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Pharmacogenomics

Identifying genetic modulators of statin response using subject-derived lymphoblastoid cell lines

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Although statins (3-hydroxy-3-methylglutaryl-CoA reductase inhibitors) have proven effective in reducing plasma low-density lipoprotein levels and risk of cardiovascular disease, their lipid lowering efficacy is highly variable among individuals. Furthermore, statin treatment carries a small but significant risk of adverse effects, most notably myopathy and new onset diabetes. Hence, identification of biomarkers for predicting patients who would most likely benefit from statin treatment without incurring increased risk of adverse effects can have a significant public health impact. In this review, we discuss the rationale for the use of subject-derived lymphoblastoid cell lines in studies of statin pharmacogenomics and describe a variety of approaches we have employed to identify novel genetic markers associated with interindividual variation in statin response.

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The most well understood mechanism by which statins reduce cardiovascular disease (CVD) risk is by lowering plasma concentrations of low-density lipoproteins (LDLs). Statins function as competitive inhibitors of HMGCR, a rate-limiting enzyme in the cholesterol biosynthesis pathway, thereby, blocking production of both cholesterol and the metabolic intermediates of the mevalonate pathway. Cholesterol depletion triggers the activation of SREBF2 (aka SREBP2) signaling pathway, resulting in the upregulation of cholesterol synthesis and uptake genes including *LDLR*, which mediates clearance of LDL particles from the plasma. LDL lowering can also be attributed to the reduction of hepatic lipoprotein secretion into the plasma as a result of inhibited cholesterol production. Statin pharmacodynamics and pharmacokinetics have been previously reviewed [1], and pathways are available on the pharmacogenetics and pharmacogenomics knowledge base (PharmGKB) [2]. Statins also exert pleiotropic cardioprotective effects such as improving endothelial function, increasing nitric oxide production, alleviating oxidative stress, reducing inflammatory markers and inhibiting platelet adhesion and the coagulation cascade [3–6].

While the clinical benefit of statin therapy is well established, there remains residual risk for CVD among statin-treated patients [7]. Contributing to this is considerable interindividual variation in LDL reduction, most commonly assessed clinically by measurement of LDL-cholesterol (LDL-C). Moreover, there is modest but significant potential for statin adverse effects, in particular myopathy [8,9] and new onset Type 2 diabetes [10,11]. However, there is limited information regarding the basis for these varying outcomes. With about half of Americans 40–75 years of age eligible for statin treatment for CVD prevention according to 2018 American Heart Association/American College of Cardiology guidelines for cholesterol management [12], there is a pressing need for the development of precision medicine standards for statin therapy. Studies of statin pharmacogenomics aim at identifying genetic biomarkers for the prediction of patients' responses to statin intervention, ultimately allowing individualization of treatment with the goals of maximizing the CVD benefit and minimizing the development of adverse effects.

A concern regarding the search for genetic predictors of statin LDL-C response is that its genetic heritability has been reported to be modest [13]. Nevertheless, identifying specific genetic biomarkers contributing to this heritability

may be of value in identifying pathways mediating variation in LDL-C response that may also be impacted by nongenetic factors. To date, genome-wide association studies (GWAS) have identified genetic variants associated with LDL-C statin response in or near *APOE*, *LPA*, *SLCO1B1*, *ABCG2* and *SORT1/CELSR2/PSRC1* [14–17]. Taken together, these account for about 5% of the variance in LDL-C statin response [17] and hence, no more than half of the estimated genetic heritability. While additional genetic variants may be detected by GWAS using larger cohort and/or rare variant analyses, the opportunities for this are limited by the need to identify sizeable and diverse study populations with available lipid measurements both before and on treatment and in the case of observational data, accurate determination of statin exposure and relevant covariates. Hence, alternative approaches for identifying clinically significant genetic determinants of statin response are needed.

Subject-derived cells and cell-lines have long been used for the identification of genetic influences on variation in drug response [18]. One system that has been extensively employed for this purpose, particularly for the study of pharmacogenomics of anticancer agents, is lymphoblastoid cell lines (LCLs) [19–21]. LCLs are immortalized B lymphocytes generated by Epstein–Barr virus (EBV) transformation of peripheral blood mononuclear cells. In this review, we will discuss novel findings derived from studies utilizing LCLs established from the participants of the Cholesterol and Pharmacogenetics (CAP) study [22], a 6-week trial of simvastatin 40 mg/day in healthy men and women, to demonstrate the utility of LCLs for studies of statin pharmacogenomics.

