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REVIEW

Role of Adenylate Cyclase 9 in the Pharmacogenomic Response to Dalcetrapib

Clinical Paradigm and Molecular Mechanisms in Precision Cardiovascular Medicine

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ABSTRACT: Following the neutral results of the dal-OUTCOMES trial, a genome-wide study identified the rs1967309 variant in the adenylate cyclase type 9 (*ADCY9)* gene on chromosome 16 as being associated with the risk of future cardiovascular events only in subjects taking dalcetrapib, a CETP (cholesterol ester transfer protein) modulator. Homozygotes for the minor A allele (AA) were protected from recurrent cardiovascular events when treated with dalcetrapib, while homozygotes for the major G allele (GG) had increased risk. Here, we present the current state of knowledge regarding the impact of rs1967309 in *ADCY9* on clinical observations and biomarkers in dalcetrapib trials and the effects of mouse *ADCY9* gene inactivation on cardiovascular physiology. Finally, we present our current model of the interaction between dalcetrapib and *ADCY9* gene variants in the arterial wall macrophage, based on the intracellular role of CETP in the transfer of complex lipids from endoplasmic reticulum membranes to lipid droplets. Briefly, the concept is that dalcetrapib would inhibit CETP-mediated transfer of cholesteryl esters, resulting in a progressive inhibition of cholesteryl ester synthesis and free cholesterol accumulation in the endoplasmic reticulum. Reduced ADCY9 activity, by paradoxically leading to higher cyclic AMP levels and in turn increased cellular cholesterol efflux, could impart cardiovascular protection in rs1967309 AA patients. The ongoing dal-GenE trial recruited 6145 patients with the protective AA genotype and will provide a definitive answer to whether dalcetrapib will be protective in this population.

Key Words: acute coronary syndrome ■ adenylate cyclase ■ dalcetrapib ■ macrophages ■ precision medicine

CETP (cholesteryl ester [CE] transfer protein) is a 74 kDa plasma glycoprotein which is secreted by liver and adipose tissue.[1] In the bloodstream, CETP is involved in the transfer of CE from HDL (high-density lipoprotein) to apo B-containing lipoproteins and is consequently involved in the modulation of HDL and non-HDL-C (HDL cholesterol) levels.[2] Accordingly, it was demonstrated that patients with CETP deficiency are characterized by markedly higher levels of HDL-C and by decreased non-HDL-C levels.[3] These effects were associated in some studies with a lower cardiovascular disease risk, paving the way for the development of drugs targeting CETP to impact cholesterol distribution within the different lipoprotein classes.

Small molecules targeting CETP, demonstrated great efficacy to reduce atherosclerosis in animal models[4,5] and have been tested in large phase 3 clinical trials. The ILLUMINATE trial (Investigation of Lipid Level Management to Understand its Impact in Atherosclerotic Events) showed that torcetrapib, the first CETPi (CETP inhibitor) tested in patients, increased all-cause mortality and major cardiovascular events.[6] Although this finding challenged the concept of raising HDL-C through CETP inhibition for cardioprotection, it was subsequently

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Nonstandard Abbreviations and Acronyms

ABCA1	ATP-binding cassette transporter
AC	adenylate cyclase
ACAT	acyl-coenzyme A: cholesterol acyltransferase
ACCELERATE	Assessment of Clinical Effects of Cholesteryl Ester Transfer Protein Inhibition With Evacetrapib in Patients at a High Risk for Vascular Outcomes
Adcy9	mouse adenylate cyclase type 9
ADCY9	adenylate cyclase type 9
Adcy9Gt/Gt	Adcy9-inactivated by genetrap
AKAP	A kinase anchoring protein
cAMP	cyclic adenosine monophopshate
CE	cholesteryl ester
CEC	cholesterol efflux capacity
CETP	cholesterol ester transfer protein
CETPi	CETP inhibitor
GPCR	G protein-coupled receptor
Gt	gene trap
HDL	high-density lipoprotein
HDL-C	HDL cholesterol
HsCRP	high-sensitivity C-reactive protein
ILLUMINATE	Investigation of Lipid Level Management to Understand its Impact in Atherosclerotic Events
LDL	low-density lipoprotein
LV	left ventricular
mAC	membrane-bound AC
PCSK9	proprotein convertase subtilisin/kexin type 9
PKA	protein kinase A
REVEAL	Randomized Evaluation of the Effects of Anacetrapib Through Lipid Modification
SNP	single nucleotide polymorphism
TG	triglycerides
WT	wild-type

demonstrated that torcetrapib had deleterious off-target effects,[6] allowing to pursue studies of CETP inhibition with other compounds. The dal-OUTCOMES (Dalcetrapib cardiovascular outcomes trial)[7] and ACCELERATE[8] trials (Assessment of Clinical Effects of Cholesteryl Ester Transfer Protein Inhibition With Evacetrapib in Patients at a High Risk for Vascular Outcomes) failed to demonstrate cardiovascular event risk reduction with dalcetrapib and evacetrapib, respectively. However, it was recently demonstrated that anacetrapib reduced cardiovascular events by 9% over 4.5 years of follow-up in the REVEAL trial (Randomized Evaluation of the Effects of Anacetrapib Through Lipid Modification).[9] Moreover, it was shown that the effects of dalcetrapib on cardiovascular outcomes varies greatly according to genotypes at the single nucleotide polymorphism (SNP) rs1967309 in the *ADCY9* (adenylate cyclase type 9) gene.[10] This finding raised many unresolved questions about the mechanism(s) of action by which dalcetrapib may modulate cardiovascular disease. In this review, we summarize recent results and current hypotheses to explain how dalcetrapib may reduce cardiovascular disease risk in patients with a specific *ADCY9* genotype, as well as ongoing research designed to demonstrate the benefit of dalcetrapib in a specific genetically defined population. Please note that throughout this review, we refer to the AC (adenylate cyclase) 9 gene as *ADCY9* and to its protein product as AC9.

