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**The *PCSK9* gene R46L variant is associated with lower plasma lipid levels and cardiovascular risk in UK healthy men**

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## ABSTRACT

We have determined the relative frequency of the R46L, I474V and E670G variants in the protein convertase subtilisin/kexin type 9 (*PCSK9*) gene and its association with plasma lipid levels and coronary heart disease (CHD) in UK healthy men and patients with clinically-defined definite familial hypercholesterolaemia (FH). Genotypes were determined using PCR and Restriction Enzyme digestion in 2444 healthy middle-aged (50-61 years) men from the prospective NPHSII Study, with 275 CHD events (15 years follow-up) and in 597 UK FH patients from the Simon Broome Register. In the NPHSII healthy men the R46L genotype distribution was in Hardy-Weinberg equilibrium and the frequency of 46L was 0.009 (95% CI 0.007–0.012), with one man homozygous for the 46L allele. There was significant association of the 46L allele with lower mean (+SD) total cholesterol (RR  $5.74 \pm 1.01$  vs RL  $5.22 \pm 1.05$ ;  $p=0.001$ ), apo B (RR  $0.87 \pm 0.24$  vs RL  $0.73 \pm 0.27$ ;  $p<0.0001$ ) and LDL-C (RR  $4.01 \pm 0.95$  vs RL  $3.55 \pm 1.01$ ;  $p=0.02$ ) levels, after adjustment for age, GP practice, smoking, BMI and SBP. As expected, 46L carriers had a low risk of definite or possible CHD HR=0.46 (0.11-1.84)) but this was not statistically significant ( $p=0.27$ ). Two other common *PCSK9* variants I474V (frequency V = 0.179, CI 0.17-0.19) and E670G (frequency G = 0.034 CI 0.03-0.04) were not associated with any significant effects on lipid levels or CHD risk. In FH patients the frequency of 46L was 0.003 (95% CI 0.00–0.01) which was significantly lower ( $p=0.037$ ) than the healthy subjects. In the four FH patients carrying 46L mean untreated total cholesterol levels were not different in carriers and non-carriers (median.10.3 mmol/l vs 10.2mmol/l  $p=0.91$  after adjustment for age, gender and mutation type).

**Conclusions.** The *PCSK9* 46L allele is more frequent in UK healthy men than in FH patients and is strongly associated with a protective plasma lipid profile risk to CHD. Its low frequency (~2% carriers) means that it is not making a major contribution to determining population CHD risk in the UK.

**Running Title** The *PCSK9* R46L variant and low lipid levels

## INTRODUCTION

The protein convertase subtilisin/kexin type 9 (*PCSK9*) gene, codes for an enzyme which has been shown to be involved in degrading the LDL-receptor protein in the lysosome of the cell and prevents it recycling to the cell surface [1,2]. Gain of function mutations in *PCSK9* that increase activity therefore cause increased degradation of LDL-receptors, reduced numbers of receptors on the surface of the cell [1,3] and hence monogenic familial hypercholesterolaemia (FH) [4]. However, loss-of function mutations that inactivate *PCSK9*, result in less degradation of the LDL-receptor and thus higher levels of on the cell surface [5]. This results in faster clearance of LDL from the plasma and has been reported to be associated with lower LDL-C levels and lower risk of Coronary Heart Disease CHD [6-8].

*PCSK9* is a highly polymorphic gene [9,10] and one of the DNA changes 137G>T in exon 1 results in the replacement of Arginine 46 by Leucine (R46L). The reported prevalence for 46L heterozygosity was 0.7% in black subjects (N=3363), and 3.2% in European-Americans (N=9524), 8 of whom were homozygous for the 46L allele [7]. Carriage of the 46L allele was associated with significantly lower plasma levels of total cholesterol and LDL-C, 9% and 15% respectively, and a 47% reduction in the rate of coronary events in those carrying the 46L allele. These results have been confirmed by others [8]. A recent study from Norway confirmed the association of R46L *PCSK9* with hypocholesterolemia, and suggested that in FH subjects, 46L carriers also showed a better lipid-lowering response to statin therapy [11].

The aims of this work are to determine the relative frequency of the *PCSK9* 46L allele and its association with plasma lipid levels and coronary heart disease (CHD) in healthy UK men [12,13] and patients with clinically-defined definite familial hypercholesterolaemia (FH), of whom 35% had evidence of premature CHD [14]. We also determined the frequency and examined the impact on lipid levels of two other reported common *PCSK9* variants, I474V and E670G. Previous studies from Japan [9] and the US [10] have also suggested that carriers of the rare alleles of these variants and haplotypes containing them have lower and higher plasma lipid levels respectively.

