ORIGINAL ARTICLE

A common variant in the *ABCA1* gene is associated with a lower risk for premature coronary heart disease in familial hypercholesterolaemia

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Familial hypercholesterolaemia (FH) is a common autosomal codominant hereditary disease caused by defects in the LDL receptor (*LDLR*) gene, and one of the most common characteristics of affected subjects is premature coronary heart disease (CHD). In heterozygous FH patients, the clinical expression of FH is highly variable in terms of the severity of hypercholesterolaemia and the age of onset and severity of CHD. Identification of mutations in the ATP binding cassette transporter 1 (*ABCA1*) gene in patients with Tangier disease, who exhibit reduced HDL cholesterol and apolipoprotein A1 concentrations and premature coronary atherosclerosis, has led us to hypothesise that *ABCA1* could play a key role in the onset of premature CHD in FH. In order to know if the presence of the R219K variant in the *ABCA1* gene could be a protective factor for premature CHD in FH, we have determined the presence of this genetic variant by amplification by PCR and restriction analysis in a group of 374 FH subjects, with and without premature CHD. The K allele of the R219K variant was significantly more frequent in FH subjects without premature CHD (0.32, 95% CI 0.27 to 0.37) than in FH subjects with premature CHD (0.25, 95% CI 0.21 to 0.29) ($p<0.05$), suggesting that the genetic variant R219K in *ABCA1* could influence the development and progression of atherosclerosis in FH subjects. Moreover, the K allele of the R219K polymorphism seems to modify CHD risk without important modification of plasma HDL-C levels, and it appears to be more protective for smokers than non-smokers.

Epidemiological studies have shown a strong inverse relationship between HDL cholesterol (HDL-C) levels and coronary heart disease (CHD),[1 2] and low concentration of HDL-C in plasma is considered an independent risk factor for premature atherosclerosis. Many genetic and environmental factors influence plasma HDL-C levels and the causes that contribute to low HDL-C values are heterogeneous. The identification of ATP binding cassette transporter 1 (ABCA1)[3 4] and the fact that mutations in the *ABCA1* gene are the cause of Tangier disease and familial HDL deficiency, both of them characterised by low plasma levels of HDL-C and apolipoprotein (apo) A-I, and increased risk of premature coronary atherosclerosis, suggests that ABCA1 is a protein that plays a key role in regulating plasma HDL-C and apo A-I metabolism.[5–7] ABCA1 is a membrane transporter protein that stimulates cholesterol and phospholipid efflux to apo A-I. This step is one of the first stages in the reverse cholesterol transport (RCT), which mediates the cholesterol catabolism from peripheral cells back to the liver. Therefore, ABCA1 has been considered as a rate limiting step in the production of HDL.[8]

Familial hypercholesterolaemia (FH) is a common autosomal codominant disease caused by defects in the LDL receptor (LDLR) gene. Affected subjects have raised plasma levels of total and LDL cholesterol and a very high risk of premature coronary heart disease.[9] In heterozygous FH patients, the clinical expression of FH is highly variable in terms of the severity of hypercholesterolaemia and the age of onset and severity of CHD, even in subjects sharing the same *LDLR* gene defect.[10 11] Therefore, the phenotype of such patients is clearly influenced by other genes and/or environmental factors[12–14] and several studies have been carried out to elucidate this issue.[15–19]

One of the genes that could be involved in the manifestation of CHD at a young age is *ABCA1*.[20] Recently, common polymorphisms in the *ABCA1* gene have been shown to affect plasma levels of HDL-C and CHD risk[21 22] and therefore they could be genetic risk factors for coronary atherosclerosis in FH.

In order to discover if the presence of the R219K polymorphism in the *ABCA1* gene plays a protective role for premature CHD in FH, we have analysed this genetic variant in a group of FH subjects. In this work, we report that the common variant R219K in the *ABCA1* gene is significantly more frequent in FH subjects without premature CHD than in FH subjects with premature CHD, suggesting that genetic variation in *ABCA1* influences the atherosclerosis process in FH subjects.

MATERIAL AND METHODS

Subjects

The Spanish FH Register was established in 1999 and currently consists of 989 confirmed FH patients using the diagnosis criteria of the MedPed programme (>8 points).[23 24] From this registry, we selected those heterozygous FH subjects with a proven premature coronary event before 55 or 65 years old for men and women, respectively, who constituted the premature CHD group (216 subjects). Coronary events included were myocardial infarction, coronary angioplasty, and coronary bypass surgery. We also selected those heterozygous FH subjects, older than 55 or 65 years for men and women, respectively, and free of any cardiovascular disease, as a control group (158 subjects).

