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ORIGINAL ARTICLE

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. The major part of this inter-individual 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 analysed in 370 (146 males) dyslipidemic patients treated with statins (46.6% simvastatin, 41.5% atorvastatin, 11.9% lovastatin, 10 or 20 mg/day) and 470 normolipidemic controls (188 males). Lipid levels were available prior to and after 8–12 weeks of therapy. There was a significant decrease both in the 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. The genotype frequencies of the three SNPs differed between patients and controls (rs646776, rs16996148, rs693). The carriers of the minor rs599838 genotype had a 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 other, multiple 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*

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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 the past two decades. Through the reduction of plasma atherogenic lipoprotein levels together with a number 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, the 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 a 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 the heterogeneity of statin effects on plasma lipid and lipoprotein levels results in the heterogeneous impact of treatment on event rates. Thus, it seems obvious that the genetic determination of the greater efficacy of particular statin type translates into the 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 a reduction in the doses necessary to achieve target levels of atherogenic lipoproteins. This should produce another benefit of such an approach – a reduction in the incidence and severity of side effects. However, nowadays this approach cannot be used in daily clinical practice as the data on the genetic background of statin action in the body is limited. Therefore, identification of the 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 that 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 the pharmacogenetics of statin therapy is the testing of the 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 also have the 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

Patient selection

Patients with primary dyslipidaemia 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, in Prague. Three hundred and seventy patients were included, the average age was 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 standardized lifestyle advice at their first visit to the clinics and were instructed to maintain a 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, individuals fulfilling the clinically and laboratory criteria of familial hypercholesterolemia were not included in 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) 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 the polymerase chain reaction (PCR) and restriction analysis. A PCR device DYAD (MJ Research, Waltham, USA) 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 an appropriate restriction enzyme at 37 °C overnight in the buffer provided by the manufacturer. For more details regarding 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 autoanalysers and conventional enzymatic methods with reagents from Boehringer Mannheim Diagnostics (Mannheim, Germany) and Hoffmann-La Roche (Basel, Switzerland) in CDC (Atlanta, USA) accredited local laboratories.

Statistical analyses

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. The Chi-square test, ANOVA and ANCOVA for adjustments were used for statistical analysis. All tests were two tailed and a significance level 2 α=0.05 was considered to be significant. Differences in lipid decreases were expressed and analysed 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 the 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) of the type of statin and the dose, most likely because of the relatively low number of patients included, and as the majority of the patients were treated with the lowest dose of statins.

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

In the entire population, the allelic frequencies of individual polymorphisms were comparable with the so far published frequencies obtained in other Caucasian populations. The Hardy-Weinberg test confirmed the independent segregation of individual alleles with two exceptions (rs16996148 in controls and rs693 in patients). These differences could easily be 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 the controls (data not shown).

Genotype differences between the groups analysed

The genotype frequencies were significantly different between the patients and controls for three out of the four analysed 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 dyslipidaemic patients. Furthermore, the frequencies of the *CELSR2/PSRC1/SORT1* (rs599838) and *APOB* (rs693) genotypes also differed significantly between the analysed groups and, thus, confirm the important

Table 1. **Basic characteristics of the analysed patients treated with statins and healthy controls.** Individual values are given for the patients before and after statin treatment.

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%

***** statistically significant vs values obtained before treatment

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

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
APOB	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 groups analysed.** Differences between the groups were calculated by chi-square. Frequencies of variants marked by * are statistically significant.

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 *	AA		AG		GG	
	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

role of these SNPs in the determination of plasma lipid levels.

Associations between the SNPs and statin treatment efficacy

The carriers of the minor rs599838 genotype within the *CELSR2/PSRC1/SORT1* cluster had a 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 a decrease similar to the common homozygotes (Δ –28.9%).

To turn to the other variants analysed, 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).

Table 4. **Changes of the lipid parameters according to 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 asterisks.

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	AA	AG	GG
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

DISCUSSION

In adult dyslipidaemic 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. The presence of the less frequent genotype was associated with an approximately 30% reduction of the 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, not even trends were detectable.

In three out of the four analysed variants (*CILP2/PBX4*, *CELSR2/PSRC1/SORT1* and *APOB* regions) we detected significant differences in genotype frequencies between the groups analysed. This confirms the role of these variants in the genetic determination of plasma lipid levels, as detected through GWA studies on (mostly) west European samples, but also in the central European Slavonic population. The highest difference was observed for the rs16996148 (*CILP2/PBX4*) variant. In this case, the minor TT homozygotes were not detected among the patients with dyslipidaemia, which suggests that these individuals could be protected against the development of dyslipidaemia. The difference in the last gene, *PCSK9*, remains just below the arbitrary recognised value for statistical significance, so it is very likely that in a study with a slightly higher number of participants, the role of this SNP in determination of plasma lipids would also be confirmed.

The 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* gene (apolipoprotein B is a major protein component of LDL particles) (Benn 2009) and the *PCSK9* gene(serine protease that reduces both hepatic and extrahepatic LDL receptor levels) (Davignon et al. 2010). 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 the 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 the therapeutic response to statin treatment. Although 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 the inter-individual variability of the final impact of statin therapy on lipoprotein concentrations seems to be negligible. 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 no physiological link to the pathway(s) involved in the metabolism or transport of statins. In general, these pathways are supposed to be more likely to affect statin treatment efficacy (or there is a greater chance of detecting 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 the processing of different lipoprotein subpopulations (mostly LDL and TG-rich particles) and not to the metabolism or transport of statins.

Another possibility is the small magnitude of the modifying effect, which could not be detected due to the relatively small sample size. However, the observed differences did not even suggest a trend in the difference between the genotypes. Thus, it seems unlikely that even a substantially increased sample size would enable the 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 the cardiovascular field (Welcome Trust Case Control Consortium 2007, Musunuru and Kathiresan 2010, Wang et al. 2010), has also its pitfalls. Surprisingly, there is so far a substantial lack of replication studies performed or published, despite the fact that original GWAs usually include 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 analysed in these studies. Therefore, we have to keep in mind that the effects of SNPs detected through the GWA approach do not need to be generally applicable. As an example of the context dependent effect of a gene, we were not able in our study to confirm with a sufficient degree

of certainty the association with the most powerful genetic determinant of plasma TG levels detected so far (Kooner et al. 2008), the *MLXIPL* variant (Vrablik et al. 2008). One of the explanations is 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 analysed 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 has been analysed in other studies with sufficient power, and even these results are far from being consistent. Other genes analysed 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 knowledge in this field is quickly expanding, but, so far, it is not sufficient to be used in clinical practice.

We are beginning to unveil the genetic determination of the efficacy of statin treatment (Ordovas and Mooser 2002, Mangravite et al. 2010). Generally, it is of outstanding interest to understand the genetic background of the efficacy of a drug, as we frequently do not have any clinical, biochemical or anthropometrical tests to predict the effects of pharmacotherapy. Assessing the individual efficacy of a drug before exposure can be carried out only by the interdisciplinary connection of human medicine and genetic analysis – through biomedical 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 economic and health benefits of this approach are evident. Given that statins are among 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 showed that statins reduce 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 the so called Residual Risk Reduction Initiative only 30% risk reduction with statin treatment is being achieved on average (Fruchart et al. 2008). Improving the efficacy and safety of statin treatment 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 the impact of combinations of gene variants is warranted to broaden our understanding of the 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 more focused, smaller study settings but with more detailed biochemical, anthropometrical and lifestyle information. Only such studies would allow an assessment of their contribution to the modulation of lipid metabolism as well as a 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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