## MATERIALS AND METHODS

### Subjects

The Second Northwick Park Heart Study (NPHS-II) recruited 3012 healthy European Caucasian men aged 50 to 61 years from nine general medical practices in the UK and has followed them for the development of CHD from 1989 [12,13]. In brief, at baseline all of the participants were free of a history of unstable angina, myocardial infarction, coronary surgery, anticoagulant therapy (including aspirin), malignancy and cardiovascular disease or any other condition precluding informed consent. Ethical approval was granted by the local institutional review committee and patients gave written, informed consent. Participants attended for examination in a non-fasting state, having avoided smoking, vigorous exercise, or heavy meals from midnight beforehand. A resting 12-lead electrocardiogram (ECG) was recorded at baseline and during the sixth year of surveillance with results coded by Minnesota criteria [15]. Weight was measured on a balance scale and height on a stadiometer to calculate the body mass index (BMI, kg/m<sup>2</sup>). Smoking habits were assessed by questionnaires [16] completed at baseline and annually thereafter for five years. Blood pressure was recorded with a random zero mercury sphygmomanometer after the subject had been seated for five minutes. Details of possible events were obtained through primary care medical practices, hospitals and coroners' offices. The clinical history, ECGs, cardiac enzymes and pathology were assessed by independent review to diagnose myocardial infarction according to World Health Organization criteria [17]. Normal limits for cardiac enzymes were those for the reporting laboratory. Other terminating events were a new major Q wave on the ECG after 5 years of follow-up (Minnesota codes 1<sub>1</sub>, 1<sub>2.1</sub> to 1<sub>2.7</sub>, 1<sub>2.8</sub> plus 5<sub>1</sub> or 5<sub>2</sub>) [15] and surgery for angina pectoris with CHD angiographically demonstrated. In the 2444 men with genotyping there had been 275 terminating events comprising 161 acute CHD events, 67 coronary artery revascularisation procedures and 22 silent myocardial infarctions and 25 possible MIs.

Patients with clinically-defined definite familial hypercholesterolaemia [18] were recruited for the Simon Broome British Heart Foundation study, which is a cross-sectional comparison of white Caucasian patients aged 18 years or more with treated heterozygous FH with and without clinically documented CHD [14,18]. Recruitment methods, inclusion and exclusion and diagnostic criteria were as defined in [14,18]. Pre-treatment cholesterol levels were obtained from patient records.

### Molecular Genetic analysis

Genomic DNA was isolated from whole blood samples using standard methods [19,20]. PCR primers were designed to introduce a recognition site for the restriction enzyme *RsaI*. Genotyping for the *PCSK9*, exon 1 c137G>T (R46L) mutation was carried out using primers and conditions as follows: Forward, 5'- CAC GGC CTC TAG GTC TTC TC -3'; Reverse, 5'- AGG CCG TCC TCC TCG GTA -3' where the forced base is underlined. The 20µl PCR reaction contained 16.6mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>; 67mM Tris-HCl, pH8.3; 6.7 mM MgCl<sub>2</sub>; 6.7 µM EDTA; 10 mM BME; 0.17mg/ml BSA; DMSO 10% and 0.2mM dATP, dGTP, dTTP, dCTP; 8pmoles of each primer and 0.2U of Taq polymerase (Invitrogen, UK); in addition to 15ng genomic DNA. The PCR protocol consisted of 94°C 3 mins; 94°C 30 secs, 55°C 30 secs, and 72°C 30 secs for 34 cycles; 72°C 5 min and 16°C 3 min. Restriction digest was carried out with *RsaI* (New England Biolabs) for 4 hours at 37°C using 3u of enzyme per digest. GG (wild type) homozygotes produce band sizes of 188bp and 17bp while heterozygotes (GT) produce band sizes of 205bp, 188bp and 17bp. The TT (homozygote) produced one band size of 205bp. Products were separated on 2.0 % agarose gel and visualised by staining with Ethidium Bromide. For R46L, all gels included a positive heterozygous control sample (confirmed by direct sequencing), and genotypes were read by two independent observers blind to case/control status and any discrepancies confirmed by PCR and digestion.