All procedures were in accordance with the Helsinki Declaration of 1975, as revised in 1983. Informed consent was obtained from every person studied.

Lipid analysis

Fasting blood samples were drawn for measurements of total plasma cholesterol, HDL-C, and triglycerides. Measurements were performed with commercially available diagnostic kits (Boehringer Mannheim, Germany), in a laboratory participating in a lipid standardisation programme. The HDL-C level

Table 1 Clinical and biochemical characteristics of the FH subjects included in this study

	Male		p	Female		p
	Control	PCHD		Control	PCHD	
No	66	144		92	72	
Age (years)	64.3 (6.6)	51.1 (10.4)	<0.001	70.5 (5.6)	57.8 (10.5)	<0.001
BMI (kg/m^2)	27.2 (3.0)	27.0 (3.5)	NS	27.0 (4.3)	28.2 (4.6)	NS
TC (mmol/l)	10.7 (1.8)	10.8 (1.9)	NS	11.1 (1.9)	11.2 (2.2)	NS
LDL-C (mmol/l)	8.7 (1.8)	8.9 (1.9)	NS	9.0 (1.9)	9.2 (2.4)	NS
TG (mmol/l)	1.55 (0.71)	1.57 (0.73)	NS	1.42 (0.66)	1.39 (0.60)	NS
HDL-C (mmol/l)	1.24 (0.36)	1.14 (0.30)	NS	1.45 (0.36)	1.42 (0.44)	NS
Lp(a) (µmol/l)	1.50 (1.52)	2.11 (2.01)	0.049	2.11 (2.07)	1.96 (2.16)	NS
Xanthomas	46.0%	41.1%	NS	28.9%	33.8%	NS
Arcus cornealis	77.8%	55.4%	0.002	63.3%	40.0%	0.003
Hypertension	27.9%	18.0%	NS	36.1%	39.3%	NS
Smoking	61.7%	79.7%	0.008	2.4%	14.8%	0.006
DM-2	11.9%	2.3%	0.006	7.3%	5.2%	NS

Values are mean (SD) for quantitative variables, and percentages for qualitative variables. PCHD, premature coronary heart disease; BMI, body mass index; TC, total cholesterol; LDL-C, low density lipoprotein cholesterol; TG, triglycerides; HDL-C, high density lipoprotein cholesterol; Lp(a), lipoprotein(a); Smoking, current and former smokers; DM-2, diabetes mellitus type 2; NS, non-significant.

was determined enzymatically in the supernatant after precipitation of apo B containing lipoproteins with dextran and magnesium sulphate. Plasma LDL-C was calculated according to the Friedewald formula.[25] Lp(a) was determined in a central laboratory by kinetic immunonephelometry with polyclonal antibodies (Beckman, USA).[26] None of the patients was being treated with lipid lowering drugs at the time of blood sampling.

R219K polymorphism analysis

Genomic DNA from peripheral blood was isolated using a previously described method, with minor modifications.[27] A 166 bp fragment of exon 7 of the *ABCA1* gene involving nucleotide 1051 of the cDNA sequence[28] was amplified by polymerase chain reaction (PCR), using the following primers: R219Ks: 5′-GCAAGGCTACCAGTTACATTTGACAAG-3′ and R219Kas: 5′-GATTGGCTTCAGGATGTCCATGTTGG-3′. Genomic DNA was subjected to 35 cycles of denaturation at 95°C for one minute, annealing at 60°C for one minute, and extension at 72°C for one minute, followed by a final extension at 72°C for 10 minutes. After amplification, an aliquot of 8 µl of PCR product was digested with 10 U of the restriction enzyme *Xag*I (MBI Fermentas, Lithuania) at 37°C for more than three hours. The fragments obtained after digestion were analysed by electrophoresis on 2% agarose gels. The bands were visualised by staining with ethidium bromide.

