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Emerging LDL Therapies: Using Human Genetics to Discover New Therapeutic Targets for Plasma Lipids

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Abstract

In humans, genetic variation occurs through different types of alleles that vary in frequency and severity of effect. Mendelian mutations, such as those in the low-density lipoprotein (LDL) receptor (*LDLR*) that result in familial hypercholesterolemia, are rare and have powerful phenotypic effects. Conversely, alleles that are common in the population (such that homozygotes for the minor allele are present even in modest sample sizes) typically have very modest phenotypic effects. In the middle of the spectrum are Goldilocks alleles such as mutations in the gene for proprotein convertase subtilisin/kexin type 9 (*PCSK9*). Loss-of-function mutations in *PCSK9* result in significantly decreased LDL-cholesterol (LDL-C) levels, and a disproportionately large reduction in coronary heart disease risk by reducing the exposure to LDL-C throughout life. Several agents to inhibit *PCSK9* are currently in development demonstrating the potential utility of translating genetics into clinical therapeutics. To date, most investigations aimed at identifying the genes responsible for hypercholesterolemia have used linkage analysis, which requires samples collected from multiple families with defects in the same gene, or common variant analysis which requires thousands of samples from the population. However, case studies have shown that with advances in whole genome sequencing or exome sequencing (targeted exome capture), the process of discovering causal genetic mutations can be significantly streamlined. Astute clinical observation of individual patients and their families with atypical lipid profiles, followed by sequencing of the affected individual, has the potential to lead to important findings regarding the genetic mutations that cause lipid abnormalities.

Keywords

familial hypercholesterolemia; LDL cholesterol; PCSK9; genetics; sequencing

Introduction

The study of genetics is primarily an examination of genetic variation, which in humans occurs through diverse alleles with varying frequencies and effect sizes (Figure 1). At one extreme are mutations that cause Mendelian diseases such as familial hypercholesterolemia (FH) arising from mutations in the low-density lipoprotein (LDL) receptor gene (*LDLR*) and familial defective Apo B arising from defects in the apolipoprotein (Apo) B gene (*APOB*).[1,2] These mutations are inevitably rare and have powerful effects on phenotype. At the other end of the mutation spectrum are common alleles, e.g., the Apo E (*APOE*) allele

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and variants in the sortilin 1 gene (*SORT1*).[3, 4] These variations are characterized by small phenotypic effects. In the middle of the spectrum is a class of alleles termed "Goldilocks alleles." The quintessential example of a Goldilocks allele is provided by the proprotein convertase subtilisin/kexin type 9 gene (*PCSK9*),[5] which has mutations that are common enough to be useful in epidemiological analyses, in contrast to Mendelian alleles, but still produce readily detectable effects in biochemical assays.

Mendelian Alleles

Mendelian alleles have traditionally been identified by linkage studies in families. The classic example of FH is shown in a pedigree in which two individuals with unusually high LDL cholesterol (-C) levels and mutations in the *LDLR* married and produced two heterozygote children and one homozygote with severe hyperlipidemia. Studies indicate that ~5% of patients who have had a myocardial infarction (MI) before 60 years of age have heterozygous FH, and ~50% of untreated FH heterozygotes will have an MI by age 60.[6, 7] These results suggest that an isolated high LDL-C level is sufficient to produce coronary heart disease (CHD).

The genetics of FH were initially investigated in Lebanon in the 1970s, prior to the era of molecular genetics, when Khachadurian recognized that there was more than one type of FH.[8] The dominant form, where at least one parent was affected, was well known, but another type of FH was suspected in a family in which two parents with normal LDL-C levels had four children with extremely high LDL-C levels. To date, there are at least five known dominant and recessive disorders of LDL metabolism. Dominant disorders include FH caused by a mutation of *LDLR*; familial defective Apo B caused by a defect in *APOB*,[9] which diminishes the ability of LDL to bind to the LDL receptor; and FH type 3 caused by mutations in *PCSK9* which increase degradation of the LDL receptor.[10]