Genotyping for *PCSK9*, exon 9 c.1420A>G (I474V) and exon 12 c.2009A>G (E670G) variants (rs562556, rs505151 respectively) was carried out using PCR and RFLP conditions as follows: Primers used for amplifying exon 9: Forward, 5'- TCC CTT CTC CCT TGT CTG TG -3'; Reverse, 5'- CTG TGG CTC TCT CCA GCA G -3'. Primers for exon 12: Forward, 5'- GAT GTC GGA GGG AGA AAT GA -3'; Reverse, 5'- GGC ACC CAG AGT GAG TGA GT -3'. The 20µl PCR reaction contained 16mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>; 67mM Tris, pH8.3; 1.5mM MgCl<sub>2</sub>; 0.01% Tween 20; 0.2mM dATP, dGTP, dTTP, dCTP; 0.032pmoles of each primer (Invitrogen, UK); 0.3U of Taq DNA Polymerase (Bioline, UK); and 15ng genomic DNA. The PCR cycling conditions consisted of 95°C 5 mins; 95°C 30 secs, 55°C 30 secs, and 72°C 30 secs for 35 cycles; and 72°C 5 min. Restriction digest for the I474V variant was carried out using 4U/sample *BccI* (NEB) and digest for the E670G variant was carried out using 2U/sample of *Sau96I* (NEB), both overnight at 37°C. The A allele of the *BccI* digest produces band sizes of 267bp, 193bp, 96bp and 25bp; the G allele produces band sizes of 267bp, 218bp and 96bp. The A allele of the *Sau96I* digest produces band

sizes of 287bp, 69bp, 51bp, 33bp, 30bp, 18bp and 6bp; the G allele produces band sizes of 215bp, 72bp, 69bp, 51bp, 33bp, 30bp, 18bp and 6bp. Products were separated on 7.5% polyacrylamide gel stained with Ethidium Bromide using the microarray diagonal gel electrophoresis (MADGE) system [21].

### **Statistical analysis**

All statistical analyses were carried out using STATA (Intercooled Stata 9.2, Stata Corporation, Texas). Differences in proportions were determined by a  $\chi^2$  test or Fisher's exact test. Lipid levels were compared by genotype after adjustment using analysis of covariance. R-squared values were used to estimate the percentage of the sample variance explained by each genotype. CHD risk was assessed by Cox proportional hazards models (which allowed for varying lengths of follow-up, producing hazard ratios (HR) with 95% confidence intervals (CI). For each variant the common homozygotes served as the reference category. Adjustments for possible confounding factors (age, BMI, smoking, cholesterol, triglyceride and systolic blood pressure (SBP)) were made by including them as covariates in the model, and differences in the baseline hazard by general practice (recruitment site) were permitted (using the strata option in STATA). Haplotype analysis was conducted using THESIAS. Pre-treatment cholesterol levels were not available for all FH patients. Throughout, a p-value of <0.05 was taken as statistically significant.

## RESULTS

### Healthy NPHSII men

The R46L genotype distribution was in Hardy-Weinberg equilibrium and there was one healthy man homozygous for the 46L allele, the rare allele frequency was 0.009 (95% CI 0.007–0.012). Table 2a shows the mean ( $\pm$  SD) baseline characteristics of lipids levels of NPHSII men by *PCSK9* R46L genotype. There was significant association of the 46L allele with lower mean ( $\pm$ SD) total cholesterol (RR  $5.74 \pm 1.01$  vs RL  $5.22 \pm 1.05$ ;  $p=0.001$ ), apo B (RR  $0.87 \pm 0.24$  vs RL  $0.73 \pm 0.27$ ;  $p<0.0001$ ) and LDL-C (RR  $4.01 \pm 0.95$  vs RL  $3.55 \pm 1.01$ ;  $p=0.02$ ) levels in NPHSII men, after adjustment for age, practice, smoking, BMI and SBP. The 46L carriers had a low risk of definite or possible CHD with a Hazard Ratio (HR) of 0.46 (0.11-1.84) but this was not statistically significant ( $p=0.27$ ).

The majority of the men had lipid levels available at baseline and for five annual visits and these are presented in Figure 1. As can be seen, the mean levels of the 46L carriers was approximately 0.5mmol/l lower at baseline and the one LL man 2.3mmol/l lower. These levels were maintained over the next five years. The 46L carriers were at the 33<sup>rd</sup> percentile of the total cholesterol distribution of the whole sample, while the LL man was at the 0.4<sup>th</sup> percentile. Over the 5 year period there was a progressive drop-out of subjects with recorded lipid levels (10 46L carriers and 659 non-carriers). During this time, there was a small but non-significant 3.8% increase in the mean levels in 46L carriers and a statistically significant 3% decrease in levels in non-carriers, the result of which was that the difference between the two groups lost statistical significance by year 4.