Statistics

The distribution of quantitative variables was tested for normality. Data without a normal distribution were log transformed before analysis. Quantitative variables were compared with the ANOVA one factor test, and qualitative variables were analysed with a chi-square test. As plasma Lp(a) levels and their log transformation were not normally distributed, comparison of this variable was done with the Mann-Whitney non-parametric test. A chi-square analysis was performed to determine the Hardy-Weinberg equilibrium of the polymorphism studied in both groups. Genotype and allele frequencies were compared by a chi-square test. Associations between R219K polymorphism and lipid data were analysed by multivariate ANOVA adjusted by age, gender, and BMI. The K allele distributions (carriers *v* non-carriers) in function of the presence of a coronary event, age of onset of the first coronary event, and smoking were compared with odds ratios and the chi-square test. In all analyses carried out, a global significance level of p=0.05 was considered. All lipid levels are expressed in SI units and all values are reported as the mean (SD). Antilogarithms and unadjusted mean values (SD) of lipid traits are presented. Statistical analyses were performed by using the SPSS v.6.1.3 program for Windows.

RESULTS

The clinical characteristics and the lipid levels of the 374 heterozygous FH subjects selected for this study are shown in table 1. There were no statistical differences between the premature CHD group and control group, concerning the body mass index (BMI), total cholesterol, LDL-C, triglycerides, HDL-C, presence of xanthomas, and hypertension. In contrast, Lp(a) levels were higher in the male premature CHD group than in the male control group ($p<0.05$). However, in females, no differences were observed for Lp(a) concentrations. FH subjects without premature CHD were older than the premature CHD group owing to the selection criteria. Arcus cornealis and diabetes mellitus type 2 were more frequent in the control group than in the premature CHD group, probably because of the difference in age between the selected groups. There were more current or former smokers in the premature CHD group than in the control group for both genders.

The R219K polymorphism is the result of a nucleotide change G→A at position 1051 of the cDNA sequence, and it results in the substitution of lysine for arginine at amino acid 219 of the ABCA1 protein. The genotype of this polymorphism for each of the 374 studied subjects was determined by amplification by PCR and restriction analysis with *Xag*I (fig 1). After digestion of the 166 bp fragment obtained by PCR, the three possible genotypes were distinguished: homozygous GG (166 bp), heterozygous GA (166, 101 and 65 bp), and homozygous AA (101 and 65 bp).

The R219K polymorphism was in Hardy-Weinberg equilibrium in the control and premature CHD groups. The genotype frequency distribution for the R219K polymorphism is shown in table 2. The frequency of the RK and KK genotypes and the frequency of K allele carriers (genotypes RK+KK) was significantly lower in the premature CHD group than in the control group ($p<0.05$). Similarly, the allele frequency distribution was significantly different between both groups. The allelic frequencies for the minor K allele of the R219K polymorphism were 0.32 (95% CI 0.27 to 0.37) and 0.25 (95% CI 0.21 to 0.29) for control and premature CHD groups, respectively ($p<0.05$). The presence of the K allele of the R219K polymorphism reduced the coronary event risk in the FH studied population (odds ratio 0.63, 95% CI 0.42 to 0.95).

The clinical and biochemical characteristics of carriers and non-carriers of the K allele for the R219K variant in the *ABCA1*

