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PCSK9 R46L, Low-Density Lipoprotein Cholesterol Levels, and Risk of Ischemic Heart Disease

3 Independent Studies and Meta-Analyses

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Objectives The aim of this study was to examine the effect of *PCSK9* R46L on low-density lipoprotein cholesterol (LDL-C), risk of ischemic heart disease (IHD), and mortality.

Background The 46L allele has been associated with reductions in LDL-C and risk of IHD, but results vary between studies.

Methods We determined the association of R46L genotype with LDL-C, risk of IHD, myocardial infarction (MI), and mortality in the prospective CCHS (Copenhagen City Heart Study) (n = 10,032) and validated the results in: 1) the cross-sectional CGPS (Copenhagen General Population Study) (n = 26,013); and 2) the case-control CIHDS (Copenhagen Ischemic Heart Disease Study) (n = 9,654). We also performed meta-analyses of present and previous studies (n = 66,698).

Results In carriers (2.6%) versus noncarriers, the 46L allele was associated with reductions in LDL-C of 0.35 to 0.55 mmol/l (11% to 16%) from 20 to 80+ years in the general population (CCHS and CGPS; p values <0.0001). Observed risk reductions for IHD in 46L allele carriers were: 6% in the CCHS study (hazard ratio [HR]: 0.94; 95% confidence interval [CI]: 0.68 to 1.31), 46% in the CGPS study (odds ratio [OR]: 0.54; 95% CI: 0.39 to 0.77), 18% in the CIHDS study (OR: 0.82; 95% CI: 0.55 to 1.21), and 30% in the studies combined (OR: 0.70; 95% CI: 0.58 to 0.86). In the CCHS study, HR for mortality was 1.18 (95% CI: 0.93 to 1.50). In meta-analyses, 46L allele carriers had a 12% (0.43 mmol/l) reduction in LDL-C and a 28% reduction in risk of IHD (HR: 0.72; 95% CI: 0.62 to 0.84), similar to results in the CCHS, CGPS, and CIHDS studies combined. However, the observed 12% (0.43 mmol/l) reduction in LDL-C theoretically predicted an only 5% reduction in risk of IHD (HR: 0.95; 95% CI: 0.92 to 0.97).

Conclusions The *PCSK9* 46L allele was associated with reductions in LDL-C from 20 to 80+ years in the general population. The reduction in risk of IHD was larger than predicted by the observed reduction in LDL-C alone. This could be because genotype is a better predictor of lifelong exposure to LDL-C than LDL-C measured in adult life. (J Am Coll Cardiol 2010;55:2833–42) © 2010 by the American College of Cardiology Foundation

Proprotein convertase subtilisin/kexin type 9 (*PCSK9*) modulates plasma levels of low-density lipoprotein cholesterol (LDL-C) by promoting the degradation of low-density lipoprotein receptors (LDLRs), the primary pathway for the removal of low-density lipoprotein (LDL) from the circulation. The *PCSK9* seems to interfere with recycling of the LDLR by targeting the receptor to the lysosome for degradation, leading to reduced clearance of LDL-C from the circulation (1–3). Gain-of-function mutations in *PCSK9* are rare and cause hypercholesterolemia and ischemic heart disease (IHD) (4), whereas loss-of-function mutations are more common and are associated with reduced plasma levels of LDL-C and protection from IHD (5,6).

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The R46L polymorphism in *PCSK9* has been associated with reductions in LDL-C of 0.28 to 0.54 mmol/l (9% to 15%) (6–10) and with a 47% reduction in risk of IHD in carriers versus noncarriers (6). This lower risk of IHD is considerably greater than that expected from statin trials, where a similar reduction in LDL-C would have caused an approximately 9% to 15% reduction in risk (11). This

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Abbreviations and Acronyms

CI = confidence interval
HDL-C = high-density lipoprotein cholesterol
HR = hazard ratio
IHD = ischemic heart disease
LDL-C = low-density lipoprotein cholesterol
LDLR = low-density lipoprotein receptor
MI = myocardial infarction
OR = odds ratio
PCSK9 = proprotein convertase subtilisin/kexin type 9

discrepancy between predicted and observed risk has been suggested to be due to the presumed life-long reduction in levels of LDL-C associated with this genetic variant, as opposed to the short-term evaluation of effect in statin trials (6). Because of this, it has been argued that early and more aggressive LDL reduction is needed (12,13). A reduction in risk of IHD could also translate into a reduced mortality, but this has never been examined for *PCSK9* R46L.

We tested the hypothesis that the *PCSK9* 46L allele is associated with lower LDL-C levels in all age groups from 20 to 80+ years, and with reduced risk of IHD, myocardial infarction (MI), and mortality. We genotyped 45,699 individuals from 3 independent Danish studies: the prospective CCHS (Copenhagen City Heart Study) (n = 10,032), the cross-sectional CGPS (Copenhagen General Population Study) (n = 26,013), and a case-control study, the CIHDS (Copenhagen Ischemic Heart Disease Study) (n = 9,654). Finally, the association of the *PCSK9* R46L genotype with LDL-C levels and risk of IHD were summarized in meta-analyses including previous and present studies.

Methods

Subjects. Studies were approved by institutional review boards and Danish ethical committees (Nos. [KF]V.100. 2039/91, [KF]01-144/01, KA93125, and KA99039) and conducted according to the Declaration of Helsinki. Informed consent was obtained from participants. All participants were white and of Danish descent. No participants appeared in more than 1 of the 3 studies, permitting independent confirmation of the findings in each group.

The CCHS trial. CCHS is a prospective study of a general population initiated in 1976 to 1978 with follow-up examinations from 1981 to 1983, 1991 to 1994, and 2001 to 2003 (14). Individuals were selected on the basis of the national Danish Civil Registration System to reflect the adult Danish population ages 20 to 80+ years. Baseline was the first examination the individuals participated in. Blood samples for deoxyribonucleic acid extraction were available on 10,032 of the 10,135 participants (99%) attending the 1991 to 1994 and/or 2001 to 2003 examinations.