To put the impact of this variant in context, the proportion of sample variance for each of the lipid traits was determined and compared to the impact of the well known common *APOE* gene E2, E3 and E4 alleles. While *APOE* explained 5.1% of the variance in LDL-C, 3.5% for ApoB and 2.9% for Total cholesterol in this sample, the *PCSK9* R46L genotype explained 0.6%, 0.9% and 0.6% respectively. There was no evidence for interaction with *APOE* genotype in NPHSII men, with effects on cholesterol levels of variation in the two genes not being different from additive ( $p$  interaction = 0.39).



The genotype was also determined for two other common variants in *PCSK9*, the I474V and E670G. For both genotype distribution was in Hardy-Weinberg equilibrium and as shown in Table 1 in healthy men the 474V frequency was 0.18 (95% CI 0.17–0.19) and for 670G the frequency was 0.034 (95% CI 0.028–0.040). Table 2b and c shows that there was no significant association with any measured plasma lipid trait. There was also no significant association with CHD risk (for I474V V allele carriers vs II men the HR= 1.16 (0.89-1.50) and for E670G G allele carriers vs EE men HR = 1.20 (0.73-1.98). These estimates were not substantially altered by adjustment for age, practice, smoking, BMI and SBP and remained non-significant (not shown). When data from these two variants was combined into haplotypes three common haplotypes were seen (V474-G670 frequency 0.79, V474-E670 frequency 2.6%, V474-E670 frequency 0.18) with significant evidence for linkage disequilibrium ( $D' = 0.93$ ). There were no significant associations between any haplotypes and plasma levels of Total- or LDL-cholesterol or with CHD risk (not shown).

### **FH patients**

Genotype was only determined for the *PCSK9* R46L variant, and was obtained in 597 FH patients. The genotype distribution was in Hardy-Weinberg equilibrium, and the 46L allele frequency was 0.03 (95% CI 0.00–0.01), which was significantly lower than in the NPHSII healthy subjects ( $p=0.037$ ). In the four FH patients carrying 46L, whose characteristics are shown in Table 3, there was no difference in mean untreated total cholesterol levels than in the FH patient group overall, (median 10.3 mmol/l vs 10.2mmol/l ( $p=0.91$ ) geometric mean 10.4 ( $\pm 1.4$ )mmol/l vs 10.3 ( $\pm 1.8$ )mmol/l ( $p=0.94$ ) after adjustment for age, gender and mutation type). None of the 46L carriers had evidence of premature CHD, compared to the reported [14] prevalence in the sample as a whole (38.5%) or in those with a detected mutation causing FH [19] (38.6%) although these differences were not statistically significant (all  $p>0.2$ ). Because of low numbers, no analysis of response to lipid-lowering therapy by R46L genotype was attempted.

### **DISCUSSION**

This study strongly confirms earlier reports [7] that in healthy middle-aged men, carriers of the 46L allele in *PCSK9* have lower plasma levels of total- and LDL-C and apo B, and demonstrate that this effect is maintained over at least 5 years of follow-up. The one individual who was homozygous for this variant had LDL-C levels that put him at the 0.5<sup>th</sup> percentile of the sample of healthy men from the UK general population. In this group of

healthy men the 46L variant was not associated with a significant effect on any other measured lipid trait. As expected, carriers also had a 54% lower risk of early CHD, which although not statistically significant in this sample is of similar magnitude to the 47% lower risk reported previously [7]. Based on data from the effect on CHD risk reduction seen with lipid lowering therapy [22], the observed reduction in LDL-C would be predicted to result in only a 10% reduction in risk. This supports the view [7] that the life-long lower LDL-C experienced by 46L carriers has a greater than expected CHD benefit than lowering in middle-age. In support of this, none of the FH patients who were carrying the 46L variant had evidence of CHD. It is well known that the age of onset of CHD varies widely in FH patients [23,24] and this suggests that variants in the *PCSK9* gene may be explaining in part this variability.

