). The carriers of minor rs599838 genotype had significantly lower response to statin treatment compared to common homozygotes (LDL cholesterol, Δ –20.3% vs. Δ –32.0%). No other significant associations with lipid changes were detected. Together with variations of multiple other gene loci the variant at *CELSR2/PSRC1/SORT1* gene cluster may be useful for individualization of statin treatment leading to better outcomes of the treatment.

Key words: dyslipidemia; statins; gene variants; pharmacogenetics; treatment efficacy; *CELSR2/PSRC1/SORT1*; *CILP2/PBX4*; *PCSK9*; *APOB*

Abbreviations: CELSR2/PSRC1/SORT1, cadherin egf lag seven-pass g-type receptor 2/ proline/serine-rich coiled-coil protein 1/sortilin1; CILP2/PBX4, cartilage intermediate layer protein 2/ pre-b-cell leukemia transcription factor 4; PCSK9, proprotein convertase subtilisin/kexin-type 9; GWA, genome wide association;

INTRODUCTION

Inhibitors of hydroxy-methylglutaryl coenzyme A reductase (statins) have become a cornerstone of cardiovascular prevention over past two decades. Through reduction of plasma atherogenic lipoprotein levels together with plenty of other pleiotropic effects statins reduce the risk of cardio- as well as cerebrovascular events in a broad spectrum of population groups (Sadowitz et al. 2010).

However, lipid lowering efficacy of statins varies widely among individuals – thus the use of the same statin in different patients produces LDL-cholesterol lowering between 8 and 55%, triglyceride (TG) reduction of 7 to 30% and HDL-cholesterol rising from 0 to 10% (Sever et al. 2003). Also the time to reach maximum efficacy differs significantly between individuals (Hachem and Mooradian 2006). On the other hand, the individual response does not significantly change over time and it is most likely to have genetic background. Gene variants impact both the pharmacokinetics (e.g. genes encoding for statin metabolism or transmembrane transport proteins) and pharmacodynamics (e.g. hydroxy-methylglutaryl coenzyme A reductase and cholesterol-7alpha hydroxylase genes, as key enzymes in cholesterol homeostasis) of statins.

It is evident that heterogeneity of statin effects on plasma lipid and lipoprotein levels results in heterogeneous impact of treatment on event rates. Thus, it seems obvious that genetic determination of greater efficacy of particular statin type translates in improved prognosis of a patient compared with another statin. Understanding the genetic determination of statin treatment efficacy would enable improved targeting of treatment and individualization of expensive therapy. Moreover, selecting the most effective statin type for an individual based on his/her genetic equipment would lead to reduction of doses necessary to achieve target levels of atherogenic lipoproteins. This should produce another benefit of such an approach-reduction in incidence and severity of side effects. However, nowadays this approach cannot be used in daily clinical practice as the data on genetic background of statin action in the body is limited. Therefore, identification of gene variants responsible for the observed heterogeneity of statin effects represents a promising strategy to shift current limits of this therapy in terms of cardiovascular risk reduction. It is evident the genetic determination of statin treatment efficacy is under polygenic control with significant influences of environmental (most importantly dietary) factors (Mangravite and Krauss, 2007; Maggo et al. 2011). Thus, the most feasible approach to studying

pharmacogenetics of statin therapy is testing multiple variants in selected genes with plausible roles in statin processing within the body. Recently published results of genome wide association studies (Kathiresan et al. 2008; Sandhu et al. 2008) have revealed several gene regions that significantly influence plasma cholesterol levels. It is possible, that these genes have also a potential to modulate the final impact of statin treatment on lipoprotein levels in the plasma. These new genes or newly detected variants within the well known and characterized genes include *CELSR2/PSRC1/SORT1* (rs646776), *CILP2/PBX4* (rs16996148), *APOB* (rs693) and *PCSK9* (rs11206510).