Discovery of a Potential Pharmacogenomic Effect of AC9 on Responses to Dalcetrapib

The dal-OUTCOMES clinical trial evaluated the effects of dalcetrapib—a modulator of circulating CETP that raises HDL-C by about 30%—on cardiovascular outcomes in 15 871 patients recruited between 1 and 3 months after an acute coronary syndrome.[7] The trial tested the hypothesis that increasing HDL-C levels through CETP inhibition reduces risk of recurrent cardiovascular events on top of best standard-of-care, including statin therapy.[11] After a median follow-up of 31 months, the study was stopped early for futility. The final reported hazard ratio for the primary end point was 1.04 (95% CI, 0.93–1.16, P=0.52).

A number of exploratory investigations were undertaken in an attempt to understand the neutral result of dal-OUTCOMES, including a genome-wide association study conducted in a subgroup of 5749 patients with demographic characteristics similar to those of the overall population enrolled in the main study.[10] Combined genotyping and imputation of SNPs allowed testing of the association of 5 543 264 common genetic variants to a prespecified composite cardiovascular end point identical to the one used in dal-OUTCOMES with the addition of unanticipated coronary revascularization to increase statistical power. A single region on chromosome 16 reached the significance threshold of $P<5\times10^{-8}$ at SNP rs1967309 in intron 2 of the *ADCY9* gene, which was associated with cardiovascular outcomes in the dalcetrapib treatment arm ($P=2.4\times10^{-8}$) but not in the subjects on placebo (P=0.25). The frequency of the minor allele A of rs1967309 was high enough at 41% in the dal-OUTCOMES genome-wide association study cohort to permit stratification by *ADCY9* genotype and comparison of event rates in the active treatment versus placebo groups. It was observed that dalcetrapib reduced cardiovascular events by 39% in carriers of the AA genotype (hazard ratio, 0.61 [95% CI, 0.41–0.92]), had no overall impact in AG heterozygotes (hazard ratio, 0.94 [95% CI,

0.77–1.16]), and increased cardiovascular risk by 27% in GG carriers (hazard ratio, 1.27 [95% CI, 1.02–1.58]).[10]

The detrimental effect caused by dalcetrapib in GG carriers does not allow for the conduct of a replication study to avoid causing harm to such patients. However, the impact of *ADCY9* genotype was evaluated in patients receiving other CETPi to determine if the pharmacogenomic interaction is specific to dalcetrapib or extends to all drugs reducing CETP activity. In a nested case-control study from the ACCELERATE trial, no significant association was found between cardiovascular outcomes and the *ADCY9* SNP rs1967309 in patients receiving evacetrapib, although a trend consistent with the dal-OUTCOMES pharmacogenomic results was observed.[12] The effect of anacetrapib was also evaluated according to the *ADCY9* genotype in the REVEAL trial. No genotype-dependent effect of anacetrapib was observed in that study.[13]

These findings raised 3 questions: (1) is the association between the *ADCY9* gene polymorphism and response to dalcetrapib a chance finding or a true pharmacogenomic interaction? (2) what is the relationship between *ADCY9* and cardiovascular disease? and (3) might AC9 itself become a relevant and novel target for the prevention of cardiovascular diseases?

Genetic Variant rs1967309 in *ADCY9* Modulates Changes in Cardiovascular Risk Biomarkers and in Plaque Burden in Patients Treated With Dalcetrapib—Supporting Clinical Data

The absence of interaction between *ADCY9* rs1967309 and both evacetrapib and anacetrapib on cardiovascular outcomes raised the possibility that the observed results with dalcetrapib might represent false-positive findings. However, a pharmacogenomic interaction specific to dalcetrapib cannot be rejected considering that its pharmacology differs from that of other CETPi. Indeed, it was proposed that dalcetrapib is a selective modulator rather than an inhibitor of CETP. While evacetrapib and anacetrapib increase HDL-C level by >100% and reduce non-HDL-C by ≈20%, dalcetrapib only increases HDL-C by 30% and has no effect on non-HDL-C. Moreover, supporting evidence points to an *ADCY9* genotype-dependent effect of dalcetrapib on cholesterol efflux to HDL.[14]