The possible mechanism of the LDL-C lowering effect of 46L has been discussed elsewhere [1,5]. The enzyme encoded by *PCSK9* is involved in degrading the LDL-receptor protein in the lysosome of the cell and preventing it recycling. Loss of function mutations in the *PCSK9* gene result in less degradation of LDL-receptors, increased numbers of receptors on the surface of the cell and hypocholesterolaemia [6]. There are two possible sites for this effect. The first is in a post-ER compartment such as the Golgi apparatus, where PCSK9 binds to the LDL-R and targets it for degradation in the acidic lysosome. A second possibility is that secreted PCSK9 binds to surface located LDL-R and, after internalisation, prevents LDL-R recycling from the endosome to the surface, and reroutes it to the lysosome. Currently it is still unclear if the LDL-R is itself cleaved directly by PCSK9. A definite proof of the effect of 46L *in vitro* has not yet been published, but a direct effect of the substitution on an aspect of PCSK9 function seems highly likely. The R46L substitution has no measurable effect on the synthesis, processing or secretion of PCSK9 in transfected HEK-293 cells [5] but modeling indicates that R46 is in a N-terminal extension of the prodomain, which may make intra-molecular contacts with the catalytical C-domain after autocatalytical cleavage. It has been suggested [5] that the substitution of a hydrophobic leucine for a polar arginine may alter this association and influence function in this way. The amino-acid at position 46 is hydrophobic in most species [8].

However, although the effect seen in the NPHSII healthy men is statistically robust, and is maintained over time, because of the relatively low frequency of 46L carriers the overall

impact on LDL-C levels in the population is low, explaining roughly 12% of the effect of the well known *APOE* gene common variants. Although the 46L-lowering effect on LDL-C of roughly 0.5mmol/l is similar to that seen in *APOE* e2 carriers compared to *APOE* e3e3 subjects [25], the population level impact of *APOE* is greater because the variants are much more common (e2 carriers ~12 % of the UK population).

The two other *PCSK9* variants examined here, I474V and E670G, were not associated with any significant impact on lipid levels in the NPHSII healthy men. The 474V variant was first reported to be present at low frequency (0.05) and associated with roughly 7% lower LDL-C levels in carriers in a Japanese study [9], but although the frequency was higher (0.18-0.20) in whites and blacks from the US [8] no association with lipid levels were detected. The frequency in UK men was similar to that reported in the US, and also no effect on lipid levels as detected. For the E670G variant, in the original study in moderately hyperlipidaemic subjects from the US, homozygosity for the 670G allele was associated with roughly 20% higher LDL-C levels than E/E subjects [10], an effect which again was not observed in either whites or blacks in the study by Kotowsky [8]. In the UK men examined here, LDL-C levels in 670G homozygotes tended to be lower, although not statistically significant. The study here has the power (80%) to be able to detect a 0.14 mmol/l difference in LDL-C levels associated with carriage of the 474V and 0.6 mmol/l difference for homozygosity of the 670G variant. The original report suggested a larger effect with a *PCSK9* haplotype [10], but no such effect was seen here (not shown). We were thus able to confirm the reports [8] of the negligible (or at the very best very modest) impact of these variants in healthy subjects and therefore did not examine their effect in FH patients.

For the FH patients examined, although the low frequency of 46L limits our ability to make strong interpretations, the lower frequency of the 46L variant and the possible protection from early CHD suggests that *PCSK9* is one of the genes that is involved in modulating the expression of FH. Thus individuals with FH (commonly due to a mutation in *LDLR* or *APOB*) who also are carrying 46L may be less likely to be diagnosed as FH, resulting in the lower than expected allele frequency. Since all the patients examined had a diagnosis of definite FH (ie a personal or family history of Tendon Xanthoma, [18], it may be that the likely lower LDL-C levels in patients carrying 46L reduces the likelihood of xanthoma development. One of the patients (SB 2825, untreated total cholesterol level 8.4mmol/l) has

an affected father with the same *LDLR* mutation (age 54 years) who did not carry the 46L variant, and his recorded untreated total cholesterol level was 13.4mmol/l (data not shown), compatible with a lowering effect of the *PCSK9* variant. Confirmation of these observation in larger patient cohorts, and particularly studies in the relatives of these patients, to determine their plasma lipid levels, will be informative.