There are two well-defined recessive disorders of hypercholesterolemia: autosomal recessive hypercholesterolemia and sitosterolemia.[11, 12] Autosomal recessive hypercholesterolemia (ARH) is caused by a defect in the *ARH* gene, also known as the LDL receptor adaptor protein 1 gene (*LDLRAP1*), which encodes an adaptor protein that is required for localization of the LDL receptor to clathrin-coated pits.[12] In the absence of functioning *ARH*, LDL receptors sit on the surface of the cell where they can bind to LDL but are unable to internalize it. Sitosterolemia is a disorder of neutral sterol excretion characterized by an accumulation of plant sterols. It is caused by mutations in two pumps, adenosine triphosphate-binding cassette sub-family G member 5 (*ABCG5*) and member 8 (*ABCG8*) which together form a complex that pumps neutral sterols out of enterocytes and hepatocytes.[11] Individuals with sitosterolemia have varying degrees of hypercholesterolemia. All five of these dominant and recessive disorders are significantly associated with premature severe CHD, demonstrating that, irrespective of cause, elevated LDL-C is a risk factor for CHD.

Common Alleles

There is a great deal of interest in finding common alleles that explain the differences in LDL-C levels in the general population. Genome wide association studies (GWAS) examine many genetic variants to determine if any are associated with a trait, typically major diseases or conditions like hypercholesterolemia. Results from a monumental meta-analysis of GWAS evaluating more than 100,000 individuals indicated that there are at least 95 different loci in the human genome that are systematically associated with plasma lipid and lipoprotein levels.[4] However, essentially all of these effects are small individually, and cumulatively these sequence variations explain only a small fraction of the variation in LDL-C or high-density lipoprotein (HDL)-C levels.

There are pros and cons of GWAS.[13, 14] In many cases the effects of the variants identified are small and difficult to interpret biologically. On the other hand, they may reveal genes that are not amenable to Mendelian genetics. .For example, it's unlikely that a Mendelian defect in 3- hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the rate limiting enzyme in cholesterol biosynthesis, will ever be detected, because no cell can live without HMG-CoA reductase. Yet more subtle mutations or variations HMG CoA reductase have been captured by a GWAS in the general population.

Epidemiological investigations have demonstrated a strong positive correlation between increased LDL-C and increased risk for CHD, and a negative correlation between HDL-C and CHD risk.[13] However, this correlation, although highly consistent, does not prove that low HDL-C causes CHD. While there is a significant body of evidence pointing to the causality of high LDL-C and CHD, whether or not a high HDL-C concentration *per se* protects from CHD is unclear.[15] To demonstrate causality, investigations are needed that assess whether sequence variations that systematically confer either a high HDL-C or a low HDL-C level, absent of any other change in the lipoprotein profile, are associated with CHD. In aMendelian randomization analysis of data from prospective studies, Kathiresan and colleagues analyzed multiple variants affecting HDL-C levels, and their correlation with CHD.[17] Using a genetic score of common polymorphisms associated with LDL-C as a positive control, a genetic score based on gene sequence variations that increased HDL-C levels was not significantly associated with reduced risk of MI [odds ratio (OR) per standard deviation increase in HDL-C due to HDL-C genetic score 0.93, 95% confidence interval (CI) 0.68–1.26, p = 0.63] (Figure 2).[17] These data support the argument that HDL-C is not causally related to CHD. Although it is clearly associated, HDL-C is more likely a marker for the disease process than a direct cause thereof. Notably the estimate from observational epidemiology that a one standard deviation increase in LDL-C was associated with increased risk for MI was concordant with that determined from the LDL-C genetic score [OR 2.13, 95% CI 1.69–2.69, p = 2x10(−10)], supporting the theory that genetic mechanisms that raise LDL-C translate directly into increased MI.[17]

Goldilocks Alleles – *PCSK9*

PCSK9 was initially implicated in lipoprotein metabolism when *PCSK9* mutations were found in two families with autosomal dominant hypercholesterolemia, but normal *LDLR* alleles. Studies in mice revealed that these mutations were, in fact, gain-of-function. An examination of the low end of the LDL-C distribution in the Dallas Heart Study found that a significant fraction of individuals had mutations in *PCSK9*.[5, 18, 19] These were loss-of-function mutations. About 2% of the African Americans evaluated had one loss-of-function allele in *PCSK9* that lowered LDL-C by 40%. Among European Americans, ~3% had a less severe mutation that resulted in 21% lower LDL-C levels.