Evidence that LCLs can be used as a cellular model of statin response

As discussed above, statins act by inhibiting cholesterol biosynthesis, with reduced cellular cholesterol levels resulting in upregulation of the transcription factor SREBF2 and consequent increase in expression of genes encoding proteins in the cholesterol synthesis and degradation pathways, including *LDLR*. While the plasma LDL lowering effect of statins administered *in vivo* is due primarily to its action in liver, where it is taken up by SLCO1B1 (aka OATP1B1) transporters [23], many features of cholesterol metabolism mediated by the components of the SREBF2 pathway are present in nearly all cell types, including LCLs.

The use of CAP LCLs for the identification of genetic factors modulating cholesterol homeostasis and statin response was supported by the demonstration that their exposure to 2 μM activated simvastatin versus sham buffer for 24 h resulted in the reduction of intracellular cholesterol ester levels by 9.6% ($n = 204$; $p = 0.002$). Moreover, genome-wide analysis of the transcriptional response revealed that SREBF2 target genes (including *HMGCR* and other genes encoding cholesterol biosynthesis enzymes), were among the most significantly upregulated [24,25]. Under these conditions, LDLR cell surface protein expression in LCLs was also increased 1.45-fold ($n = 193$; $p = 0.002$) [26]. Notably, the degree of *in vitro* statin-induced LDLR upregulation in LCLs was correlated with *in vivo* statin-mediated reductions in plasma APOB (the major apolipoprotein in the LDL particle) in the donor individuals. Together these findings demonstrated that cholesterol homeostatic pathways are present and functioning in the LCLs, and importantly that these cells reflect genetic variation that influences these pathways in ways that can be related to clinical response to statins *in vivo*.

Nevertheless, there are limitations of using LCLs as a model to study statin pharmacogenomics. Using GTEx data [27], only about 55% of the lipid metabolic process GO term related genes expressed in human livers are present in LCLs. Moreover, the SLCO1B1 transporters and enzymes involved in statin activation and metabolism such as CYP3A4 and CYP3A5 are absent in LCLs, rendering LCLs poorly suited for studying statin pharmacokinetics. LCLs do not express the genes for APOB and bile acid production, and hence cannot model the synthesis or secretion of APOB-containing lipoproteins or cholesterol degradation and excretion, respectively. Another caveat is the influence of EBV transformation on the LCL transcriptome. Using RNA-seq data from simvastatin- and sham-exposed CAP LCLs, we found that 64% of EBV genes tested were significantly upregulated by statin treatment ($n = 150$; $p < 0.0001$) [24]. By direct comparison of the transcriptome of statin-exposed LCLs and native B-lymphocytes derived from the same subjects, we showed that statin effects on cell cycle, apoptosis and alternative splicing in LCLs may be affected by transcription factors encoded by the EBV genome, such as EBNA2 [28]. These effects may be of particular significance in LCLs with high EBV copy numbers [29].

Despite these limitations, as described in the following sections, LCLs derived from the CAP study population have proven to be informative for identifying genetic differences among individuals that are associated with variation in cholesterol and lipoprotein metabolism and statin response.

Use of LCLs for studying statin pharmacogenomics

Functionalization of candidate gene genotypes or haplotypes

As suggested by the mechanism for statin-induced LDL lowering described above, two of the most important genes contributing to this effect are *HMGCR* and *LDLR*. In candidate gene association studies, we identified haplotypes in these genes associated with endogenous (untreated) plasma LDL-C concentrations and LDL-C change in response to simvastatin treatment in the CAP study [26,30]. For example, African–American carriers (n = 89) of the combined *HMGCR* H2/H7 and *LDLR* L5 haplotypes had significantly attenuated (p = 0.0002) *in vivo* simvastatin-mediated LDL-C reductions compared with noncarriers (n = 78) [26]. Consistent with this finding, we observed significantly lower (p = 0.01) *in vitro* simvastatin-mediated cell-surface LDLR induction in statin and sham-exposed LCLs derived from African–American carriers (n = 57) of the combined haplotypes compared to noncarriers (n = 39) [26]. The H2/H7 haplotype contains rs3846662, the *HMGCR* variant known to impact statin inhibition of HMGCR enzyme activity, while the L5 haplotype is defined by several SNPs in the *LDLR* 3′ UTR and thus is thought to function primarily by modulating regulation of *LDLR* [26,30–32].