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## References

1. Horton, J. D, Cohen, J. C, and Hobbs, H. H. (2006) Molecular biology of PCSK9: its role in LDL metabolism. *Trends Biochem Sci* 32(2).
2. Maxwell,K.N., Fisher,E.A. and Breslow,J.L. (2005) Overexpression of PCSK9 accelerates the degradation of the LDLR in a post-endoplasmic reticulum compartment. *Proc.Natl.Acad.Sci.U.S.A* **102**, 2069-2074.
3. Cameron,J., Holla,O.L., Ranheim,T., Kulseth,M.A., Berge,K.E. and Leren,T.P. (2006) Effect of mutations in the PCSK9 gene on the cell surface LDL receptors. *Hum.Mol.Genet.* **15**, 1551-1558.
4. Abifadel,M., Varret,M., Rabes,J.P. et al. (2003) Mutations in PCSK9 cause autosomal dominant hypercholesterolemia. *Nat.Genet.* **34**, 154-156.
5. Zhao,Z., Tuakli-Wosornu,Y., Lagace,T.A. et al. (2006) Molecular characterization of loss-of-function mutations in PCSK9 and identification of a compound heterozygote. *Am.J.Hum.Genet.* **79**, 514-523.
6. Cohen,J., Pertsemlidis,A., Kotowski,I.K., Graham,R., Garcia,C.K. and Hobbs,H.H. (2005) Low LDL cholesterol in individuals of African descent resulting from frequent nonsense mutations in PCSK9. *Nat.Genet.* **37**, 161-165.
7. Cohen,J.C., Boerwinkle,E., Mosley,T.H., Jr. and Hobbs,H.H. (2006) Sequence variations in PCSK9, low LDL, and protection against coronary heart disease. *N.Engl.J.Med.* **354**, 1264-1272.
8. Kotowski,I.K., Pertsemlidis,A., Luke,A. et al. (2006) A spectrum of PCSK9 alleles contributes to plasma levels of low-density lipoprotein cholesterol. *Am.J.Hum.Genet.* **78**, 410-422.
9. Shioji,K., Mannami,T., Kokubo,Y. et al. (2004) Genetic variants in PCSK9 affect the cholesterol level in Japanese. *J.Hum.Genet.* **49**, 109-114.
10. Chen,S.N., Ballantyne,C.M., Gotto,A.M., Jr., Tan,Y., Willerson,J.T. and Marian,A.J. (2005) A common PCSK9 haplotype, encompassing the E670G coding single nucleotide polymorphism, is a novel genetic marker for plasma low-density lipoprotein cholesterol levels and severity of coronary atherosclerosis. *J.Am.Coll.Cardiol.* **45**, 1611-1619.
11. Berge,K.E., Ose,L. and Leren,T.P. (2006) Missense mutations in the PCSK9 gene are associated with hypocholesterolemia and possibly increased response to statin therapy. *Arterioscler.Thromb.Vasc.Biol.* **26**, 1094-1100.
12. Miller,G.J., Bauer,K.A., Barzegar,S. et al. (1995) The effects of quality and timing of venepuncture on markers of blood coagulation in healthy middle-aged men. *Thromb Haemost* **73**, 82-86.

13. Miller,G.J., Bauer,K.A., Barzegar,S., Cooper,J.A. and Rosenberg,R.D. (1996) Increased activation of the haemostatic system in men at high risk of fatal coronary heart disease. *Thromb.Haemost.* **75**, 767-771.
14. Neil,H.A., Seagroatt,V., Betteridge,D.J. et al. (2004) Established and emerging coronary risk factors in patients with heterozygous familial hypercholesterolaemia. *Heart* **90**, 1431-1437.
15. Prineas, R. J., Crow, R. S., and Blackburn, H. (1982) *The Minnesota Code Manual of Electrocardiographic Findings: standards and procedures for measurement and classification.* Boston,USA, John Wright PSC.
16. Medical Research Council's Committee on Chronic Bronchitis and Questionnaire on respiratory symptoms (1995). 20 Park Crescent, London W1N 4AL, UK, Publications Group, Medical Research Council.
17. WHO - World Health Organisation Regional Office for Europe. (1976) *Myocardial Infarction Community Registers.* Public Health in Europe No. 5.
18. The Simon Broome Register Group. Scientific Steering Committee on behalf of The Simon Broome Register Group: (1991) The risk of fatal coronary heart disease in familial hypercholesterolaemia. *British Medical Journal* 303, 893-896.
19. Humphries,S.E., Whittall,R.A., Hubbart,C.S. et al. (2006) Genetic causes of familial hypercholesterolaemia in patients in the UK: relation to plasma lipid levels and coronary heart disease risk. *J.Med.Genet.* **43**, 943-949.
20. Heath,K.E., Humphries,S.E., Middleton-Price,H. and Boxer,M. (2001) A molecular genetic service for diagnosing individuals with familial hypercholesterolaemia (FH) in the United Kingdom. *Eur.J.Hum.Genet.* **9**, 244-252.
21. Day,I.N. and Humphries,S.E. (1994) Electrophoresis for genotyping: microtiter array diagonal gel electrophoresis on horizontal polyacrylamide gels, hydrolink, or agarose. *Anal.Biochem.* **222**, 389-395.
22. Baigent,C., Keech,A., Kearney,P.M. et al. (2005) Efficacy and safety of cholesterol-lowering treatment: prospective meta-analysis of data from 90,056 participants in 14 randomised trials of statins. *Lancet* **366**, 1267-1278.
23. Slack,J. (1969) Risks of ischaemic heart-disease in familial hyperlipoproteinaemic states. *Lancet* **2**, 1380-1382.
24. Kotze,M.J., De Villiers,W.J., Steyn,K. et al. (1993) Phenotypic variation among familial hypercholesterolemics heterozygous for either one of two Afrikaner founder LDL receptor mutations. *Arterioscler.Thromb.* **13**, 1460-1468.