An examination of the Atherosclerosis Risk in Communities (ARIC) population showed that among African Americans, the average LDL-C reduction in carriers of the *PCSK9* mutation (heterozygotes) was 28%. This was associated with an 88% reduction in incident CHD over a period of 15 years.[5] In European Americans, the LDL-C reduction was 15%, leading to a 46% reduction in CHD. Of note, more than half of the African Americans studied were hypertensive, one-third smoked, and almost 20% had diabetes (risk factors which occurred with the same frequency in mutation carriers and non-carriers). Nonetheless, the *PCSK9* mutation conferred marked protection from CHD up to ~age 70, the upper age limit of the study participants at the time of screening.

The magnitude of protection against CHD provided by the lower LDL-C level observed in those with *PCSK9* loss-of-function mutations in the Dallas Heart and ARIC studies is

greater than might be expected based on results from statin trials.[20, 21] This discrepancy holds true in other populations, and is not peculiar to *PCSK9*. Variants such as *APOB*, *SORT1*, and *LDLR* also confer a disproportionate increase in CHD risk protection relative to the level of LDL-C, based on predictions from statin trials (Figure 3).[1, 22] The reason for this may be that genotypes capture another dimension – time. The phenotype of LDL-C concentration gives a snapshot of the cholesterol concentration at the present time, but genotypes integrate the "tendency" of an individual's LDL-C concentration since birth.[21]

Pragmatically, it appears that complex diseases, particularly CHD, may not actually be so complex. In the case of CHD it is clear there is a single predominant risk factor. While the value of other risk factors should not be ignored, the cardinal risk factor is high LDL-C concentration combined with the amount of time the patient has been exposed to increased LDL-C (or atherogenic LDL particles). A measure of cumulative exposure, analogous to pack-years for smokers, would provide a more accurate prediction of risk of CHD. Currently, therapeutic steps are not taken until an individual has an increased 10-year risk beyond some threshold; typically this involves a treatment delay until patients are at least 50 years of age.[15] Genetic data suggest that starting treatment earlier might produce "more bang for the therapeutic buck" whether that therapy is diet or statins.

Early experiments raised considerable interest in the utility of *PCSK9* for LDL-C-lowering and CHD prevention, but questions remained regarding the safety of manipulating *PCSK9*, particularly because it is expressed in several tissues in addition to the liver (e.g., kidneys, brain). In order to investigate whether the absence of PCSK9 would be compatible with good health, family members of individuals in the Dallas Heart Study with a single mutation in *PCSK9* were identified. In one of these families, the proband was a 53-year-old woman with an LDL-C level of 49 mg/dL. Her 32-year-old daughter had an unusually low LDL-C level (14 mg/dL).[23] The daughter had inherited her mother's mutation in *PCSK9*, Y142X, which is a premature stop code in the protein transcript, and another mutation from her father which deleted an arginine at codon 97. For practical purposes, these mutations rendered both copies of the gene non-functional resulting in total deficiency of PCSK9. The deficiency in PCSK9 did not appear to have any adverse effects. Her intelligence was normal (she was a college graduate); she had normal kidney and liver functions and normal blood pressure. PCSK9 is expressed at fairly high levels in the cerebellum, particularly during development, but neurological examination and magnetic resonance imaging results, plus her vocation as an aerobics instructor, confirmed that the PCSK9 deficiency did not adversely affect her motor control. A follow-up six years later verified that after nearly 40 years of total absence of PCSK9 and extremely low LDL-C (14–29 mg/dL), this individual continued to be in good health. Subsequently, a 21-year-old pregnant Zimbabwean woman was identified as a compound heterozygote with two *PCSK9* mutations.[24] Little is known about this patient, other than she had an extremely low LDL-C level (16 mg/dL) when she visited an antenatal clinic.