In dal-OUTCOMES, there were no differences in major cardiovascular risk factors at baseline and serum HDL-C changes produced by dalcetrapib between patients with the 3 rs1967309 genotypes AA, AG, and GG.[10] However, the response for other specific biomarkers to dalcetrapib differed by genotype. First, in AA patients, circulating hsCRP (high-sensitivity C-reactive protein) was not altered significantly by dalcetrapib after 3 months of treatment and still showed no change at the end of the trial, while AG and GG carriers had significant 15% to 20% increases in circulating hsCRP at 3 months that persisted throughout the trial (Table 1). These data suggest that the AA genotype at rs1967309 in the *ADCY9* gene protected patients from the rise in systemic inflammation induced by dalcetrapib in patients with the other genotypes. Second, dalcetrapib-treated GG patients had a greater reduction in body mass index and weight compared with AA patients, the latter presenting a nonclinically significant change (Table 1). Third, those with the GG genotype had greater reductions in LDL (low-density lipoprotein)-C and triglycerides (TG) after 1 month compared with those who were AA.

In a different population, a set of 20 SNPs in the *ADCY9* gene nominally associated ($P<0.05$) with cardiovascular outcomes following dalcetrapib treatment in dal-OUTCOMES were tested in subjects with stable coronary artery disease who were participants in the dal-PLAQUE-2 trial (Effect of Dalcetrapib on Atherosclerotic Disease in Patients With Coronary Artery Disease). As for dal-OUTCOMES, the patients' demographic characteristics in the pharmacogenomics sub-study were similar to those in the main study. In this imaging study, changes in carotid intima-media thickness were assessed by ultrasonography (N=386).[10] Genotypes identified in dal-OUTCOMES as protective in the dalcetrapib arm were associated with reduction in carotid intima-media thickness after 6 and 12 months of dalcetrapib therapy. Although all 20 SNPs tested showed the same trend and half of them achieved nominal statistical significance at $P<0.05$ (adjustment for significance threshold was not applied because of the highly correlated nature of the selected SNPs), the association between SNP rs1967309 and the change in carotid intima-media thickness was not statistically significant. However, marker rs2238448, which is in high linkage disequilibrium with rs1967309 and is associated with cardiovascular events in dal-OUTCOMES, was significantly associated with changes in carotid intima-media thickness. In the absence of a formal replication study, these results provide supporting evidence to the pharmacogenomics study from dal-OUTCOMES. However, it will be important in the future to confirm the impact of dalcetrapib in patients with the AA genotype at SNP rs1967309.

Serum HDL samples from genotyped patients of the dal-PLAQUE-2 trial were tested for their ability to modify cholesterol efflux capacity of serum HDL (HDL-CEC) from cultured macrophages. Lower HDL-CEC has been associated with increases in both incident and recurrent cardiovascular events in several studies.[15,16] HDL-CEC was increased significantly by 22.3% in the dalcetrapib group compared with 3.5% in those given placebo after 1 year of treatment in the rs1967309 AA carriers.[14] Smaller changes were observed in the AG carriers, while GG carriers demonstrated no increase in HDL-CEC with dalcetrapib, and there was no difference at 1 year

Table 1. Summary of Clinical Evidence for the Cardiovascular Effects of Dalcetrapib According to rs1967309 Genotypes in the *ADCY9* Gene

Clinical trial	Variable, unit	Observations according to rs1967309 genotype		
		AA	AG	GG
dal-OUTCOMES	Primary composite end point*	39% risk reduction for dal vs pbo	Unchanged for dal vs pbo	27% risk increase for dal vs pbo
		HR, 0.61 (95% CI, 0.41–0.92)	HR, 0.94 (95% CI, 0.77–1.16)	HR, 1.27 (95% CI, 1.02–1.58)
	Event rates, %	Dal: 7.8	Dal: 12.8	Dal: 18.0
		Pbo: 12.4	Pbo: 13.5	Pbo: 14.5
	Relative change in hsCRP (end of trial), placebo-adjusted, %	−1.0 (−13.9–13.9)	18.7 (9.9–28.3)	18.1 (7.1–30.2)
	Change in BMI (1 mo), mean± SD, kg/m²	Dal: 0.04±1.09	Dal: −0.05±0.68	Dal: −0.10±0.78
		Pbo: 0.01±0.73	Pbo: −0.01±0.73	Pbo: 0.03±0.64
dal-PLAQUE-2	Change in cIMT (12 mo), mean±SD, mm	Dal: −0.021±0.083	Dal: −0.001 ±0.048	Dal: 0.005±0.042
		Pbo: 0.001±0.021	Pbo: 0.007±0.042	Pbo: 0.002±0.054
	Change in HDL-CEC (12 mo), mean±SD, %	Dal: 22.3±22.3	Dal: 12.9±16.9	Dal: 7.8±18.0
		Pbo: 3.5±12.3	Pbo: 1.7±13.6	Pbo: 7.4±10.8

AA indicates homozygous AA carriers for rs1967309; BMI body mass index; cIMT, carotid intima-media thickness; dal, dalcetrapib; dal-OUTCOMES, Dalcetrapib CV oucomes trial; dal-PLAQUE-2, Effect of Dalcetrapib on Artherosclerotic Disease in Patients With Coronary Artery Disease; GG, homozygous GG carriers for rs1967309; HDL, high-density lipoprotein; HDL-CEC, cholesterol efflux capacity of serum HDL; HR, hazard ratio; hsCRP, high-sensitivity C-reactive protein; and Pbo, placebo.