To evaluate the putative role of gene variants within these newly identified gene regions in the modification of individual treatment response to statins we conducted a retrospective study in a cohort of lipid clinic patients treated with statins.

PATIENTS AND METHODS

Patients selection

Patients with primary dyslipidemia indicated to statin treatment were retrospectively selected from databases of Lipid Clinics of the 3rd Department of Internal Medicine of the 1st Faculty of Medicine, Charles University and the Institute for Clinical and Experimental Medicine, Prague, the Czech Republic. Three hundred seventy patients were included, average age 59.3 ± 12.7 years (146 males, aged 56.3 ± 12.6 years and 224 females, aged 61.4 ± 12.3 years), 23.0% were diabetics and 48.9% had hypertension. All patients received a standardized lifestyle advice at their first visit to the clinics and were instructed to maintain low-cholesterol diet according to the standardized education provided by an experienced dietitian. Table 1 shows the baseline characteristics of the study group. We compared the pre-treatment lipid levels with the first values obtained after initiation of statin treatment, usually after 12 weeks (range 10 to 13 weeks) of therapy. Patients taking simvastatin (46.6%), atorvastatin (41.5%) and lovastatin (11.9%) in doses of 10 (~90% of individuals) or 20 mg/day were enrolled in the study. We did not include subjects on combination lipid-lowering therapy (e.g. statin-fibrate, statin-ezetimibe) and those who experienced weight loss of more than 5% between visits suggesting a substantial impact of lifestyle changes. Also, the individuals fulfilling the clinically and laboratory criteria of familial hypercholesterolemia were not included to the study.

Controls selection

As a control group, a subset of 470 individuals (188 males and 282 females) selected from the Czech post-MONICA (MONItoring of CArdiovacular disease) study (2559 individuals, 1191 males, average age 49 years) was used (Thunsdall-Pedoe et al. 2003). The selection criteria were i) no history of cardiovascular disease, ii) no lipid-lowering treatment and iii) the plasma lipid values below 5.0 mmol/L for total cholesterol, below 2.0 mmol/L for plasma TG and over 0.75 mmol/L (for males) or 0.8 mmol/L (for females) for HDL-cholesterol (Table 1).

All participants of the study were of Caucasian ethnicity from the Central European Czech population. Written informed consent was obtained from all the study participants and the local ethics committee approved the design of the study according to the Declaration of Helsinki of 1975.

Genotype analysis

Three millilitres of whole blood collected into EDTA tubes for DNA isolation were stored at -20˚C.

The DNA was isolated using the standard salting out method (Miller et al. 1988) and individual variants of four gene loci (rs646776 - *CELSR2/PSRC1/SORT1*, rs16996148 - *CILP2/PBX4*, rs11206510 - *PCSK9*, rs693 - *APOB*) were genotyped using polymerase chain reaction (PCR) and restriction analysis. PCR device DYAD (MJ Research, Waltham, MA) was used to perform the PCR reaction in a total volume of 25 μL. DNA was amplified under the following conditions: initial denaturation of 96°C for 3 min, followed by 35 cycles of 95°C for 15 sec, appropriate annealing temperature for 30 sec and 72°C for 30 sec. The last amplification step was extended for 3 min at 72°C. A 10 μl of PCR product was digested in a total volume of 25 μl with appropriate restriction enzyme at 37°C overnight in the buffer provided by the manufacturer. For more details regarding the PCR conditions, oligonucleotides and restriction enzymes used, see Table 2. Restriction fragments were separated on 10% PAA gel using the MADGE technique (Day and Humphries 1994).

Analysis of plasma lipids

The lipoprotein parameters in fasting plasma samples were assessed using autoanalyzers and conventional enzymatic methods with reagents from Boehringer Mannheim Diagnostics and Hoffmann-La Roche in CDC Atlanta accredited local laboratories.