The culmination of these genetic efficacy and safety studies has been the development of agents designed to inhibit PCSK9. It has proved difficult to target PCSK9 with small molecules, but several companies have developed monoclonal antibodies that inhibit PCSK9. The results of the first trial demonstrated that inhibition of PCSK9 caused a sharp, prompt, and relatively isolated reduction in LDL-C, without obvious side effects, exactly as would be predicted from the genetics.[25] The rapid course of this work illustrates the utility of genetics in the development of therapeutic agents. From the initial discovery to clinical trials has taken seven years: the initial discovery of *PCSK9* in 2003, to the discovery of LDL-C-lowering variants in the gene and target validation, and completion of phase one trials four years later. Several trials of PCSK9 inhibitors are now in progress.

Whole Genome Sequencing

New developments in sequencing technology have made it possible to screen the entire genome (or exome), potentially allowing the identification of disease-causing mutations in novel genes in individual patients. The power of this approach for patients with lipid disorders was illustrated by the discovery of a family with atypical hypobetalipoproteinemia in which four of the offspring had very low levels of LDL-C, HDL-C, and triglycerides. These individuals were distinguished from carriers of typical *APOB* mutations that prevent secretion of very low-density lipoprotein because they did not have steatosis, which almost invariably occurs in individuals with conventional familial hypobetalipoproteinemia.[26] Exome sequencing revealed that the affected offspring were homozygous for a mutation in *ANGPTL3*, a gene which encodes angiopoietin-like 3 – an inhibitor of lipoprotein lipase and endothelial lipase.[27] This finding demonstrates that astute clinical observation of individual patients and their families with atypical and unexplained lipid profiles, followed by sequencing of the affected individuals, has the potential to provide major new insights into the molecular basis of lipid diseases.

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List of Abbreviations

ABCG5	adenosine triphosphate-binding cassette sub-family G member 5
ABCG8	adenosine triphosphate-binding cassette sub-family G member 8
ANGPTL3	angiopoietin-like 3 gene
Apo	apolipoprotein
APOB	apolipoprotein B gene
APOE	apolipoprotein E gene
ARH	autosomal recessive hypercholesterolemia
CHD	coronary heart disease
CI	confidence interval
FH	familial hypercholesterolemia
GWAS	genome wide association studies
HDL-C	high-density lipoprotein cholesterol
HMG CoA Reductase	3-hyroxy-3-methylglutaryl coenzyme A reductase
LDL-C	low-density lipoprotein cholesterol
LDLR	low-density lipoprotein receptor gene
MI	myocardial infarction
OR	odds ratio
PCSK9	proprotein convertase subtilisin/kexin type 9