*The prespecified primary composite end point of the pharmacogenomic analysis of dal-OUTCOMES included death from coronary heart disease, myocardial infarction, unstable angina, resuscitated cardiac arrest and ischemic stroke, plus unanticipated coronary revascularization.

between dalcetrapib- and placebo-treated GG subjects (Table 1). Increased HDL-CEC may contribute to the potential cardiovascular protective effect of dalcetrapib in AA patients. Favorable changes in cardiovascular outcomes, atherosclerotic disease, serum hsCRP, and HDL-CEC that appear limited to only those patients with the AA genotype who received dalcetrapib in these separate clinical trials suggest a true pharmacogenomic interaction. Replication of the favorable cardiovascular response to dalcetrapib in AA patients is tested in the dal-Gene trial, as described in the last section of this article.

Role of ACs in Risk of Disease and Potential as Therapeutic Targets

AC9 is the ninth member of the family of mAC (membrane-bound ACs) that catalyze the formation of cAMP (cyclic adenosine monophosphate) from ATP. The 9 mACs constitute signaling hubs that receive, transmit, and generate signals for their associated complexes, including AKAPs (A kinase anchoring proteins), PKA (protein kinase A), and nucleotide phosphodiesterases.[17] Human AC9 was cloned in 1998 as a membrane protein of 1353 amino acids and was shown to be the most divergent member of this family.[18] Human AC9 is expressed in multiple tissues at the mRNA level including heart, brain, placenta, lung, liver, skeletal muscle, kidney, pancreas, spleen, thymus, prostate, testis, ovary, small intestine, colon, and peripheral blood leukocytes.[17,19] In brain and heart tissues, AC9 is found in complexes with the scaffold protein AKAP9/Yotiao.[20,21] AC9-Yotiao complexes in cardiomyocytes have been well documented and facilitate PKA-mediated phosphorylation of the potassium channel subunit KCNQ1 (potassium voltage-gated channel subfamily Q member 1), which in turn favors I_{Ks} currents involved in β-adrenergic receptor-mediated cardiac repolarization.[22,23] Among members of the mAC family, the unique forskolin-insensitivity of AC9 allowed its identification as the major mAC in the myeloid lineage, that is, in neutrophils and monocytes.[24,25] Indeed, knockdown or overexpression of AC9 in human neutrophils modulates chemotaxis.[26] AC9 is also a major AC isoform in splenocytes, a reservoir of myeloid and lymphoid cells, as AC activity in forskolin-stimulated splenocytes from Adcy9-inactivated mice decreases by 50%.

mACs are recognized as potential therapeutic targets for drug development.[27] For instance, AC3 is a current anti-obesity drug target based on human genome-wide association study, epigenetic studies, and mouse models.[28] Loss-of-function mutations in *ADCY3* gene are associated with morbid obesity.[29] Activation of AC3 is sought for its protective effects against diet-induced obesity and triggers lipolysis of TG stores through activation of PKA and hormone-sensitive lipase.[30,31] Selective inhibitors of mACs have been identified; for example ST034307, a selective inhibitor of AC1, reduced pain responses in a mouse model of inflammatory pain induced by complete Freund's adjuvant injection.[32] In the cardiovascular field, a selective AC5 inhibitor (AC90), reduced myocardial infarct size in mice when injected after coronary artery

reperfusion, and this effect was abolished in AC5 knockout mice.[33] Genetic polymorphisms in the *ADCY9* gene have been associated with different diseases and traits, including asthma,[34] susceptibility to malaria,[35] mood disorders,[36] pancreatic cancer,[37] and body weight.[38] At least one functional variant in the AC9 protein catalytic domain, Ile172Met (rs2230739 A/G), is linked to altered activity and activation by β2-adrenergic receptors, suggesting that it could modulate GPCR (G protein-coupled receptors) response in multiple tissues.[34] Indeed, carriers of the AC9 Ile172Met variant had a positive change in 1-second forced expiratory volume after 8-week inhaled combination therapy with budesonide (corticosteroid) and formoterol, a long-acting β2-agonist.[39] It is thus expected that changes in expression or activity of AC9 may impact physiological response to pharmacological therapy in tissues where AC9 is present.

Inactivation of Adcy9 in Mice Imparts Cardiovascular Protection Which Is Reversed by CETP Expression