LCLs were also key to characterizing a GWAS-identified locus for statin LDL-C response in the JUPITER clinical trial of rosuvastatin 20 mg/day (rs6924995) that was of subgenome-wide statistical significance ($p = 5.3 \times 10^{-7}$) [14]. This signal was initially attributed to a strong biological candidate, *MYLIP* (aka *IDOL*), due to its close proximity and the established role of *MYLIP* in mediating LDLR decay [33]. However, rs6924995 itself is located within *RP1-13D10.2*, a long noncoding RNA gene. In an RNA-seq analysis performed on statin-exposed CAP LCLs taken from the upper and lower tails of the *in vivo* LDL-C statin response distributions in European–Americans and African–Americans, we found that statin-induced expression change of *RP1-13D10.2* (but not *MYLIP*) differed significantly between the two LDL-C response groups in both the European–American and African–American subsets [34]. With subsequent functional studies in human hepatoma cell lines, we showed that *RP1-13D10.2* expression level is sterol regulated and its overexpression increased transcript levels and activity of LDLR, demonstrating its novel role in cholesterol regulation [34]. To our knowledge, *RP1-13D10.2* is the first long noncoding RNA that has been implicated in LDL-C response to statin treatment.

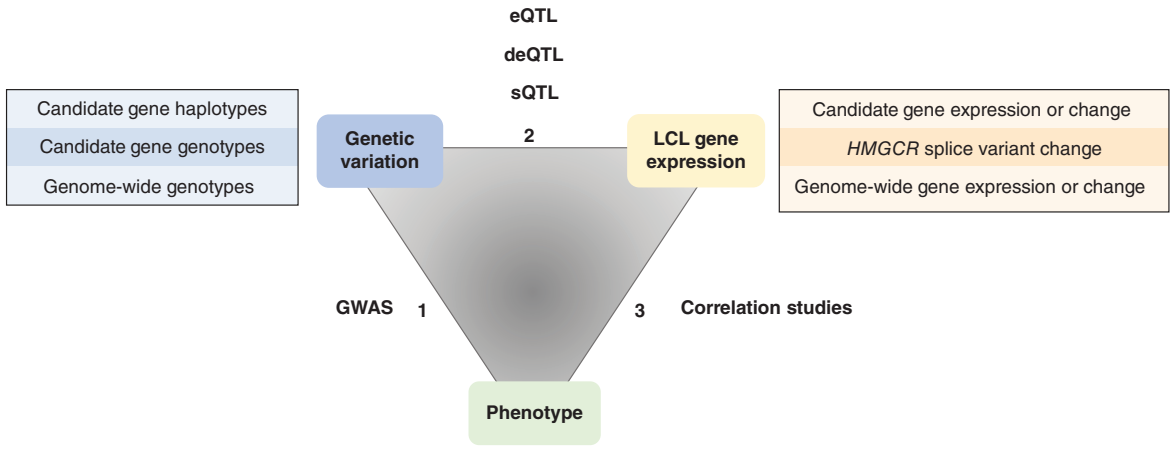
Correlation of statin-induced candidate gene transcript expression with *in vivo* statin response

We pioneered the use of subject-derived LCLs for statin pharmacogenetic discovery with the finding that in CAP LCLs, statin-induced upregulation of expression of an *HMGCR* splice variant that lacked exon 13, *HMGCR13*(-), was strongly correlated ($p < 0.0001$; r = -0.3) with attenuated plasma LDL-C response to statin treatment in the cell line donors [31]. Based on this observation, we evaluated the functional impact of *HMGCR* exon 13 skipping and found that HMGCR enzyme activity in HEK-293 cells after *HMGCR13*(-) enrichment led to resistance to statin inhibition. Variation in *HMGCR* alternative splicing remains the single most informative molecular marker of LDL-C response to statin identified to date, explaining >6% of the variance [32]. This use of subject-derived LCLs to test for direct correlation between *in vitro* and *in vivo* phenotypes revealed the significant discovery potential of LCLs to identify genetic drivers of interindividual variation that had not been previously recognized.