SORT1	sortilin 1

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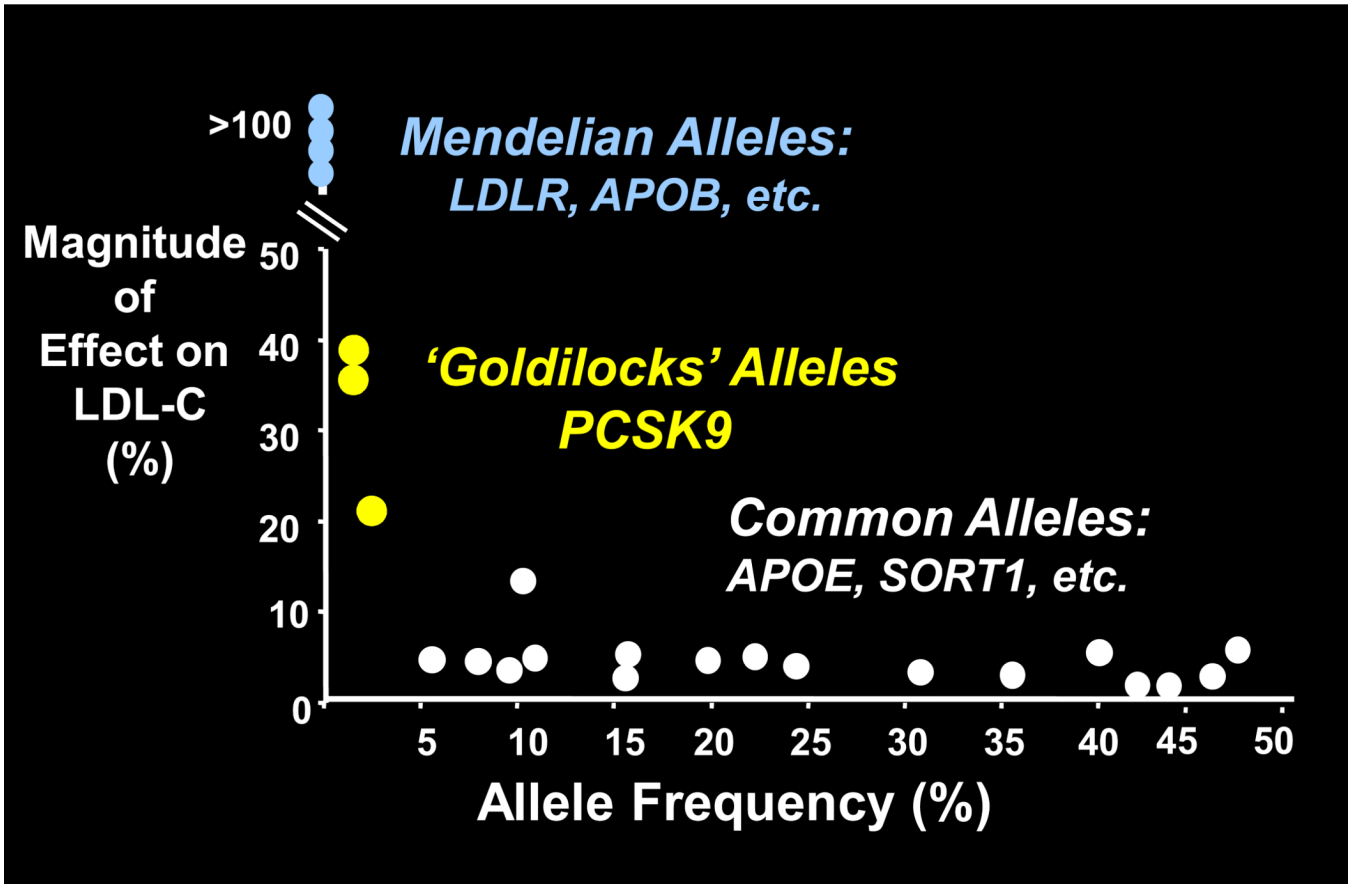

Figure 1.
Schematic of the relationship between allele frequency and effect size
Abbreviations: *APOB* = gene for apolipoprotein B, *APOE* = gene for apolipoprotein E, LDL-C = low-density lipoprotein cholesterol, *LDLR* = gene for low-density lipoprotein receptor, *PCSK9* = gene for proprotein convertase subtilisin/kexin type 9, *SORT1* = gene for sortilin 1