Information on diagnosis of IHD (World Health Organization International Classification of Diseases-8th edition: codes 410 to 414; 10th edition: I20 to I25) was collected and verified from 1976 until July 2007 by reviewing all hospital admissions and diagnoses entered in the national Danish Patient Registry and all causes of death entered in the national Danish Causes of Death Registry. A diagnosis of MI required the presence of at least 2 of the following criteria: characteristic chest pain, elevated cardiac enzymes, and electrocardiographic changes indicative of MI (15). Follow-up was 100% complete for both morbidity (IHD, MI) and mortality (i.e., no individual was lost to follow-up). Median follow-up time was 28.7 years (range 0 to 31.4 years). Individuals diagnosed with an end point before entry were excluded from Cox regression analyses, and those dying during follow-up were censored at their death date.

The CGPS study. CGPS is a cross-sectional study initiated in 2003 with ongoing enrollment. Participants were ascertained from the general population exactly as at enrollment in the CCHS study: data were obtained from a questionnaire, physical examination, and blood samples. At the time of genotyping 31,013 had been included; of these, 5,000 were used as control subjects in the CIHDS study (see the following section), leaving 26,013 for analyses in the CGPS study. Diagnoses of IHD and MI were established exactly as in the CCHS study, collected from registers, and verified from 1976 until July 2007.

The CIHDS trial. CIHDS comprised 4,654 patients from the greater Copenhagen area referred for coronary angiography to Copenhagen University Hospital during the period 1991 to 2007 and 5,000 unmatched control subjects randomly selected from individuals without IHD in the CGPS study. The unmatched design was selected to avoid introduction of a selection bias among the remaining participants in the CGPS study. Control subjects were excluded from data analysis in the CGPS study. Cases had documented IHD on the basis of characteristic symptoms of angina pectoris (15) plus at least 1 of the following: stenosis or atherosclerosis on coronary angiography, a previous MI, or a positive exercise electrocardiography test. The diagnosis of MI was established with the same criteria as in the CCHS and CGPS studies. Data were available from enrollment and from registers from 1976 until July 2007.

Laboratory analyses. Genotyping of R46L (rs11591147) and 3 additional nonsynonymous single-nucleotide polymorphisms in *PCSK9* (A53V, rs11583680; I474V, rs562556; and E670G, rs505151) (16) was by TaqMan, ABI Prism 7900HT (Applied Biosystems, Foster City, California). Each run included a positive control verified by sequencing. Genotypes were verified by sequencing of all R46L homozygotes, 20 randomly selected R46L heterozygotes, and 20 noncarriers.

Colorimetric and turbidimetric assays were used to measure nonfasting plasma levels of total cholesterol, high-density lipoprotein cholesterol (HDL-C), and triglycerides. The LDL-C was calculated with the Friedewald equation when plasma triglycerides were ≤4.0 mmol/l and otherwise measured directly by a colorimetric assay (14). Non–HDL-C was total cholesterol minus HDL-C.

Other covariates. Information on diabetes mellitus, smoking, and hypertension were dichotomized and defined as diabetes (self-reported disease, use of antidiabetic medica-

tion, and/or a nonfasting plasma glucose >11.0 mmol/l), smoking (current smokers), and hypertension (systolic blood pressure ≥140 mm Hg, diastolic blood pressure ≥90 mm Hg, and/or use of antihypertensive medication prescribed specifically for hypertension). Body mass index was weight (kg) divided by height squared (m^2).

Statistical analysis. Data were analyzed with Stata/S.E. version 10.1 (StataCorp, College Station, Texas). Two-sided probability values <0.05 were considered significant. Exact uncorrected p values are given in the tables, whereas values corrected for multiple comparisons with the Bonferroni method (significance level = 0.05/number of comparisons) are given with symbols. Student *t* test and Pearson's chi-square test were used in 2-group comparisons.

In the prospective CCHS study, with the use of left truncation and delayed entry, Cox proportional hazards regression models with age as time scale were used to estimate risk of IHD and MI. Multivariate models were adjusted for age, sex, body mass index, smoking, hypertension, diabetes mellitus, and *PCSK9* A53V, I474V, and E670G genotypes. Because data were available from both the 1991 to 1993 and 2001 to 2003 examinations of the CCHS study, we were able to take changes in risk factors over time into account with time-dependent covariates in analyses; however, results were similar when using baseline data only (fixed model). In the cross-sectional CGPS study and in the case-control CIHDS study, in which cases and control subjects were unmatched, unconditional logistic regression analysis was used to estimate odds ratios (ORs). Multivariate models were adjusted for age, sex, body mass index, smoking, hypertension, and diabetes mellitus.

The decrease in hazard ratio (HR) for IHD for a 1-mmol/l decrease in LDL-C level observed in the CCHS study was used to predict theoretical HRs for IHD and mortality (only in the CCHS study) associated with the decreases in LDL-C levels caused by the 46L allele in the 3 studies, in the studies combined, and in meta-analyses. The decrease in HR was estimated with Cox regression analysis with LDL-C as a continuous variable. The observed mean difference in LDL-C for 46L allele carriers versus noncarriers in the studies was multiplied by the beta-coefficient for the HR for IHD or mortality (only in the CCHS study) observed for a 1-mmol/l decrease in LDL-C and subsequently transformed into a theoretically predicted HR, assuming that the change in risk is due to the change in LDL-C alone. Observed and theoretically predicted HRs as a function of plasma LDL-C levels were corrected for regression dilution bias with a nonparametric method (17). Risk estimates were compared with Altman's method for estimating differences in effect sizes (18). The HR/OR for IHD, which could be detected with 80% power and p = 0.05, was 0.73 or lower in the CCHS study, 0.74 in the CIHDS study, and 0.80 in the CGPS study.

Meta-analyses. To summarize results from previous studies on *PCSK9* R46L genotype, LDL-C levels, and risk of IHD, 2 separate meta-analyses were performed: 1 summarizing association between R46L genotype and LDL-C levels in studies of general populations only, and 1 summarizing association between R46L genotype and risk of IHD. Relevant peer-reviewed articles were identified by searching the PubMed EMBASE and the Genetic Association Database, limiting the search to the period January 2000 (*PCSK9* was first described in 2003) until June 31, 2009. Keywords used were "PCSK9 OR R46L OR pro-protein convertase subtilisin kexin 9 OR NARC-1" in combination with words related to LDL-C levels ("cholesterol," "low-density lipoprotein," and "LDL") and the end point ("ischemic heart disease," "myocardial infarction," "death," "morbidity," and "mortality"). All reported cases were included in subgroups by ethnicity, if relevant information was available. The studies included in the 2 meta-analyses differ somewhat, because not all studies reported both lipid levels and risk of IHD. Funnel plots were both visually inspected and statistically tested for signs of publication bias with adjusted rank correlation analysis (19) and by linear regression analysis (20). I^2-statistics evaluated study heterogeneity. Fixed effects as well as random effects of standardized mean difference and OR were estimated.