Statistical analysis

The Hardy-Weinberg test (http://www.tufts.edu/~mcourt01/Documents/Court%20lab%20-%20HW%20calculator.xls) was applied to confirm the independent segregation of the alleles. Chi-square test, ANOVA and ANCOVA for adjustments were used for statistical analysis. All tests were two tailed and the significance level 2 alpha = 0.05 was considered to be significant. Differences in lipid decreases were expressed and analyzed in per cent of the decrease. The changes of plasma lipids were compared between subjects with different genotypes for individual polymorphisms.

RESULTS

Basic characteristics

As expected, there was a significant decrease both in total (7.36 ± 1.28 → 5.43 ± 1.01 mmol/L) and LDL-cholesterol (4.72 ± 1.35 → 3.19 ± 0.98 mmol/L) after treatment (Table 1). The cholesterol decrease was independent (ANCOVA; both for total cholesterol and for LDL-cholesterol) on the type of statin and the dose, most likely because of the relative low number of patients included, and as the majority of the patients was treated with the lowest dose of statins.

The call rates for the individual variants vary between 94.1% for the rs11206510 within the *PCSK9* gene in patients and 98.5% for the rs16996148 within the *CILP2/PBX4* cluster in controls.

In the entire population, the allelic frequencies of individual polymorphisms were comparable with the so far published frequencies obtained in other Caucasian populations. Hardy-Weinberg test confirmed independent segregation of individual alleles with two exceptions (rs16996148 in controls and rs693 in patients). These differences could be easily explained as the groups had not been selected as representative general population samples. No gender differences in genotype frequencies were observed either in the patients or in controls (data not shown).

Genotype differences between the analyzed groups

The genotype frequencies were significantly different between the patients and controls for three out of four analyzed variants (Table 3). The largest difference was observed for the rs16996148 variant within the *CILP2/PBX4* gene cluster. We did not detect (chi-square) any homozygous carriers of the less common T allele among the dyslipidemic patients. Furthermore, also the frequencies of the *CELSR2/PSRC1/SORT1* (rs599838) and *APOB* (rs693) genotypes differed significantly between the analysed groups and, thus, confirm the important role of these SNPs in determination of plasma lipid levels.

Associations between the SNPs and statin treatment efficacy

The carriers of minor rs599838 genotype within the *CELSR2/PSRC1/SORT1* cluster had significantly lower response to statin treatment compared to common homozygotes (LDL cholesterol, Δ –20.3% vs. Δ –32.0 %, significant both for unadjusted and adjusted for sex and age values, ANOVA) with heterozygotes having similar decrease to the common homozygotes (Δ –28.9%).

For any of the other variants analyzed, we did not find a significant association between the genetic polymorphism and changes of plasma lipid levels induced by statin therapy (decrease of total or LDL- cholesterol, triglycerides; increase of HDL-cholesterol) (Table 4).

DISCUSSION

In adult dyslipidemic patients of Slavonic Caucasian descent we have detected a significant effect of the SNP within the *CELSR2/PSRC1/SORT1* gene cluster on statin treatment efficacy. Presence of the less frequent genotype was associated with approximately 30% reduction of LDL-lowering efficacy of statins. No significant effect of the variants within genes/gene clusters for *CILP2/PBX4*, *PCSK9* and *APOB* on statin mediated lipid decrease was observed. In subgroups divided according to genotypes of these SNPs, even no trends were detectable.

In three out of the four analyzed variants (*CILP2/PBX4*, *CELSR2/PSRC1/SORT1* and *APOB* regions) we have detected significant differences in genotype frequencies between analyzed groups. This confirms the role of these variants in genetic determination of plasma lipid levels, as detected through the GWA studies on (mostly) west European samples, also in the central European

Slavonic population. The highest difference was observed for the rs16996148 (*CILP2/PBX4*) variants. In this case, the minor TT homozygotes were not detected among the patients with dyslipidemia, which suggests that these individuals could be protected against the development of dyslipidemia. The difference within the last gene, *PCSK9*, remains just below the arbitrary recognised value for statistical significance, so it is very likely in a study with slightly higher number of participants, also the role of this SNP in determination of plasma lipids would be confirmed.