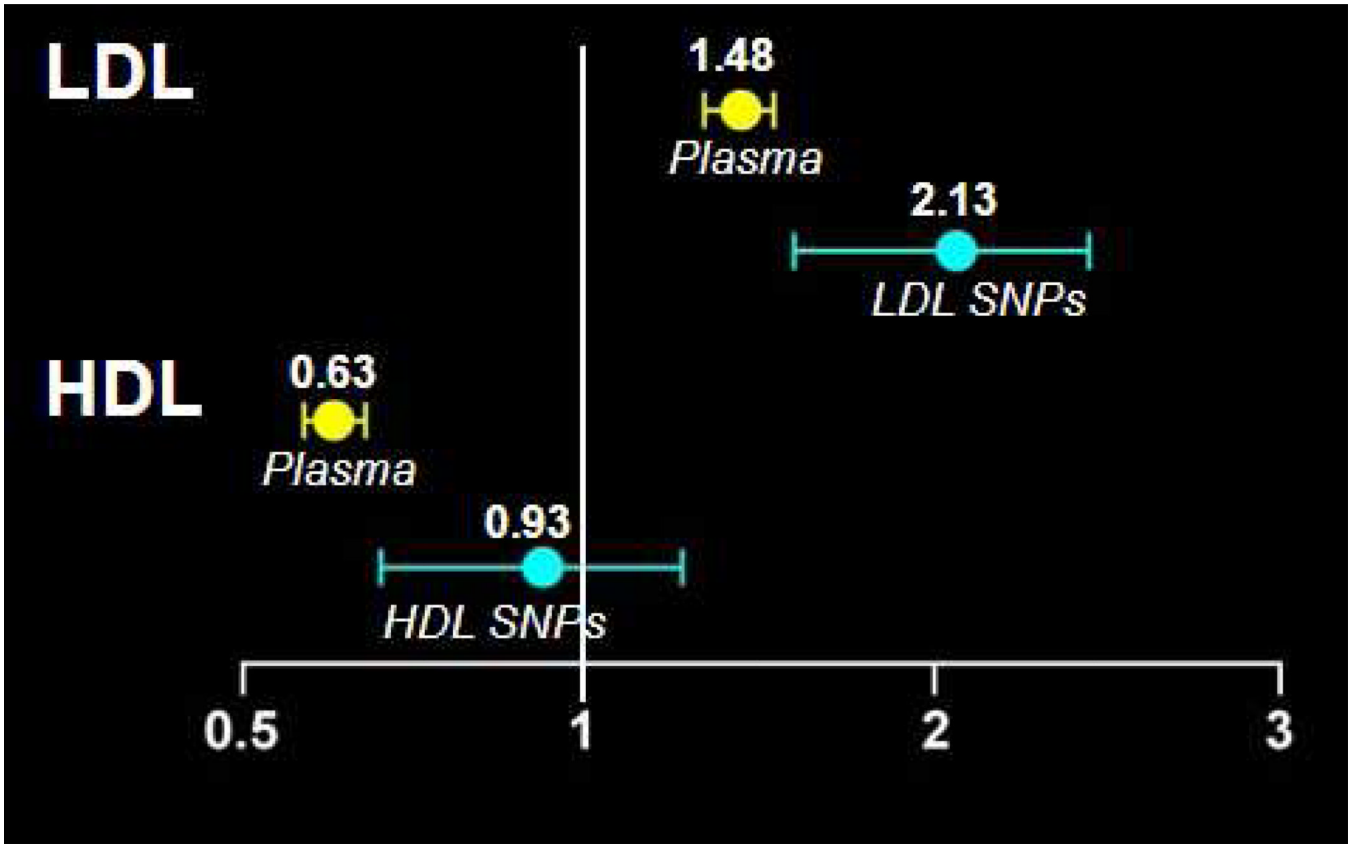

Figure 2.
Hazard ratios of myocardial infarction in prospective studies (n = 25,000) per standard deviation increase in lipid level. Plasma denotes predicted hazard ratios for the change in plasma levels of each lipoprotein that would be caused by the SNPs.
Abbreviations: HDL = high-density lipoprotein, LDL = low-density lipoprotein, SNPs = single nucleotide polymorphisms
[17]Voight BF, et al. Plasma HDL cholesterol and risk of myocardial infarction: a Mendelian randomization study. *Lancet.* 2012; 380:572–580.