The power to detect an OR of 0.5 or less at p = 0.05, corresponding to the OR initially observed for 46L allele carriers versus noncarriers in the ARIC (Atherosclerosis Risk in Communities) study (6), ranged from 19% in the Northwick Park Heart Study II study to 98% to 99% in the CCHS, CGPS, and CIHDS studies.

Results

We identified 241, 727, and 230 heterozygote 46L allele carriers and 2, 3, and 1 homozygotes in the CCHS, CGPS, and CIHDS studies, respectively (Table 1). The frequency of the 46L allele was 1.3% in the 45,699 individuals examined. Genotype distributions were in Hardy-Weinberg equilibrium (p > 0.74) (Table 1). The RL heterozygotes and LL homozygotes were pooled in subsequent analyses, now called 46L allele carriers, due to the low number of LL homozygotes. In the CCHS study the following risk factors for IHD were increased compared with the CGPS study (Table 1): LDL-C (difference 0.4 mmol/l), total cholesterol (0.3 mmol/l), non–HDL-C (0.4 mmol/l), and HDL-C inversely (−0.1 mmol/l), whereas the following risk factors were decreased: male sex (−4%), age (−1.8 years), body mass index (−0.9 kg/m^2), smoking (−7%), and hypertension (−16%). Furthermore, statin use was very low in the CCHS study (3.9% vs. 9.6%), whereas the prevalence of IHD and MI was 2-fold in the CGPS study (18% vs. 9% for IHD, and 9% vs. 4% for MI). In the CIHDS study, the following risk factors were increased in cases versus randomly selected control subjects from the CGPS study (Online Table 2): male sex (27%), age (1.6 years), LDL-C (0.3 mmol/l), total cholesterol (0.2 mmol/l), non–HDL-C (0.3 mmol/l), remnant cholesterol (0.1 mmol/l), HDL-C inversely (−0.4 mmol/l), triglycerides (0.2 mmol/l), body

Table 1 Characteristics of Participants in the 3 Independent Studies

	CCHS (n = 10,032)	CGPS (n = 26,013)	CIHDS (n = 9,654)
Enrollment	1991–1994 and 2001–2003	2003–ongoing	1991–ongoing
Sex, women (%)	55%*	51%	42%†
Age (yrs)	58.0 (44–69)*	59.8 (51–69)	60.0 (51–69)
LDL-C (mmol/l)	3.6 (2.9–4.4)*	3.2 (2.6–3.9)	3.1 (2.5–3.9)
Total cholesterol (mmol/l)	6.0 (5.1–6.9)*	5.7 (5.0–6.4)	5.4 (4.6–6.2)†
Non–HDL-C (mmol/l)	4.4 (3.5–5.3)*	4.0 (3.2–4.8)	4.0 (3.3–4.8)
Remnant cholesterol (mmol/l)	0.7 (0.5–1.0)	0.7 (0.5–1.0)	0.7 (0.5–1.0)
HDL-C (mmol/l)	1.5 (1.2–1.8)*	1.6 (1.3–2.0)	1.4 (1.1–1.8)†
Triglycerides (mmol/l)	1.5 (1.0–2.2)	1.5 (1.0–2.2)	1.5 (1.0–2.4)
Body mass index (kg/m^2)	24.9 (22.5–27.9)*	25.8 (23.4–28.6)	26.0 (23.6–28.8)
Smoking	48%*	55%	52%†
Hypertension	52%*	68%	48%†
Diabetes mellitus	4.1%	4.5%	6.5%†
Statin use	3.9%*	9.6%	24.2%†
IHD	18%*	9%	48.2%†
MI	9%*	4%	23.1%†
PCSK9 R46L genotype (no. of individuals)			
RR	9,789	25,283	9,581
RL	241	727	230
LL	2	3	1

Data are from the 1991 to 1994 or 2001 to 2003 examinations of the CCHS study (Copenhagen City Heart Study) when deoxyribonucleic acid was collected, from study enrollment 2003 to 2007 for the CGPS study (Copenhagen General Population Study), and from study enrollment 1991 to 2007 in the CIHDS study (Copenhagen Ischemic Heart Disease Study). Values are median and interquartile range or percentages unless otherwise indicated. In the CCHS study (1991 to 1994 examination), low-density lipoprotein cholesterol (LDL-C), total cholesterol, and non–high-density lipoprotein cholesterol (HDL-C) and the relative frequencies of women, ischemic heart disease (IHD), and myocardial infarction (MI) were higher than in the CGPS study (2003–ongoing); whereas age; HDL-C; body mass index; and the relative frequencies of current smoking, hypertension, and individuals treated with statin were lower. In the CIHDS study (1991–ongoing), total cholesterol and HDL-C and the relative frequencies of smokers and hypertension were lower than in the CGPS study, whereas the relative frequencies of diabetes mellitus, statin use, IHD, and MI were higher. *$p < 0.001$, comparing the 2 general population studies, the CCHS versus the CGPS. †$p < 0.001$, comparing the CIHDS versus the CGPS.

mass index (0.7 kg/m^2), and diabetes mellitus (10.8%), whereas the frequency of hypertension was decreased (−22%). Statin use was almost 10-fold increased in cases versus control subjects (39.2% vs. 4%) (Online Table 2).

LDL-C. In 46L allele carriers versus noncarriers, the 46L allele was associated with 0.55 mmol/l (15%) lower LDL-C levels in the CCHS study, 0.35 mmol/l (11%) in the CGPS study, 0.50 mmol/l (16%) in the CIHDS study, and 0.43 mmol/l (13%) in the 3 studies combined (all $p < 0.0001$) (Table 2). Carriers of the 46L allele also had lower levels of total cholesterol (6% to 9%), non–HDL-C (9% to 13%), and apolipoprotein B (8% to 13%) than noncarriers ($p < 0.001$). The LDL-C levels in 46L allele carriers were consistently lower than in noncarriers from 20 to 80+ years of age in both studies of general populations (Fig. 1).