Loci we have studied include both well known genes with a clear link to plasma lipid values and also newly detected loci without well established mechanisms influencing plasma lipid regulation. The first group is represented by the *APOB* (apolipoprotein B is a major protein component of LDL particles) (Benn 2009) and *PCSK9* (serine protease that reduces both hepatic and extrahepatic LDL receptor levels) (Davignon et al. 2010) genes. The second group of genes studied, with rather unclear mechanisms affecting plasma lipid concentrations, was represented by two gene clusters – the variants being located within the intergenic regions of the *CELSR2* (Waterworth et al. 2010) and *CILP2* (Seki et al. 2005) gene clusters. At time of their first description, the genes located within these clusters had no known association with the metabolism of plasma lipids.

However, only very recently, SORT1, a member of the CELSR2 gene cluster (in which we have detected a potential to influence the treatment efficacy of statins), was described as an intracellular receptor for the APOB. It interacts with APOB at the apparatus of Golgi and facilitates the hepatic transport of APOB containing lipoproteins (Kjolby et al. 2010).

Variation of the three new gene loci modulating concentrations of plasma lipoproteins (and thus contributing to the development of dyslipidemia) did not significantly influence therapeutic response to statin treatment. Despite the new gene loci have been repeatedly shown to determine plasma lipid levels (Kathiresan et al. 2008; Sandhu et al. 2008; Aulchenko et al. 2009), their contribution to interindividual variability of the final impact of statin therapy on lipoprotein concentrations seems to be neglectable. This holds true not only for the individual variants but also for their combinations.

The observed lack of association could be explained by the fact, that there is not a physiological link to the pathway(s) involved in the metabolism or transport of statins. In general, these pathways are supposed to be more likely affecting statin

treatment efficacy (or there is a greater chance to detect such an effect), as they are less prone to environmental modifications. Variations we have studied potentially impact pathways that involve transport proteins or enzymes directly linked to processing of different lipoprotein subpopulations (mostly LDL and TG-rich particles) and not to metabolism or transport of statins.

Another possibility is a small magnitude of the modifying effect, which could not have been detected due to the relatively small sample size. However, the observed differences did not suggest even a trend towards a difference between the genotypes. Thus, it seems unlikely even a substantially increased sample size could enable identification of, possibly modest, modifying effects.

The increasing popularity of genome wide association studies (Rosenberg et al. 2010) leading to identification of some very interesting and powerful genetic determinants not just in cardiovascular field (Wang et al. 2010; Musunuru and Kathiresan 2010; Welcome Trust Case Control Consortium 2007), has also its pitfalls. Surprisingly, there is so far a substantial lack of the replication studies performed or published, despite the fact, that original GWAs include usually very high numbers of individuals, but without detailed analyses of interethnic or even international differences. Also, the gene-gene or gene-environment interactions have never been analyzed in these studies. Therefore, we have to keep in mind even the effects of SNPs detected through the GWAs approach do not need to be generally applicable. As an example of the context dependent effect of a gene, we were not able to confirm the association between the most powerful genetic determinant of plasma TG levels detected so far (Kooner et al. 2008), the *MLXIPL* variant, in a study with sufficient power (Vrablik et al. 2008). One of the explanations maybe the different genetic and/or environmental background between the west European/German and central European/Slavonic populations. On the other hand, the same variant was associated with plasma TG levels in a Japanese population (Nakayama et al. 2009). However, the generally higher plasma TG levels in the Czech population at large could be the reason why the attempts to replicate the original results in other studies have failed.