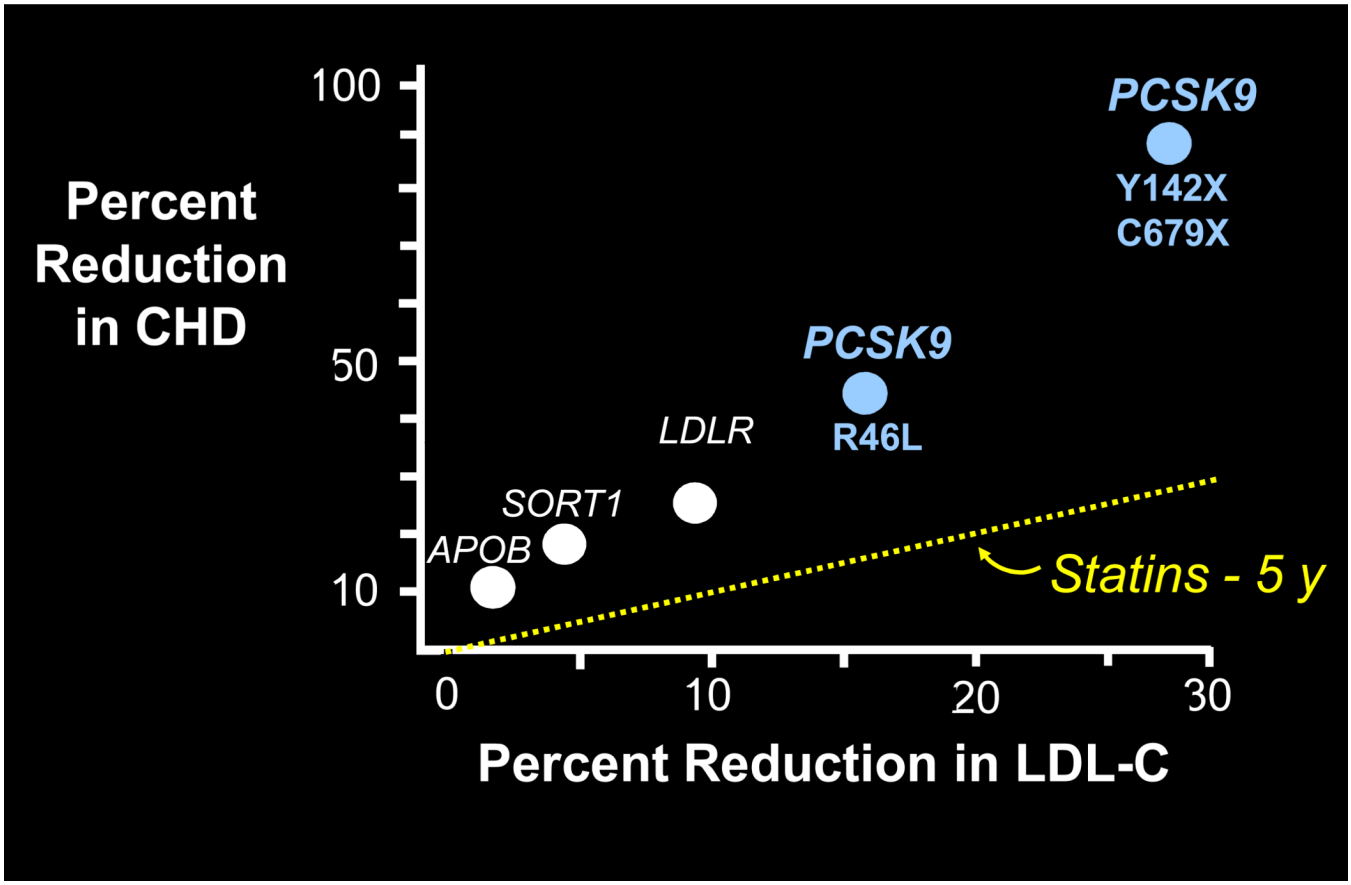

Figure 3.

Reduction in coronary heart disease associated with LDL-C lowering according to gene variants or statin use

Abbreviations: *APOB* = gene for apolipoprotein B, CHD = coronary heart disease, LDL-C = low-density lipoprotein cholesterol, *LDLR* = gene for low-density lipoprotein receptor, *PCSK9* = gene for proprotein convertase subtilisin/kexin type 9, *SORT1* = gene for sortilin 1

[1]Linsel-Nitschke P, et al. Lifelong reduction of LDL-cholesterol related to a common variant in the LDL-receptor gene decreases the risk of coronary artery disease – a Mendelian Randomisation study. *PLoS One*. 2008;3:e2986.

[22]Willer CJ, et al. Newly identified loci that influence lipid concentrations and risk of coronary artery disease. *Nat Genet*. 2008;40:161–169.