IHD, MI, and mortality. In the prospective CCHS study, comprising 10,032 individuals, *PCSK9* R46L genotype did not predict risk of IHD (multifactorially adjusted HR: 0.94; 95% confidence interval [CI]: 0.68 to 1.31; $p = 0.73$) or MI (HR: 1.13; 95% CI: 0.73 to 1.75; $p = 0.57$) (Table 3). In

Table 2 LDL-C as a Function of *PCSK9* R46L Genotype

	Number of Individuals (46L Allele Carriers)	LDL-C (mmol/l)		LDL-C Difference (mmol/l)	LDL-C Difference (%)	*p Value
		Noncarriers	46L Allele Carriers			
All						
CCHS	10,032 (243)	3.7 (0.01)	3.1 (0.07)	−0.55 (0.08)	15%	<0.0001
CGPS	26,013 (730)	3.3 (0.006)	2.9 (0.03)	−0.35 (0.04)	11%	<0.0001
CIHDS	9,654 (231)	3.2 (0.01)	2.7 (0.07)	−0.50 (0.08)	16%	<0.0001
All	45,699 (1,204)	3.4 (0.005)	2.9 (0.03)	−0.43 (0.03)	13%	<0.0001
Statin users excluded						
CCHS	9,634 (238)	3.7 (0.01)	3.2 (0.07)	−0.50 (0.08)	14%	<0.0001
CGPS	23,521 (695)	3.4 (0.006)	3.0 (0.04)	−0.40 (0.006)	12%	<0.0001
CIHDS	7,431 (222)	3.4 (0.01)	2.8 (0.08)	−0.58 (0.09)	17%	<0.0001
All	40,586 (1,155)	3.4 (0.005)	3.0 (0.03)	−0.46 (0.03)	14%	<0.0001

Values are mean and SEM or percentages. Noncarriers = RR homozygotes; 46L allele carriers = RL heterozygotes and LL homozygotes. *46L allele carriers versus noncarriers. Abbreviations as in Table 1.

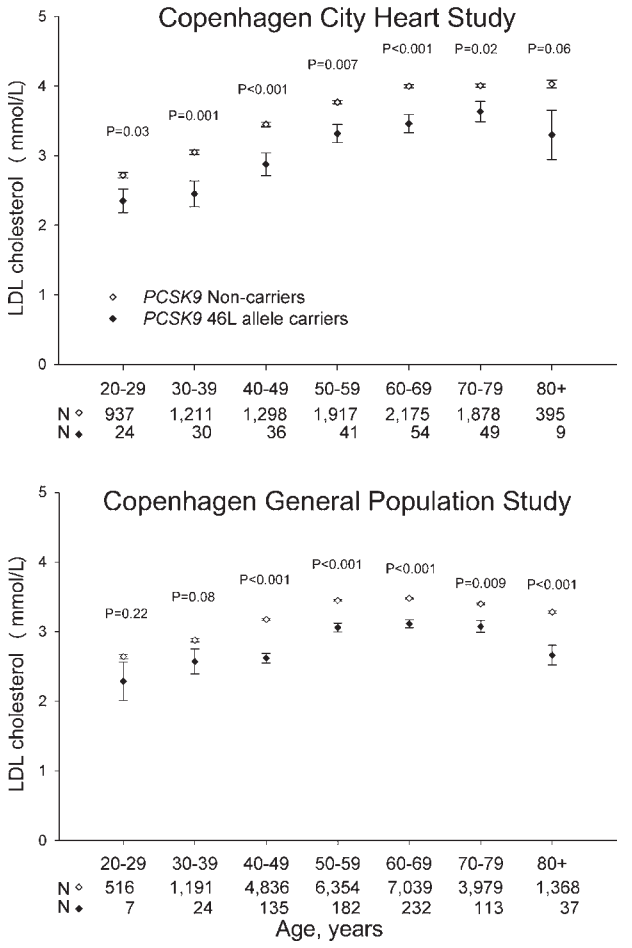

Figure 1 **LDL Cholesterol Levels by Age and *PCSK9* R46L Genotype**

Low-density lipoprotein (LDL) cholesterol levels as a function of age by *PCSK9* R46L carrier status in 2 general population samples, The Copenhagen City Heart Study, n = 10,032 **(top)**, and the Copenhagen General Population Study, n = 26,013 **(bottom)**. Noncarriers = RR homozygotes; 46L carriers = RL heterozygotes and LL homozygotes. Number of noncarriers **(open diamonds)** and 46L allele carriers **(closed diamonds)** are given below the x-axis of each study.

the cross-sectional CGPS study, comprising 26,032 individuals, *PCSK9* 46L allele carriers had a 46% reduction in risk of IHD (OR: 0.54; 95% CI: 0.39 to 0.77; p = 0.001) and a 58% reduction in risk of MI (OR: 0.42; 95% CI: 0.23 to 0.76; p = 0.004) compared with noncarriers (Table 3). In the case-control CIHDS study, comprising 4,654 cases versus 5,000 unmatched control subjects, R46L genotype was not associated with risk of IHD (OR: 0.82; 95% CI: 0.55 to 1.21; p = 0.32) or MI (OR: 1.11; 95% CI: 0.72 to 1.73; p = 0.63) (Table 3). Risk of IHD for 46L allele carriers was lower in the CGPS than in the CCHS study (p = 0.05), and risk of MI in the CGPS study was lower than in both the CCHS study (p = 0.004) and the CIHDS study (p = 0.005) (Table 3). After exclusion of statin users or after adjustment for PCSK9 A53V, I474V, and E670G (16), results were similar.

Combining the 3 studies into 1 large study comprising 8,830 cases and 36,869 control subjects, 46L allele carriers had a 30% reduction in risk of IHD compared with noncarriers (OR: 0.70; 95% CI: 0.58 to 0.86; p = 0.001) (Table 3). In a post-hoc subgroup analysis, the cardioprotective effect of the 46L allele was similar in individuals at or below the median age of 60 years (OR: 0.70; 95% CI: 0.50 to 0.95), and in individuals above the median age of 60 years (OR: 0.71; 95% CI: 0.56 to 0.91; p value for interaction between age and genotype on risk of IHD = 0.49).