The impact of different genetic polymorphisms on statin induced changes of lipid levels has been analyzed in several clinical trials. So far, single nucleotide polymorphisms (SNPs) in more than 30 different genes have been examined (Mangravite and Krauss, 2007; Maggo et al. 2011) but the results were not replicated

in larger patient groups and also the magnitude of impact on statin efficacy was small. It needs to be mentioned, that only the impact of the apolipoprotein E gene on statin treatment efficacy was analyzed in more studies with sufficient power, and however, even these results are far from being consistent. Other genes analyzed include for example apolipoprotein A5 (Hubacek et al. 2009), cholesterol 7 alpha hydroxylase (Kajinami et al. 2005) and apolipoprotein E (Hubacek and Vrablik, 2011). The expanding knowledge in this field is quickly growing, but, so far, it is not sufficient to be used in clinical practice.

At present, we are beginning to unveil the genetic determination of statin treatment efficacy (Ordovas and Mooser, 2002; Mangravite et al. 2010). Generally, it is of outstanding interest to understand the genetic background of a drug efficacy, as we frequently do not have any clinical, biochemical or anthropometrical tests to predict the effects of pharmacotherapy. Assessing individual efficacy of a drug before exposure can be done only by interdisciplinary connection of human medicine and genetic analysis – through the biomedicine research (Berger, 2011). Such examination would have a potential to detect the hyper- and hypo- responders and, moreover, identification of those at high risk of side effects. Thus, the results of genetic analyses will help us select the most effective and, at the same time, safest treatment alternative for the individual patient. The economical and health benefits of this approach are evident. Given the statins belong to the most widely used drugs worldwide improved targeting of their use and identification of the most suitable statin type using a genetic test represents a very attractive approach. A recent meta-analysis shown statins reduce the cardiovascular risk by approximately 20% per each 1 mmol/L reduction of LDL-cholesterol levels. This should translate to 40-50% risk reduction when 2-3 mmol/L LDL-cholesterol decreased is achieved (Baigent et al, 2010). However, as highlighted recently by so called Residual Risk Reduction Initiative on average only 30% risk reduction with statin treatment is being achieved (Fruchart et al., 2008). Improving statin treatment efficacy and safety by genetic testing might be another way of shifting the current limits of the treatment towards greater reduction in event rates, cardiovascular morbidity and, most importantly, also mortality.

To accomplish this ultimate goal, comprehensive research in large populations studying impact of combinations of gene variants is warranted to broaden our understanding of determination of statin treatment efficacy.

Our results confirm the notion that the roles of new gene loci identified through genome wide association studies should be replicated in focused smaller study settings but with more detailed biochemical, anthropometrical and lifestyle information. Only such studies allow assessing their contribution to modulation of lipid metabolism as well as determination of their role in pharmacogenetics, nutrigenetics or actigenetics. Our study has detected a potential of the variant within the *CELSR2/PSRC1/SORT1* gene cluster, but not within *CILP2/PBX4*, *PCSK9* and *APOB* gene loci to significantly impact on statin treatment efficacy.

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Table 1. **Basic characteristics of the analyzed patients treated with statins and healthy controls.** Individual values are given for the patients before and after the statin treatment. Asterics indicate statistical significant differences between the values obtained before and after statin treatment in the patients group only.

Character	Patients		Controls
Number	370		470
Age	59.3 ± 12.7		42.5 ± 10.2
% of males	35.4		40
	Before	After	
Total cholesterol *	7.36 ± 1.29	5.43 ± 1.00	4.35 ± 0.42
LDL cholesterol *	4.72 ± 1.35	3.18 ± 0.98	n.a.
HDL cholesterol *	1.53 ± 0.48	1.49 ± 0.40	1.38 ± 0.35
Triglycerides *	2.16 ± 1.22	1.66 ± 0.92	1.03 ± 0.37
Smoking prevalence	25.7 %		24.9 %
Diabetes prevalence	23.0 %		1.5 %
Hypertension	48.9 %		16.8 %

Table 2 **Primer sequences, restriction enzymes and size of the restriction fragments used for detection of polymorphisms of interrest.**