The role of AC9 in cardiovascular physiology and disease in humans is unknown presently. The information available is the result of mouse models studies that are relevant to better characterize the function(s) of AC9 in cardiovascular diseases in humans, given the low interspecies variation between *ADCY9* sequences.[40] Indeed, sequence alignment with blastn suite-2 sequences from National Center for Biotechnology Information indicated an homology of 85% between the human and mouse sequences. We have tested the hypothesis that inactivation of the mouse adenylate cyclase 9 (*Adcy9*) gene impacts the development of atherosclerosis. As mice do not express CETP,[41] the effects of *Adcy9* inactivation were tested in a model that recapitulates a condition of complete CETP inhibition. Our recently published studies[42] used mice that have a gene trap (Gt) insertion in intron 2 of the *Adcy9* gene. In this study, homozygous *Adcy9*-inactivated ($Adcy9^{Gt/Gt}$) mice (n=15) and wild-type (WT) mice (n=21) were given a single injection of adeno-associated virus expressing a gain-of-function mutant of PCSK9 (proprotein convertase subtilisin/kexin type 9) to stimulate chronic degradation of the hepatic LDL receptor and induce hypercholesterolemia, and then administered an atherogenic diet (0.75% cholesterol) for 16 weeks.[42] At the end of the experimental period, the extent of atherosclerosis was quantified across the excised aorta. Compared with WT counterparts, a 65% decrease in plaque area was observed in whole aortas prepared en face from *Adcy9*-inactivated mice, which was associated with reduced lipid content, fewer CD68-positive macrophages (foam cells) and proliferating macrophages in aortic root sections. Splenocyte (immune cell) adhesion to histamine-stimulated aortic endothelium was also reduced in *Adcy9*-inactivated compared with WT mice. Thus, loss of Adcy9 protects the vascular wall against immune cell adhesion and lipid deposition (Table 2).

Table 2. Summary of the Impact of AC9 Deletion in Mice

Topic	Impact of AC9 deletion in mice (without CETP)
Atherosclerosis	Decreased plaque area in whole aortas of mice under atherosclerotic diet
	Reduced lipid content
	Reduced CD68-positive macrophages (foam cells)
	Reduced proliferating macrophages
	Reduced splenocyte (immune cell) adhesion
Cardiac function	Lower heart rate
	Improved recovery after myocardial infarction
	Improved LV systolic and diastolic function
	Smaller myocardial infarct size
	Reduced LV dilation
	Reduced LV hypertrophy
	Reduced cardiomyocyte size
Body weight and adiposity	Increased weight gain under atherosclerotic diet
	Increase in whole-body adipose tissue volume
	Increased daily calorie intake
	Increased feed efficiency

AC9 indicates adenylate cyclase 9; CETP, cholesterol ester transfer protein; and LV, left ventricular.

Favorable effects of *Adcy9* inactivation were also observed on endothelium-dependent vasorelaxation. *Adcy9*-inactivated mice had increased vasodilation of femoral arteries in response to acetylcholine and shear stress.[42] Similarly, femoral arteries of *Adcy9*-inactivated mice have increased endothelium-dependent vasorelaxation in response to vasoactive intestinal peptide, which in fact increases cAMP levels in mouse lung and human coronary artery endothelial cells.[43] Overall, these results suggest that absence of Adcy9 in the context of absent or low CETP activity is antiatherosclerotic.

The impact of mouse *Adcy9* gene inactivation on myocardial function was also studied. Li et al[44] reported a slower heart rate without structural heart defect in *Adcy9*-inactivated mice. Echocardiographic finding were consistent with mild left ventricular (LV) diastolic dysfunction and preserved LV ejection fraction in *Adcy9*-inactivated mice.[44] More recently, we showed significantly better LV systolic and diastolic function in *Adcy9*-inactivated mice compared with WT animals 28 days after myocardial infarction induced by permanent ligation of the left anterior descending artery.[45] Smaller myocardial infarct size and reduced LV dilation, hypertrophy, and cardiomyocyte size were also observed in these $Adcy9^{Gt/Gt}$ mice compared with WT animals (Table 2). The protective effects of *Adcy9* inactivation on the vascular wall and myocardium in mice devoid of CETP suggest that reduced ADCY9 activity in humans might be beneficial during reduction of

CETP activity with dalcetrapib. The reason for the emergence of this benefit only in the presence of reduced CETP activity is unclear, but possible mechanisms are emerging as outlined below.

In our experimental atherosclerosis study, Adcy9-inactivated mice gained more weight (10 grams) than WT animals throughout the 16 weeks of atherogenic diet.[42] This was due to an increase in whole-body adipose tissue volume and specifically in both white adipose tissue (epididymal and inguinal) and brown adipose tissue (interscapular) depots (Table 2). Because adrenergic tone has a major influence on both cardiac and adipose tissue physiology, we searched for indications of changes in autonomic nervous system activity on the feeding behavior, lipolysis response to isoproterenol, and cardiac rhythm. Indeed, *Adcy9* inactivation increased daily calorie consumption and doubled feed efficiency (grams of weight gained per 100 kilocalories ingested; Table 2), suggesting reduced energy expenditure and adipose tissue accretion. Moreover, these *Adcy9*-inactivated mice have reduced lipolytic response to adrenergic receptor stimulation with isoproterenol, in agreement with reduced energy expenditure in absence of *Adcy9* and *CETP* expression. The lack of weight and body mass index reductions in AA patients treated with dalcetrapib is consistent with the observations in *Adcy9*-inactivated mice. Regarding ECG effects, R-R intervals were longer in *Adyc9*-inactivated mice, with corresponding lower heart rates compared with WT mice, in agreement with observations from Li et al.[44] Reduced sympathetic autonomic nervous system activity is considered a protective factor against cardiovascular diseases.[46] This may have contributed to the cardioprotective effects of dalcetrapib in patients with the AA genotype at rs1967309 in the *ADCY9* gene.