In the prospective CCHS study, carrying the 46L allele was not associated with a reduction in all-cause mortality (OR: 1.18; 95% CI: 0.93 to 1.50; p = 0.17) or with mortality due to IHD (OR: 1.35; 95% CI: 0.72 to 2.54; p = 0.34) (Table 3).

Meta-analyses. In a meta-analysis including 7 general population studies with a total of 1,639 46L allele carriers and 59,198 noncarriers, standardized mean differences in LDL-C levels between 46L allele carriers and noncarriers were −0.43 mmol/l (95% CI: −0.48 to −0.38 mmol/l) and −0.44 mmol/l (95% CI: −0.51 to −0.37 mmol/l) in fixed and random effect models, respectively (Fig. 2), corresponding to a 12% lower LDL-C level in 46L allele carriers. I^2, a measure of heterogeneity between studies, was 38% (p = 0.14).

In a meta-analysis combining studies on risk of IHD (11,339 cases and 55,359 control subjects), 46L allele carriers versus noncarriers had a reduction in risk of IHD of 28% with a fixed effect OR of 0.72 (95% CI: 0.62 to 0.84) and random effect of 0.72 (95% CI: 0.57 to 0.93) and an I^2 of 40% (p = 0.06) (Fig. 3). In a meta-analysis including the 3 studies from Copenhagen, 46L allele carriers versus noncarriers had a reduction in risk of IHD of 23% with a fixed effect OR of 0.77 (95% CI: 0.65 to 0.92) and random effect of 0.77 (95% CI: 0.55 to 1.07). However, I^2 was 72% (p = 0.03), suggesting variability between studies (Fig. 3).

In both meta-analyses (Figs. 2 and 3), Begg's test demonstrated an equal distribution of studies above and below the log OR line (p = 0.88 and p = 0.65, respectively), and there was also no indication of asymmetry of studies distributed at higher variances (p = 0.86 and p = 0.30, respectively, by Egger's test), suggesting absence of publication bias in meta-analyses.

Theoretically predicted versus observed risk of IHD and mortality. The observed mean reductions in plasma LDL-C of 15% (0.55 mmol/l) in the CCHS study, 11% (0.35 mmol/l) in the CGPS study, and 16% (0.53 mmol/l) in the CIHDS study theoretically predicted risk reductions for IHD of 5% (OR: 0.95; 95% CI: 0.92 to 0.98), 4% (OR: 0.96; 95% CI: 0.94 to 0.98), and 6% (OR: 0.94; 95% CI: 0.91 to 0.97), respectively (Fig. 4, left and middle panels), and a nonsignificant reduction of 4% (OR: 0.96; 95% CI: 0.93 to 1.01) in all-cause mortality, assuming that the reduction in risk was due to a long-lasting lower LDL-C level alone. The corresponding observed risk reductions for IHD were 6% (OR: 0.94; 95% CI: 0.68 to 1.31) in the CCHS study, 46% (OR: 0.54; 95% CI: 0.39 to 0.77) in the

Table 3 Risk of IHD, MI, and Mortality in *PCSK9* 46L Allele Carriers Versus Noncarriers in the CCHS, CGPS, and CIHDS

	46L Allele Carriers		Age- and Sex-Adjusted		Multifactorially Adjusted	
	Control Subjects	Events/Cases	HR/OR (95% CI)	p Value	HR/OR (95% CI)	p Value
IHD						
All						
CCHS	8,269 (204)	1,763 (39)	0.94 (0.69–1.30)	0.71	0.94 (0.68–1.31)*	0.73
CGPS	23,600 (690)	2,413 (40)	0.56 (0.40–0.78)	0.001†	0.54 (0.39–0.77)	0.001†
CIHDS	5,000 (118)	4,654 (113)	0.85 (0.65–1.12)	0.62	0.82 (0.55–1.21)	0.32
All	36,869 (1,012)	8,830 (192)	0.74 (0.61–0.89)	0.001†	0.70 (0.58–0.86)	0.001
Statin users excluded						
CCHS	8,081 (202)	1,553 (36)	0.98 (0.71–1.36)	0.93	0.97 (0.69–1.37)	0.88
CGPS	22,133 (673)	1,388 (22)	0.51 (0.33–0.78)	0.002†	0.47 (0.29–0.74)	0.001†
CIHDS	4,801 (116)	2,630 (106)	0.88 (0.64–1.21)	0.44	0.79 (0.48–1.30)	0.35
All	35,015 (991)	5,571 (164)	0.75 (0.61–0.91)	0.002†	0.66 (0.52–0.84)	0.001†
MI						
All						
CCHS	8,269 (204)	858 (24)	1.22 (0.81–1.83)	0.34	1.13 (0.73–1.75)‡	0.57
CGPS	23,600 (690)	1,011 (12)	0.41 (0.23–0.74)	0.003†	0.42 (0.23–0.76)	0.004†
CIHDS	5,000 (118)	1,764 (42)	1.02 (0.71–1.46)	0.93	1.11 (0.72–1.73)§	0.63
All	36,869 (1,012)	3,633 (78)	0.79 (0.63–1.00)	0.05	0.82 (0.63–1.06)	0.12
Statin users excluded						
CCHS	8,081 (202)	730 (23)	1.35 (0.89–2.04)	0.16	1.23 (0.79–1.92)	0.36
CGPS	22,133 (673)	452 (4)	0.29 (0.11–0.77)	0.01	0.30 (0.11–0.82)	0.02
CIHDS	4,801 (116)	613 (13)	0.87 (0.49–1.53)	0.62	0.94 (0.51–1.73)	0.84
All	35,015 (991)	1,795 (40)	0.79 (0.57–1.09)	0.16	0.80 (0.58–1.11)	0.19
All-cause mortality						
All						
CCHS	7,087 (169)	3,031 (74)	1.11 (0.88–1.40)	0.38	1.18 (0.93–1.50)	0.17
Statin users excluded						
CCHS	6,775 (166)	2,945 (72)	1.08 (0.85–1.37)	0.53	1.14 (0.90–1.45)	0.27
IHD mortality						
CCHS	9,550 (224)	368 (19)	1.24 (0.67–2.34)	0.49	1.35 (0.72–2.54)	0.34

The p values are not corrected for multiple comparisons. Multifactorial adjustment was for sex, age, body mass index, smoking, hypertension, and diabetes mellitus. *CCHS versus CGPS, p = 0.05. †p < 0.008 corrected for multiple comparisons with the Bonferroni method (required significance level = 0.05/number of comparisons/end point [= 6]). ‡CCHS versus CGPS, p = 0.004. §CGPS versus CIHDS, p = 0.005.