HMGCR alternative splicing was subsequently shown to be regulated by an intron 13 SNP, rs3846662, which was also reported to be associated with plasma LDL-C levels [35,36]. This SNP was computationally predicted to modulate binding of HNRNPA1, a sterol-responsive splicing repressor that we identified using expression data from CAP LCLs [37]. Functional studies showed that overexpression of *HNRNPA1* increased the ratio of *HMGCR13*(-) to total *HMGCR* transcripts, diminished HMGCR enzyme activity, enhanced LDL-C uptake and increased cellular APOB [37]. Notably, another SNP (rs1920045) near *HNRNPA1* was found to be associated with *HNRNPA1* exon 8 alternative splicing in CAP LCLs and with statin-induced cholesterol reduction in two independent clinical trials [37]. These findings demonstrate the role of *HNRNPA1* in modulating statin response, and highlight the role of alternative splicing as an orchestrated mechanism of regulating key genes in cholesterol metabolism. Importantly, they illustrate how observations made in patient-derived LCLs can be used to elucidate new biology.

Transcriptomic correlation – cellular: cellular phenotype

The 'triangle method' is a three-stage approach incorporating gene expression data for assessing pharmacogenomic findings by first identifying the genetic variation associated with the phenotype, then determining the association of that genetic variation with gene expression and finally, testing the significant results from that analysis for association with the phenotype (Figure 1) [38]. In one such example of this approach, we utilized CAP clinical phenotypes, DNA genotypes and microarray gene expression data from 480 simvastatin- and sham-exposed CAP



in vivo clinical phenotypes	LCL cellular phenotypes
APOB level	LDLR cell surface protein level
LDL-C level or LDL-C change	HMGCR enzyme activity
Triglyceride change	Cholesterol ester level
High vs low LDL-C response categories	*HMGCR* transcript change
Myopathy incidence	

Figure 1. Different datasets utilized in variations of the triangle method employed in studies reviewed here. A classic triangle method utilizes all three arms of the triangle; however, there are many discovery opportunities using just one or two arms as we have summarized. While integration of all three arms might be expected to provide the most robust results, such analyses are subject to limitations in each of the datasets incorporated and thus may inadvertently miss associations that are detectable using only two arms.
deQTL: Differential eQTL; eQTL: Expression quantitative trait loci; GWAS: Genome-wide association studies; LCL: Lymphoblastoid cell line; LDL-C: Low-density lipoprotein-cholesterol; sQTL: Splicing quantitative trait loci.

LCLs to identify *RHOA* as a novel gene associated with LDL-C statin response [39]. In this analysis, the variation in statin-induced *HMGCR* upregulation in LCLs was used as a marker of variation in statin-induced intracellular cholesterol reduction. Among the 45 candidate genes whose statin-induced expression change in LCLs was highly correlated with change in *HMGCR* expression, *RHOA* was selected for follow-up because it was known to mediate some of the pleiotropic but not the lipid-lowering effects of statin treatment [40]. We then showed significant associations between statin-mediated *RHOA* expression in LCLs and changes in plasma LDL-C ($p = 0.04$) and APOB ($p = 0.007$) induced by statin treatment of the individuals from whom the LCLs were derived. Furthermore, the minor (T) allele of rs11716445, a SNP located in a novel *RHOA* exon, was found to be associated with higher inclusion of this exon (cis-splicing quantitative trait loci, or splicing quantitative trait loci), as well as a smaller LDL-C reduction in response to statin treatment *in vivo* [39]. These results together with additional functional studies in hepatoma cell lines [39] demonstrated that *RHOA* may contribute to variation in LDL-C response to statin treatment, as well as illustrated the value of utilizing the triangle approach for discovery and validation.

Transcriptomic correlation – *in vivo*: cellular phenotype

We have also sought to identify novel modulators of statin-induced change in plasma triglyceride through correlations with genome-wide RNA-seq profiling of gene expression in 150 statin-exposed LCLs. *INSIG1* emerged from this analysis as an attractive candidate gene based on its central role in cholesterol metabolism, promoting degradation of HMGCR and inhibiting activation of SREBF signaling [24]. The minor (A) allele of a common SNP near *INSIG1*, rs73161338, was associated with both smaller statin-induced increases in *INSIG1* expression levels in LCLs and greater *in vivo* plasma triglyceride reduction in two statin clinical trials, suggesting a role of *INSIG1* in modulating triglyceride statin response [24]. Notably, there was a sex-specific correlation of *INSIG1* with triglyceride statin response (interaction $p = 0.0055$), and a combined model of *INSIG1* expression level and splicing changes accounted for 29.5% of the variation in statin-induced triglyceride response in men ($p = 5.6 \times 10^{-6}$) [24]. This was another successful example employing all three components of the triangle method.