To model the placebo group of clinical trial patients in which CETP activity is at normal levels, *Adcy9*-inactivated mice were crossed with human CETP transgenic mice. Human CETP transgenic mice have a human *CETP* minigene that allows its expression in tissues that normally express human CETP and are thus considered a humanized model.[47] The human CETP transgenic background did not allow for the *Adcy9* inactivation-induced changes in atherosclerosis, endothelial-dependent vasorelaxation, weight, adipose tissue, and feed efficiency.[42] Thus, normal CETP expression level blocks the cardiovascular protection procured by the inactivation of *Adcy9* expression in mice. Similarly, the improvement of LV function after experimental myocardial infarction in *Adcy9*- inactivated mice compared with WT animals was blunted by the human CETP transgenic background. The potential implication of these findings is that the presence of fully functional CETP in humans, such as in the placebo arm of the dalcetrapib trials, does not allow the beneficial effects of low *ADCY9* expression to occur.

Conceptual Model of AC9—Dalcetrapib Interaction

The classical view of the mechanism of action of CETP inhibitors is that they inhibit circulating CETP activity and so block partially or nearly completely the transfer of CE and TG between lipoprotein particles. The agents differ in the extent to which they reduce CETP activity. Although evacetrapib and anacetrapib provide virtually complete inhibition with a subsequent almost doubling of HDL-C levels, dalcetrapib has been shown to permit neutral lipid transfer to continue between HDL particles while reducing the exchange between HDL and apolipoprotein B-containing lipoproteins.[48] As noted above, dalcetrapib increases HDL-C by 30% and has been usefully termed a CETP modulator rather than a complete inhibitor of CETP-mediated lipid transfers.[48] Moreover, dalcetrapib is different from other CETP inhibitors as it covalently binds to cysteine 13 on plasma CETP and changes its conformation.[49] This covalent binding requires that dalcetrapib undergoes hydrolysis by esterase to produce its thiol active form that will react with a cysteine.[50] This reaction is needed since thiols are unstable and requires administration of dalcetrapib as a thioester form.[5] Interestingly, contrary to other CETPi the covalent binding to CETP does not lead to the formation of a complex between HDL and CETP as observed on native gels.[51] Thus, dalcetrapib presents several distinct properties compared with other drugs inhibiting CETP.

The role of CETP in lipid transport within cells is less widely known and characterized. CETP has an important function in the intracellular metabolism of cholesterol, CE and TG.[52–56] Full-length CETP, corresponding to the plasma protein, and a shorter isoform are detected in all tissues that express this gene. The shorter CETP protein lacking exon 9 is produced in cells but seems poorly secreted and reduces full-length CETP secretion.[57–59] Full-length CETP appears to be involved in the transfer of CE and TG from their sites of synthesis in the endoplasmic reticulum to lipid droplets in adipocytes.[53] Reducing CETP expression with antisense oligonucleotides impaired TG and CE storage in human adipocytes, and this was associated with reduced transport into droplets and slower hydrolysis of lipid stores.[53]

It is noteworthy that *CETP* and *ADCY9* genes are both expressed in cell types with important roles in lipid metabolism and atherosclerosis such as adipocytes, hepatocytes, and macrophages. A critical function of each of these cells is the ability to store neutral lipids in the short-term or for prolonged periods and then release it in a regulated fashion. Given the observations detailed above, especially from mice models, it is tempting to speculate that the CETP-ADCY9 interaction, and by extrapolation the effect of dalcetrapib, occurs at an intracellular location rather than in the blood circulation. Key findings on which our proposed mechanistic model

is built are that (1) CETP has a role in transporting CE and TG within cells between sites of synthesis and storage[53,55]; (2) *ADCY9* deficiency is associated with higher cellular cAMP levels[43]; (3) cAMP is a key regulator of CE hydrolysis from lipid droplets[60–63] and of ABCA1 (ATP-binding cassette transporter)-mediated cholesterol efflux to HDL[64,65]; and (4) dalcetrapib can act intracellularly to inhibit CE synthesis in various cell types (unpublished data). Our conceptual model as it relates to macrophage/foam cell lipid metabolism is presented schematically in Figure and described hereafter. Analogous pathways are likely to operate in adipocytes and hepatocytes, although it remains possible that other mechanisms could be involved according to the function of each cell type or tissue.

Intracellular Model

Endoplasmic reticulum is the site of synthesis of complex lipids including CE by the action of acyl-coenzyme ACAT (A: cholesterol acyltransferase) on free (unesterified) cholesterol. These lipids are normally transported efficiently to sites of storage (droplets in adipocytes and macrophages) or utilization (VLDL assembly in hepatocytes and enterocytes). If CE begins to accumulate in the endoplasmic reticulum, a negative feedback response is triggered that leads to reduced CE synthesis and a build-up of free cholesterol in the endoplasmic reticulum membranes. Since free cholesterol is especially toxic to cells (reviewed in[66]), negative feedback pathways are activated leading to reduced cholesterol biosynthesis, reduced LDL receptor expression and cellular uptake of cholesterol, and enhanced expression of ABCA1 protein to allow efflux of free cholesterol from cells.[53] Accumulation of free cholesterol has been shown to lead to the generation of lipid structures within macrophages that are rich in free cholesterol and phospholipids. If the accumulation of intracellular free cholesterol persists, then microcrystalline deposits could occur leading to an inflammatory response.[66]