CI = confidence interval; HR = hazard ratio; OR = odds ratio; other abbreviations as in Table 1.

CGPS study, and 18% (OR: 0.82; 95% CI: 0.55 to 1.21) in the CIHDS study (Fig. 4, right panel). The p values for differences between predicted and observed risks were 0.48 for the CCHS study, <0.001 for the CGPS study, and 0.25 for the CIHDS study. The observed risk for all-cause mortality was 1.18 (95% CI: 0.93 to 1.50; p = 0.06 for predicted vs. observed risk).

For all 3 studies combined, the corresponding predicted and observed risk reductions for IHD were 5% (OR: 0.95, 95% CI: 0.93 to 0.98) and 30% (OR: 0.70; 95% CI: 0.58 to 0.86) (p value for difference = 0.004). For studies included in the meta-analysis on risk of IHD, predicted and observed risk reductions were 5% (OR: 0.95; 95% CI: 0.93 to 0.98) and 28% (OR: 0.72; 95% CI: 0.62 to 0.84) (p value for difference <0.001) (Fig. 4).

Discussion

The main findings of the present studies were: 1) the *PCSK9* 46L allele was consistently associated with a 0.35- to 0.55-mmol/l lower LDL-C from 20 to 80+ years; 2) the 46L allele was associated with reduced risk of IHD in the largest study only, the CGPS study, and in the studies combined; 3) the 46L allele did not predict mortality; and 4) in the CGPS study and in the combined analyses, the association of the 46L allele with IHD risk seemed disproportionately greater than expected from the effect of the polymorphism on LDL-C alone.

In the 2 studies of the general population, the CCHS and the CGPS studies, we show that the 11% to 15% reduction in LDL-C levels in 46L allele carriers versus noncarriers is present from the age of 20 years and persists throughout life. These results are in accordance with the 9% to 15% reduction found in previous studies of adult populations (6,7,10,21,22). The Bogalusa Heart Study, comprising 1,564 school children (mean age 9.4 years) reported similar 12% lower LDL-C levels, suggesting that the reduction in LDL-C might be lifelong (8).

Despite a consistent reduction in LDL-C levels in 46L allele carriers versus noncarriers, risk estimates for IHD seem to vary somewhat (6,7,21). In the 3 present studies,

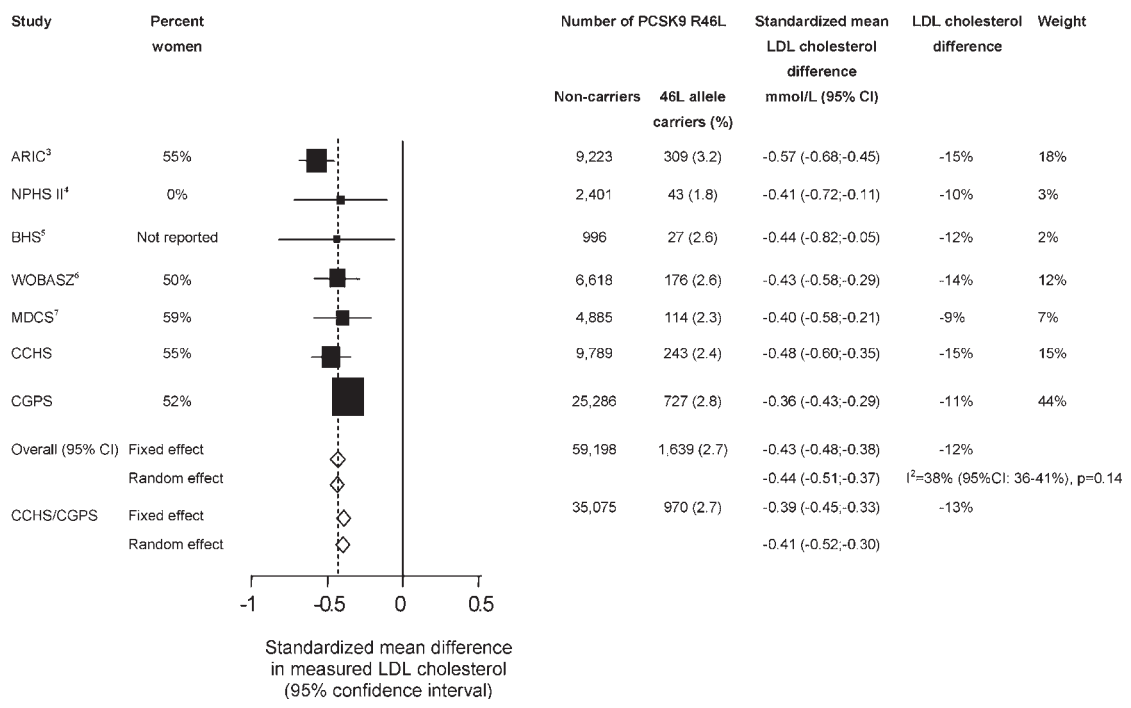

Figure 2 **Meta-Analysis of *PSCK9* R46L Genotype and LDL Cholesterol Levels**

Meta-analysis of general population studies of whites of *PSCK9* R46L genotype and low-density lipoprotein (LDL) cholesterol levels. On the forest plot, the **box area** is proportional to the weight (= 1/[standard error of effect size]^2) of each study, and the **horizontal lines** correspond to the 95% confidence intervals (CIs). The **diamonds** and **dashed vertical line** represent the summary estimate of standardized mean differences, and the CI for the summary estimate corresponds to the width of the **diamond**. The **solid vertical line** corresponds to difference in LDL cholesterol = 0 between 46L carriers and noncarriers. Test for heterogeneity: all studies, I^2 = 38%. Noncarriers = RR homozygotes; 46L allele carriers = RL heterozygotes and LL homozygotes. ARIC = Atherosclerosis Risk in Communities Study (lipid levels by R46L genotype were not reported for blacks in this study); BHS = Bogalusa Heart Study; CCHS = Copenhagen City Heart Study; CGPS = Copenhagen General Population Study; MDCS = Malmö Diet and Cancer Study; NPHSII = Northwick Park Heart Study II; WOBASZ = National Health Survey in Poland.