25. Davignon,J., Cohn,J.S., Mabile,L. and Bernier,L. (1999) Apolipoprotein E and atherosclerosis: insight from animal and human studies. *Clin.Chim.Acta* **286**, 115-143.
26. Webb,J.C., Patel,D.D., Shoulders,C.C., Knight,B.L. and Soutar,A.K. (1996) Genetic variation at a splicing branch point in intron 9 of the low density lipoprotein (LDL)-receptor gene: a rare mutation that disrupts mRNA splicing in a patient with familial hypercholesterolaemia and a common polymorphism. *Hum.Mol.Genet.* **5**, 1325-1331.

**Table1**

Baseline characteristics of NPHSII men divided on the basis of absence or presence of subsequent CHD (definite plus possible)

|                                       | No event<br>( <i>n</i> = 2169) | CHD<br>( <i>n</i> = 275) | <i>p</i> -value |
|---------------------------------------|--------------------------------|--------------------------|-----------------|
| Age (years)                           | 56.1 (3.4)                     | 56.6 (3.5)               | 0.02            |
| SBP (mmHg) <sup>a</sup>               | 136.6 (18.8)                   | 141.7 (19.2)             | <0.0001         |
| DBP (mmHg)                            | 84.3 (11.3)                    | 87.1 (11.1)              | <0.0001         |
| BMI (kg/m <sup>2</sup> ) <sup>d</sup> | 26.2 (3.4)                     | 26.9 (3.4)               | 0.001           |
| Smoking % (N)                         | 27.1 (588)                     | 37.5 (103)               | <0.0001         |
| Cholesterol (mmol/l)                  | 5.68 (1.00)                    | 6.08 (1.05)              | <0.0001         |
| Triglyceride (mmol/l) <sup>a</sup>    | 1.76 (0.92)                    | 2.09 (1.11)              | <0.0001         |
| ApoA (g/l)                            | 1.63 (0.32)                    | 1.62 (0.30)              | 0.64            |
| ApoB (g/l) <sup>a</sup>               | 0.86 (0.24)                    | 0.92 (0.23)              | 0.0001          |
| HDL(mmol/l) <sup>a</sup>              | 0.80 (0.24)                    | 0.75 (0.22)              | 0.03            |
| Genotype Distribution                 |                                |                          |                 |
| RR/RL/LL                              | 2128/40/1                      | 273/2/0                  | 0.31            |
| L allele frequency<br>(95% CI)        | 0.010<br>(0.007-0.013)         | 0.004<br>(0.0-0.013)     | 0.23            |
| Genotype Distribution                 |                                |                          |                 |
| II/IV/VV                              | 1428/618/64                    | 175/86/11                | 0.44            |
| V allele frequency<br>(95% CI)        | 0.18<br>(0.17-0.19)            | 0.20<br>(0.17-0.23)      | 0.21            |
| Genotype Distribution                 |                                |                          |                 |
| EE/EG/GG                              | 1712/115/3                     | 218/16/1                 | 0.44            |
| G allele frequency<br>(95% CI)        | 0.03<br>(0.028-0.039)          | 0.04<br>(0.023-0.060)    | 0.55            |

<sup>a</sup> Geometric mean given for log-transformed data (approx sd)



**Table 2: Mean ( $\pm$  SD) Baseline lipids levels of NPHSII men by *PCSK9* genotype a) R46L, b) I474V and c) E670G genotypes**