Transcriptomic correlation – DNA variation: cellular phenotype (eQTLs & deQTLs)

Using genome-wide transcriptomic profiling (expression array) of *in vitro* statin response in 480 Caucasian CAP LCLs, we identified six expression quantitative trait loci (eQTLs) that interacted with simvastatin exposure, in other words, eQTLs with different effects in simvastatin versus sham-exposed LCLs or differential eQTLs (deQTL) [25]. These included rs9806699, a *cis*-eQTL and deQTL for *GATM*, the gene that encodes the rate-limiting enzyme in creatine synthesis. We found this locus to be significantly associated with incidence of statin-induced myopathy in two independent populations (meta-analysis odds ratio [OR]: 0.60; 95% CI: 0.45–0.81; $p = 6.0 \times 10^{-4}$) [25]. Notably, *GATM* knockdown in hepatoma cell lines attenuated the upregulation of *HMGCR*, *LDLR* and *SREBF2* by sterol depletion [25], demonstrating that *GATM* may act as a functional link between statin-mediated lowering of cholesterol and energy metabolism.

Following our report, a similar protective effect of the rs9806699 minor A allele was observed in a study of rosuvastatin-induced myopathy in Han Chinese patients (OR: 0.617; 95% CI: 0.406–0.939; p = 0.024) [41], although several other studies did not replicate this association [42–45]. However, a recent meta-analysis of six studies with a total of 707 statin-induced myopathy cases and 2321 controls concluded that *GATM* rs9806699 G>A was indeed associated with decreased risk of statin-induced myopathy (OR: 0.80; 95% CI: 0.68–0.94; p = 0.006) [46]. Additional functional studies are needed to elucidate the underlying mechanism linking GATM to statin-induced myopathy.

In a second comprehensive deQTL analysis, this time using RNA-seq data from both 259 European ancestry and 153 African–American ancestry CAP LCLs, we identified significant *cis*-deQTLs for 15 genes, many of which have clinically relevant functions related to the pleiotropic effects of statin intervention, such as defense from viruses, glucose regulation and response to chemotherapy drugs [47].

Testing for differences in transcript profiles between the tails of *in vivo* statin response

Since studies of individual genes have only explained a small proportion of the variation in LDL-C statin response, we have also employed a more comprehensive approach by seeking to identify a transcriptomic profile consisting of a panel of genes that could be better powered for predicting response. Using expression array data from sham-treated LCLs derived from 372 Caucasian CAP participants, we identified 100 signature genes that were differentially expressed between high and low LDL-C statin responders [48]. A radial-basis support vector machine prediction model of these signature genes explained 12.3% of the variance in statin-mediated LDL-C change [48]. Addition of SNPs either associated with expression levels of the signature genes (eQTLs) or previously reported to be associated with statin response by GWAS resulted in a combined model that predicted 15% of the variance [48]. This is the largest proportion of variance in statin LDL-C lowering efficacy explained by molecular biomarkers to date, suggesting that the multigenic transcriptomic information generated from this approach may provide a framework for future studies identifying novel biomarkers and pathways involved in cholesterol metabolism and statin response.

Using the same approach, but this time with RNA-seq data from both simvastatin and sham-treated CAP LCLs, we sought to identify genes whose statin-induced expression changes were most different between individuals in the tails of the LDL-C statin response distribution. We created a classification model of 82 signature gene expression changes that distinguished high versus low LDL-C statin response [49]. One of the most differentially changing signature genes was *ZNF542P*, a pseudogene whose expression change was found to be most strongly correlated with statin-induced change in LCL cellular cholesterol ester, an *in vitro* marker of statin response [49]. These results, together with those for *RP1-13D10.2* described above, point to the value of RNA-seq of statin and sham-exposed LCLs for identifying nonprotein coding transcripts with regulatory functions that may contribute to variation in response to statin.

Conclusion & future perspective

LCLs have proven to be a valuable cellular model in statin pharmacogenomics research for identifying genetic markers associated with both cellular and clinical statin response, providing a unique resource for assessment of functional consequences of genetic variation. In addition, LCLs retain donors' genetic background and reflect all genomic loci involved in statin response, enabling the study of genetic contributor without knowing the exact causal SNP or gene. Based on this experience, it is likely that future studies of statin pharmacogenomics will be further advanced by the use of subject-derived induced pluripotent stem cells (iPSCs). As a cellular model, iPSCs overcome several limitations of LCLs (Table 1), including the elimination of EBV during cell line establishment and, therefore, its effect on transcriptome. iPSCs express additional genes relevant to statin pharmacodynamics,

Table 1. Key advantages and disadvantages of lymphoblastoid cell lines versus induced pluripotent stem cells as cellular models for statin pharmacogenomic discovery.