although the reduction in LDL-C in 46L allele carriers versus noncarriers is consistent between studies, risk estimates for IHD cover a spectrum from nonsignificant reductions of 6% and 18% in the CCHS and CIHDS studies, respectively, to a significant 46% reduction in risk in the CGPS study. Part of this variability could be due to differences in study designs and thus in ascertainment of individuals for studies (CCHS, prospective; CGPS, cross-sectional; CIHDS, case-control). Other factors that could influence risk associated with the 46L allele are changes in risk factors for IHD and changes in treatment and in risk of IHD in the population over time, from the start of enrollment in the CCHS study and the CIHDS study in 1991 to enrollment in the CGPS study in 2003 and onwards. During the study period there has been a reduction in dietary fat intake in the Danish general population (44 energy% in 1985 vs. 34 energy% in 2002), an increase in statin treatment, and a reduction in risk of IHD from 326/100,000 in 1985, to 173/100,000 in 2002 (Danish National Board of Health). These trends are also reflected in a general reduction in lipid risk factors for IHD in the CGPS study versus the CCHS study, an almost 150% increase in statin treatment, and a 50% reduction in risk of both IHD and MI, although other risk factors increased (body mass index, current smokers, and hypertension). This general reduction in lipid risk factors, especially LDL-C, and in risk of IHD in the general population over time changes the context in which the *PCSK9* 46L allele might exert its effect on risk, from the start of enrollment of the CCHS participants and the CIHDS cases (1991) to the start of enrollment of CGPS participants (2003) or perhaps more generally from high-risk to lower-risk populations, despite a consistent effect of the 46L allele on plasma levels of LDL-C.

The 5% theoretically predicted risk reduction for IHD in 46L allele carriers versus noncarriers, estimated from the risk reduction associated with a 1-mmol/l reduction in LDL-C, was less than the 30% reduction observed in our 3 studies combined and less than the 28% risk reduction observed in meta-analysis, suggesting either that: 1) the *PCSK9* 46L allele might reduce risk by an additional mechanism unrelated to the LDL-C lowering effect (3), although no lipid independent effects of PCSK9 have so far been demonstrated in functional studies in vitro or in vivo; or 2) *PCSK9* genotype is a better predictor of the effect on risk of IHD of lifelong exposure to LDL-C than LDL-C measured in adult life. Genotypes are present from conception, and as Cohen et al. (6) and Brown and Goldstein (13)

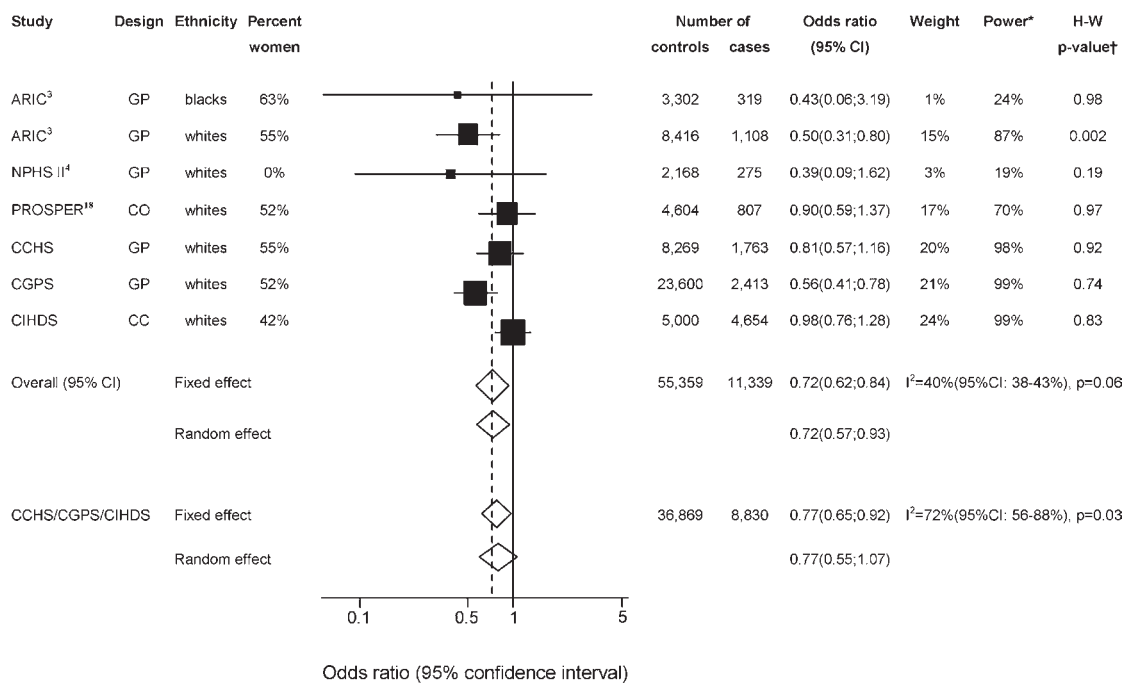

Figure 3 **Meta-Analysis of *PSCK9* R46L Genotype and Risk of Ischemic Heart Disease**

Meta-analysis of studies of the association between *PSCK9* R46L genotype and risk of ischemic heart disease. On the forest plot, the **box area** is proportional to the weight (= /[standard error of effect size]^2) of each study, and the **horizontal lines** correspond to the 95% CIs. The **diamonds** and **dashed vertical line** represent the summary estimate, and the CI for the summary estimate corresponds to the width of the **diamond**. The **solid vertical line** corresponds to an odds ratio (OR) = 1 and is equivalent to no effect of genotype. Test for heterogeneity: all studies, I^2 = 40%; CCHS, CGPS, and CGPS studies, I^2 = 72%. *The power to detect an OR of 0.5 or lower for the individual study (p = 0.05); an OR of 0.5 corresponds to the OR observed in the first study on the *PCSK9* R46L genotype (6); it follows that the probability of a false negative result for a given study is 100%-power. †A p value <0.05 indicates that the genotype is not in Hardy-Weinberg (H-W) equilibrium. CC = case-control, CIHDS = Copenhagen Ischemic Heart Disease Study; CO = case-only; GP = general population; PROSPER = Prospective Study of Pravastatin in the Elderly at Risk; other abbreviations as in Figure 2.

have pointed out, genotypes associated with LDL-C might therefore most accurately reflect lifelong exposure to LDL-C. If the lower risk seen in R46L carriers is due to the lifelong reduction in LDL-C, early long-term lipid-lowering therapy might be more beneficial than therapy instituted later in life (13).