Polymorphism	Primer sequence	Annealing temperature	PCR product	Enzyme	Size (bp)	Allele
CILP2/…	5’ tgg ctc ttg tcc act ggc cac atc ccc	70°C	135 bp	Hin1II	137	G
rs16996148	5’ ttc tcc cat gcc tcc agg ccc cca ag				82+54	T
Apo B	5’aga gga aac caa ggc cac agt tgc	57.5°C	163 bp	XhoI	136	C
rs693	5’ tac att cgg tct cgt gta tct tct				110+26	T
CELSR2/…	5’ atc cag cta ttt ggg agc agt gtc ctg g	66°C	137 bp	Hin1II	139	A
rs646776	5’aag gtc tgg tct ctg gaa aac aga ag				107+32	G
PCSK9	5’ tcc agc att gcc agc ttc tct gtc tc	68.9°C	130 bp	Hin6I	130	T
rs11206510	5’ agc caa aga cgg cca cca cag aca gc				104+26	C

Table 3 **Genotype distributions within the analyzed groups**. Differences between the groups were calculated by chi-square. Frequencies of variants marked by * are signicicantly different between the groups.

CILP2/PBX4						
Rs16996148 *	GG		GT		TT	
	N	%	N	%	N	%
Patients	322	89.4	38	10.6	0	0.0
Controls	368	79.5	81	17.5	14	3.0

Apolipoprotein B						
rs693 *	CC		CT		TT	
	N	%	N	%	N	%
Patients	72	20.2	204	57.3	80	22.5
Controls	126	27.9	238	52.8	87	19.3

CELSR2/PSRC1/SORT1						
rs646776 *	TT		TC		CC	
	N	%	N	%	N	%
Patients	242	67.8	102	28.5	13	3.6
Controls	256	57.5	176	39.6	23	5.2

PCSK9						
rs11206510	TT		TC		CC	
	N	%	N	%	N	%
Patients	236	67.8	95	27.3	17	4.9
Controls	295	65.1	147	32.5	11	2.4

Table 4 **Changes of the lipid parameters according the individual genotypes.** Percentages of the decrease of plasma cholesterol in different fractions and plasma TG levels were calculated from the baseline values (before treatment) for each patient. Significant difference was observed for the rs646776 variant and is indicated by asterix.

CILP2/PBX4		
rs16996148	GG	GT
T-C	25.5 ± 11.9	25.1 ± 10.9
LDL-C	29.3 ± 17.0	31.6 ± 15.5
HDL-C	0.3 ± 18.8	-1.8 ± 20.2
TG	17.5 ± 29.1	16.4 ± 27.5

Apolipoprotein B			
rs693	CC	CT	TT
T-C	23.5 ± 12.4	26.4 ± 11.6	24.5 ± 10.8
LDL-C	29.4 ± 16.3	31.2 ± 17.8	30.3 ± 15.6
HDL-C	-3.3 ± 20.7	0.9 ± 19.5	0.4 ± 16.7
TG	16.3 ± 27.9	16.1 ± 32.6	21.4 ± 26.6

CELSR2/PSRC1/SORT1			
rs646776	TT	TC	CC
T-C	25.8 ± 11.3	25.1 ± 12.3	23.0 ± 13.4
LDL-C *	32.0 ± 16.5	28.9 ± 17.7	20.3 ± 20.1
HDL-C	0.4 ± 19.2	0.0 ± 20.3	-3.0 ± 16.1
TG	17.2 ± 31.7	18.5 ± 25.9	22.6 ± 25.2

PCSK9			
rs11206510	TT	TC	CC
T-C	24.9 ± 11.6	24.2 ± 10.9	24.7 ± 11.9
LDL-C	29.1 ± 17.4	29.5 ± 16.8	24.9 ± 16.1
HDL-C	0.9 ± 17.6	-1.4 ± 19.8	3.9 ± 20.8
TG	18.9 ± 26.5	13.9 ± 30.1	25.5 ± 24.3