	LCLs	iPSCs
Advantages	• Cost effective • Ease of preparation and handling • Large repositories available from a variety of racial and ethnic backgrounds with publicly available genetic and phenotypic data	• Can be derived from a number of different tissue sources using ever-improving protocols • Can be differentiated into tissue-specific cell types • Even in undifferentiated state, express more statin pharmacodynamic relevant genes than LCLs
Disadvantages	• Require the handling of biohazardous EBV • Gene expression could be confounded by EBV transcription factors	• Labor-intensive • Higher cost to establish and culture

EBV: Epstein–Barr virus; iPSC: Induced pluripotent stem cell; LCL: Lymphoblastoid cell line.

such as *APOE* and most importantly, they can be further differentiated into other cell types, enabling evaluation of cell type specific pathways and functions. For example, pharmacokinetic pathways that are not represented in LCLs or human hepatoma HepG2 cells can be probed using iPSC-derived hepatocyte-like cells (iPSC-Heps) and statin-induced myopathy can be investigated using iPSC-derived skeletal myoblasts. The number of iPSCs available from unique donors is growing within public repositories [50–52].

With carefully selected patient-specific cellular models and appropriate validation study designs, future statin pharmacogenomics studies are likely to identify additional genetic markers of statin response, with the goals of further delineating mechanisms modulating cholesterol metabolism and ultimately identifying a comprehensive panel of markers for determining those individuals for whom the likelihood of clinical benefit significantly exceeds the risk for adverse outcomes.

Executive summary

Background

- There is a wide range of interindividual variation in statin efficacy for cardiovascular disease prevention and risk for adverse treatment outcomes, and studies of statin pharmacogenomics are needed to identify markers of statin response.
- Approaches beyond genome-wide association studies are needed for identifying markers of statin response.

Evidence that LCLs can be used as a cellular model of statin response

- Cholesterol homeostatic pathways are present and functioning in lymphoblastoid cell lines (LCLs), and subject-derived LCLs reflect genetic variation that influences these pathways in ways that can be related to clinical response to statins *in vivo*.

Use of LCLs for studying statin pharmacogenomics

- The identification of *HMGCR* and *HNRNPA1* alternative splicing as determinants of statin response through the use of LCLs highlighted how studies of pharmacogenomics can lead to the discovery of new biology, in this case, the role of alternative splicing as an orchestrated mechanism of regulating cholesterol metabolism.
- Using genome-wide transcriptomic profiling and differential expression quantitative trait loci approaches in statin and sham-exposed LCLs, we have shown the potential role of GATM as a link between statin-mediated low-density lipoprotein-cholesterol (LDL-C) lowering and statin-induced myopathy susceptibility.
- Transcriptomic profiling from statin and sham-exposed LCLs identified panels of signature genes with better power for predicting *in vivo* LDL-C statin response than individual genes or genome-wide association studies identified SNPs.
- Using RNA-seq data of statin and sham-exposed LCLs we have identified nonprotein coding transcripts *RP1-13D10.2* and *ZNF542P* with regulatory functions that may contribute to variation in response to statin.

Conclusion & future perspective

- Future studies of statin pharmacogenomics will be further advanced by the use of subject-derived induced pluripotent stem cells.
- The ultimate goal of statin pharmacogenomics studies, when achieved, will improve the prediction of those individuals for whom the likelihood of clinical benefit significantly exceeds the risk for adverse outcomes of statin therapy.

Author contributions

Y-L Kuang and MW Medina acquired and analyzed data on intracellular cholesterol ester levels in 204 LCLs exposed to simvastatin versus sham buffer and drafted the manuscript. E Theusch and RM Krauss reviewed and revised the manuscript critically. All authors read and approved the final manuscript.

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•• **Illustrates the utility of expression quantitative trait loci and differential expression quantitative trait loci analyses of statin-exposed lymphoblastoid cell lines (LCLs) in identifying genetic modulators of statin response, and thus for the first time defined drug x SNP interactions.**

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