In the prospective CCHS study, neither the HRs for IHD or MI nor the HRs for mortality predicted by the 46L allele were different from those of noncarriers (HRs overlapped 1.00). In this study, a 1-mmol/l decrease in LDL-C predicted a 6% decrease in all-cause mortality (OR: 0.94; 95% CI: 0.90 to 0.98), and the 0.55-mmol/l decrease in LDL-C observed in the CCHS study predicted a nonsignificant 4% decrease (96%) (OR: 0.96; 95% CI: 0.93 to 1.01) in total mortality, which was not different from what we observed. However, if we believe the HR for IHD from the meta-analysis of our 3 studies and from most of the previous studies on IHD risk, mortality—like risk of IHD—is also likely to be reduced more than predicted by the effect on LDL-C, especially in populations at lower risk, such as the CGPS study.

Study limitations. The present studies have limitations (i.e., selection bias and misclassification of the diagnosis of IHD). However, the general populations used were selected with the Danish Central Person Registry, drawing 2 random samples from the Danish adult general population without knowledge of genotypes, plasma LDL-C levels, or IHD status. Response rates in the CCHS study were comparable to those of other large epidemiologic studies (23,24). In the CCHS study, blood samples for deoxyribonucleic acid analysis were not collected until the 1991 to 1994 examination and the subsequent 2001 to 2003 examination. Thus, the present study is based on participants who survived until the 1991 to 1994 examination (or who were invited for the first time at the time of this examination or for the 2001 to 2003 examinations). Survivor bias might potentially limit the generalizability of the results but not the internal validity. A number of observations argue against survivor bias: 1) the original cohort was supplemented with younger individuals at all 3 follow-up examinations; 2) the genotype distribution for *PCSK9* R46L did not differ from that predicted by the Hardy-Weinberg equilibrium; 3) HR for all-cause mortality in 46L-allele carriers was similar to noncarriers; and 4) the 46L allele frequency was similar to that found in other studies of Caucasians. We

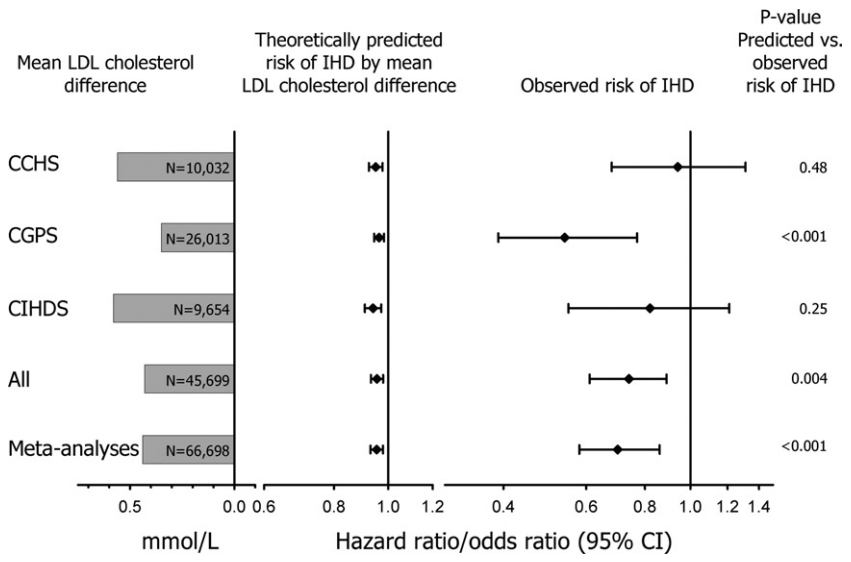

Figure 4 **LDL Cholesterol, Predicted and Observed Risks for IHD**

Differences in mean LDL cholesterol levels in *PCSK9* 46L allele carriers (RL heterozygotes plus LL homozygotes) versus noncarriers (RR homozygotes) **(left panel)**, the corresponding theoretically predicted risks **(middle panel)**, and the observed risks for ischemic heart disease (IHD) **(right panel)**. Shown for the following studies **(top to bottom)**: CCHS, n = 10,032; CGPS, n = 26,032; CIHDS, n = 9,654; the 3 studies combined; and in meta-analysis (n = 66,698). The theoretically predicted hazard ratios/ORs (age- and sex-adjusted) for IHD corresponding to the observed mean reduction in LDL cholesterol levels in 46L allele carriers versus noncarriers is based on the risk reduction for IHD observed for a similar LDL reduction in the CCHS study as a whole. Abbreviations as in Figures 2 and 3.

have complete follow-up on morbidity and mortality for all participants; however, the definition of IHD and background therapy might have varied over time or might have been incorrectly coded in the Danish National Patient Registry or some individuals might not have had IHD yet. Such potential limitations can be assumed to be evenly distributed between carriers and noncarriers and therefore would result in more conservative risk estimates for the association of genotype with risk of IHD. Finally, our 3 studies differ in number of participants, and therefore all estimates from the combined studies or from meta-analyses are mainly driven by the comparatively large CGPS study.

Conclusions

The *PCSK9* 46L allele was associated with reductions in LDL-C of 11% to 16% in all age groups from 20 to 80+ years in the general population. The 30% reduction in risk of IHD observed in our 3 studies combined was larger than predicted by the reduction in LDL-C alone. This could be because genotype is a better predictor of lifelong exposure to LDL-C than LDL-C measured in adult life.

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Key Words: cholesterol ■ epidemiology ■ genetics of cardiovascular disease ■ LDL cholesterol ■ lipids ■ *PCSK9*.

APPENDIX

For supplementary tables, please see the online version of this article.