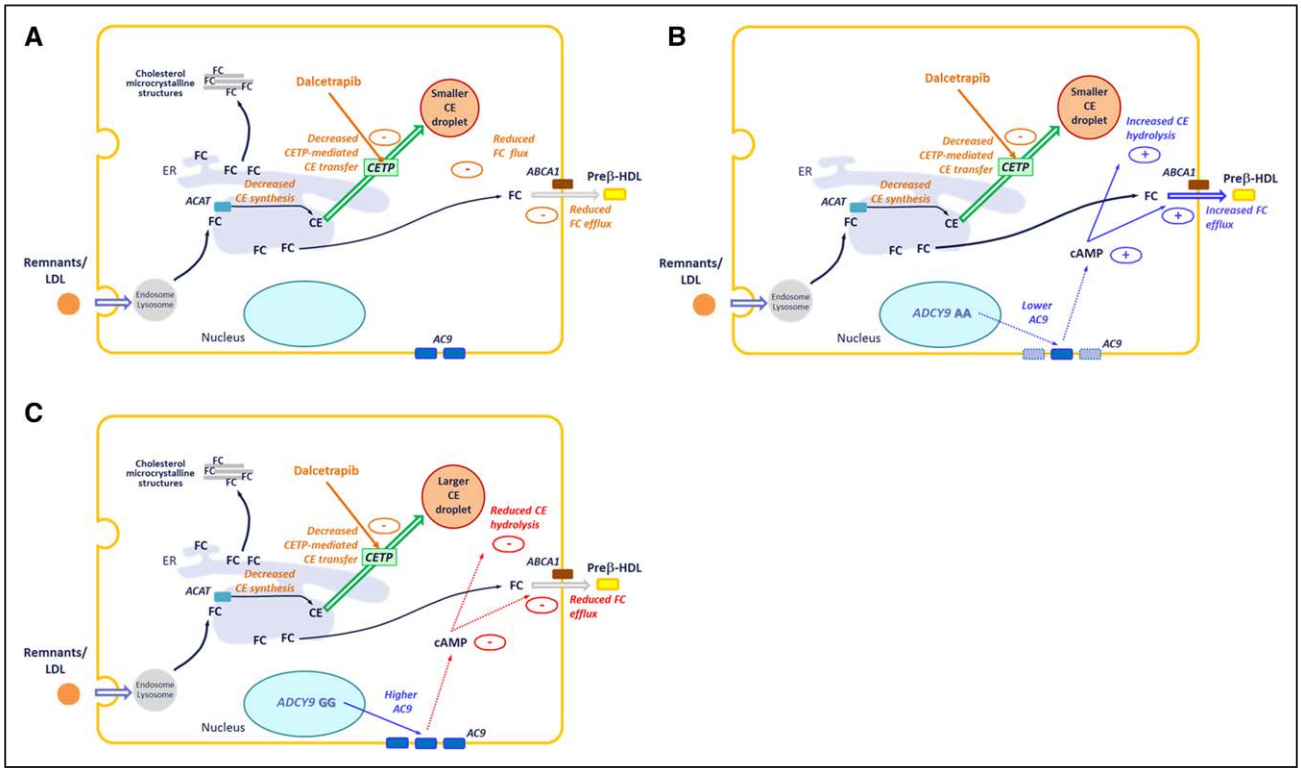

Figure. Conceptual model for the interaction of rs1967309 genotype in the adenylate cyclase 9 (*ADCY9*) gene and dalcetrapib in macrophages.

In this schematic, it is postulated that the arterial wall macrophage is continuously exposed to cholesterol-rich lipoproteins, such as lipoprotein remnants and LDL (low-density lipoprotein), ingests these particles and hydrolyzes their cholesteryl ester (CE) cargo to free cholesterol (FC) in the endo-lysosomal system. FC migrates to the endoplasmic reticulum (ER) where it can be re-esterified via acyl-coenzyme A : cholesterol acyltranferase (ACAT) into CE that is transferred to cytoplasmic lipid droplets for safe storage via the intracellular action of CETP (cholesterol ester transfer protein). **A**, Dalcetrapib inhibits this transfer of CE to lipid droplets and prevents their safe storage, with secondary reduction in CE formation by ACAT in the ER and a consequent rise in the FC concentration in the ER, which may stimulate the formation of cholesterol microcrystal structures and a rise in cholesterol export pathways that are modulated by genetic variants. **B**, Individuals with the *ADCY9* AA genotype and in turn higher cellular levels of cyclic adenosine monophosphate (cAMP) can upregulate ABCA1-mediated free cholesterol efflux to HDL (high-density lipoprotein) and prevent accumulation of free cholesterol. **C**, Those with the GG genotype have lower cAMP and cannot mount the same protective response. Free cholesterol accumulates and increases the possible formation of proinflammatory cholesterol micro-crystal structures that trigger atherogenesis. AA indicates homozygous AA carriers for rs1967309; GG, homozygous GG carriers for rs1967309.