**a) R46L**

|                                   | <b>GG<br/>N=2401</b> | <b>GT<br/>N=42</b> | <b>TT<br/>N=1</b> | <b>P value<br/>GG vs<br/>GT+TT</b> | <b>Adjusted p<br/>value<sup>\$</sup></b> |
|-----------------------------------|----------------------|--------------------|-------------------|------------------------------------|--|
| <b>Total cholesterol (mmol/l)</b> | 5.74 (1.01)          | 5.26 (1.03)        | 3.40 (0)          | 0.0008                             | <b>0.001</b>                             |
| <b>Triglyceride* (mmol/l)</b>     | 1.80 (0.95)          | 1.85 (0.96)        | 1.33 (0)          | 0.77                               | 0.32                                     |
| <b>ApoB* (g/l)</b>                | 0.87 (0.24)          | 0.75 (0.26)        | 0.37 (0)          | 0.0003                             | <b>&lt;0.0001</b>                        |
| <b>HDL-C* (mmol/l)</b>            | 0.79 (0.24)          | 0.85 (0.20)        | 1.01 (0)          | 0.16                               | 0.18                                     |
| <b>LDL-C (mmol/l)</b>             | 4.01 (0.95)          | 3.62 (0.97)        | 1.79 (0)          | 0.01                               | <b>0.02</b>                              |

**b) I474V genotype**

|                                   | <b>AA<br/>N=1603</b> | <b>AG<br/>N=704</b> | <b>GG<br/>N=75</b> | <b>P value</b> | <b>Adjusted p<br/>value<sup>\$</sup></b> |
|-----------------------------------|----------------------|---------------------|--------------------|----------------|--|
| <b>Total cholesterol (mmol/l)</b> | 5.72 (0.99)          | 5.76 (1.03)         | 5.80 (1.05)        | 0.56           | 0.56                                     |
| <b>ApoB* (g/L)</b>                | 0.87 (0.24)          | 0.87 (0.24)         | 0.83 (0.23)        | 0.44           | 0.29                                     |
| <b>HDL* (mmol/l)</b>              | 0.80 (0.24)          | 0.81 (0.24)         | 0.82 (0.24)        | 0.53           | 0.62                                     |
| <b>LDL (mmol/l)</b>               | 3.98 (0.98)          | 4.02 (0.88)         | 4.02 (0.99)        | 0.71           | 0.60                                     |

**c) E670G genotype**

|                                   | <b>AA<br/>N=1930</b> | <b>AG<br/>N=131</b> | <b>GG<br/>N=4</b> | <b>P value</b> | <b>Adjusted p<br/>value<sup>\$</sup></b> |
|-----------------------------------|----------------------|---------------------|-------------------|----------------|--|
| <b>Total cholesterol (mmol/l)</b> | 5.73 (0.99)          | 5.66 (0.97)         | 5.35 (0.19)       | 0.56           | 0.46                                     |
| <b>ApoB* (g/L)</b>                | 0.86 (0.23)          | 0.88 (0.23)         | 0.84 (0.06)       | 0.61           | 0.37                                     |
| <b>HDL* (mmol/l)</b>              | 0.81 (0.24)          | 0.83 (0.26)         | 0.77 (0.10)       | 0.72           | 0.87                                     |
| <b>LDL (mmol/l)</b>               | 3.98 (0.94)          | 3.93 (0.95)         | 3.55 (0.30)       | 0.73           | 0.76                                     |

\*geometric mean (approximate sd)

\$ adjusted for age, practice, smoking, BMI, SBP

**Table 3: Characteristics of FH patients carrying the *PCSK9* 46L variant**

| SB No | M/<br>F | Age at recruitment*<br>(years) | Untreated Chol<br>(mmol/l) | Age onset<br>CHD | <i>LDLR</i><br>mutation |
|-------|---------|--------------------------------|----------------------------|------------------|-------------------------|
| 2825  | F       | 30                             | 8.6                        | No CHD           | splice i9 <sup>§</sup>  |
| 3049  | F       | 57                             | 9.7                        | No CHD           | C337Y                   |
| 3154  | M       | 62                             | 12.6                       | No CHD           | P664L                   |
| 8     | M       | 63                             | 11.3                       | No CHD           | none                    |

\* in 1999

§ mutation is intron 9 c.1359-23del9/ins5 [26]

In 169 FH patients with a detected *LDLR* mutation the recorded mean ( $\pm$ SD) untreated total cholesterol levels were 10.3 (1.9) mmol/l, and the average age of onset of CHD was 47.3 (11.3) years, while in 113 patients with no detected mutation these values were 9.85 (1.9) mmol/l and 47.7 (12.9) years respectively (data from [19]).

**Figure 1**

Change in cholesterol levels over five years by *PCSK9* R46L genotype in NPHSII men