Because dalcetrapib enters into cells and binds covalently to CETP, we surmise that it can inhibit intracellular CETP-mediated lipid transfers and so modulate lipid metabolism in a range of cell types including those relevant for atherosclerosis. Indeed, dalcetrapib has been found to inhibit complex lipid synthesis (CE and TG) in human adipocytes, hepatocytes and macrophages (unpublished data), possibly as a result of a reduction in transfer from the endoplasmic reticulum to droplets (Figure). We speculate that free cholesterol then accumulates and the response of the cell should be to prevent its further build-up, which in the macrophage means stimulating cholesterol efflux since there is no ability to assemble and secrete lipoproteins or export cholesterol as lipoproteins in these cells. Since cAMP is a key regulator of ABCA1-mediated cholesterol efflux in macrophages, higher intracellular cAMP levels because of altered AC9 activity (AA patients) may lead to greater cholesterol efflux and reduced macrophage cholesterol burden upon treatment with dalcetrapib (Figure [B]). In contrast, patients with the GG genotype (Figure [C]) and limited ability to raise cAMP levels would be consequently less able to upregulate ABCA1-mediated efflux and make CE and store it safely in droplets, and ultimately become burdened with excessive free cholesterol. The consequences of these dalcetrapib-induced changes in GG carriers is increased systemic inflammation reflected by increased hsCRP, as seen in the dal-OUTCOMES trial, and with time increased cardiovascular risk.

The dal-GenE Trial—Testing Dalcetrapib as a Pharmacogenomics-Based Precision Medicine in Postacute Coronary Syndrome Patients With rs1967309 AA Genotype

ADCY9 genotype-dependent effects of dalcetrapib on cardiovascular outcomes were observed in the post hoc pharmacogenomic analysis of dal-OUTCOMES.[7] This hypothesis is now being prospectively tested in the dal-GenE study (URL: https://www.clinicaltrials.gov; Unique identifier: NCT02525939). dal-GenE is a phase 3 morbidity and mortality randomized trial that has recruited 6149 postacute coronary syndrome patients with the rs1967309 AA genotype in the *ADCY9* gene (AG and GG subjects were excluded at the time of screening). By approximating the design of dal-OUTCOMES, dal-GenE will provide an answer to the question as to whether dalcetrapib reduces cardiovascular events in patients with that specific genotype. The trial started in 2016 and is expected to report early in 2021. dal-GenE is the first pharmacogenomics precision medicine-based study targeting the reduction of cardiovascular events in patients with a recent acute coronary syndrome and as such constitutes a ground-breaking trial. In January 2020, DalCor Pharmaceuticals announced that dal-GenE will continue as planned following the recommendation of the independent Data Safety Monitoring Board, based on the results of an interim futility analysis. It is thus expected that a definitive answer is within our reach.

Limitations

This new field of research arising from the discovery of a potential interaction between *ADCY9* genotype and the effects of dalcetrapib raises several questions that are unresolved because of information missing in the literature. Among these, the inability to replicate the pharmacogenomic results of dal-OUTCOMES in a cohort with the whole spectrum of genotypes at rs1967309 constitutes an important limitation to ascertain the interaction of dalcetrapib with ADCY9 variants. Although association studies have tried to replicate the pharmacogenomic finding in patients receiving evacetrapib[12] or anacetrapib,[13] no significant association between *ADCY9* SNP (rs1967309) and cardiovascular outcomes was found. Even if these results raised concern about the possibility of false-positive findings with dalcetrapib, the effects of the latter agent and CETPi on lipoprotein cholesterol levels are very different. Moreover, differences in patient characteristics among phase 3 clinical trials may have contributed to the divergent pharmacogenomic results obtained between dalcetrapib and other CETPi. In that context, the results of dal-GenE are particularly important and will provide the definitive answer as to whether dalcetrapib improves cardiovascular outcomes in AA patients.

The intronic location of rs1967309 is another aspect raising important questions. More investigations are required to define whether effects of dalcetrapib in AA patients are associated with the *ADCY9* gene. Indeed, the involvement of at least one other gene modulated by the SNP cannot be excluded. The possibility that ADCY9 modulation depends on the tissues studied increases the challenge to understand the involvement of this gene in the effects of dalcetrapib.

Although results in mice are very promising, this model does not allow the possibility to study directly the interaction between ADCY9 and dalcetrapib. Indeed, it was demonstrated that administration of dalcetrapib to mice, expressing or not CETP, reduces HDL-C level instead of the expected rise caused by CETP inhibition.[67] This suggests that dalcetrapib has off-target effects in mice. Thus, other models are required to determine whether the effects of dalcetrapib are modulated by the level of *ADCY9* expression. Moreover, mice models do not allow the study of the impact of SNP rs1967309, since no genetic variation was reported at that location in the mouse genome. However, despite these limitations for the study of dalcetrapib's effects, the Gt/Gt mouse remains an excellent model to characterize the physiological role of ADCY9.

CONCLUSIONS

The discovery of an association between *ADCY9* gene polymorphism rs1967309 and responses to dalcetrapib represents the first step in cardiovascular precision medicine based on pharmacogenomics, even if several unresolved questions remain. In that context, the ongoing dal-GenE study will provide the definitive answer as to whether an interaction between dalcetrapib and this SNP truly exists.

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