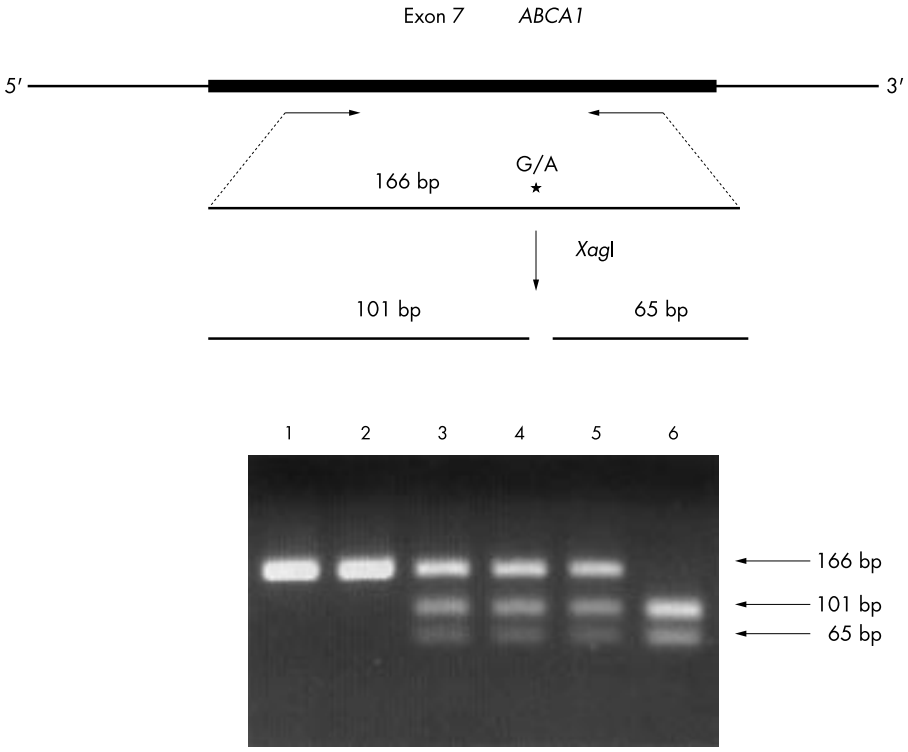

Figure 1 Determination of the R219K genotype by PCR amplification and restriction analysis. In the upper part, the G/A polymorphism position is indicated by an asterisk. When the nucleotide A is present, a *Xag*I restriction site is created. In the lower part, 2% agarose gel electrophoresis of *Xag*I digested PCR products is shown. After cleavage with *Xag*I, either a 166 bp fragment (homozygous GG, lanes 1 and 2), 166, 101, and 65 bp fragments (heterozygous GA, lanes 3, 4 and 5), or 101 and 65 bp fragments (homozygous AA, lane 6) are produced.

Table 2 Genotype and allele frequencies distribution for the R219K polymorphism in the control and premature coronary heart disease groups of FH subjects studied

	Genotype frequencies							Allele frequencies		
	RR	RK	KK	p	RR	RK+KK	p	R	K	p
Control	45.6%	44.9%	9.5%	0.037	45.6%	54.4%	0.029	0.68	0.32	0.036
PCHD	56.9%	36.1%	6.9%		56.9%	43.1%		0.75	0.25	

PCHD, premature coronary heart disease.

gene are shown in table 3. Male subjects with the RR genotype had higher LDL-C levels than K allele carriers of the R219K polymorphism (p=0.04). However, this difference was not observed in females or in all subjects as a whole. On the other hand, subjects not carrying the K allele of the R219K polymorphism had more xanthomas than subjects with the RK or KK genotypes (p=0.04). This difference was also observed when subjects were analysed by gender, although in this case the difference did not reach statistical significance. The remaining variables, age, BMI, lipid and lipoprotein levels, and presence of arcus cornealis, did not show differences between carriers and non-carriers of the K allele of the R219K polymorphism.

To assess whether the presence of the K allele of the R219K variant has an effect on the age of onset of the first coronary event in the premature CHD group, we analysed the distribution of carriers and non-carriers of the K allele in subjects who suffered their first coronary event before 40 years old (first quartile) (PCHD<40 group, n=53), in subjects who suffered their first coronary event after 40 years old (PCHD≥40 group, n=163), and in the control group. The percentages of non-carriers and carriers of the K allele in each of the three groups of FH subjects analysed are shown in fig 2. A different distribution was observed, with fewer carriers of the K allele of the R219K polymorphism in the PCHD<40 group than in the PCHD≥40 and fewer than in the control group. The difference between the PCHD<40 and control groups was statistical significant (p=0.035). In this case, the odds ratio of carrying the K allele in PCHD<40 *v* the control group was 0.51 (95% CI 0.27 to 0.96).

The premature CHD group as a whole has a higher percentage of smoking subjects than the control group (59% *v* 27%, p<0.0001). In the FH population studied, smoking increases the coronary event risk to 3.88 (95% CI 2.44 to 6.19). However, when the age of onset of the first coronary event was taken into account and the two groups, PCHD<40 and PCHD≥40, were analysed separately, no statistical differences in the percentage of smoking subjects between the PCHD<40 and PCHD≥40 groups were observed (63% and 58%, respectively). The effect of the K allele of the R219K polymorphism on a premature event of CHD (before 40 years) was analysed in smokers and non-smokers separately. In smoking (current and former) subjects, the odds ratio of onset of premature CHD before 40 years old in carriers *v* non-carriers of the K allele was 0.45 (95% CI 0.16 to 1.25, p=0.123). In non-smoking subjects, the odds ratio of having a premature coronary event before 40 years old in carriers *v* non-carriers of the K allele was 0.76 (95% CI 0.28 to 2.08, p=0.595). In subjects with premature CHD before 40 years old, the odds ratio of carrying the K allele in smokers *v* non-smokers was 0.35 (95%

Table 3 Clinical and biochemical characteristics of non-carriers and carriers of the K allele of the R219K polymorphism in FH subjects studied

	Male			Female			All		
	RR	RK+KK	p	RR	RK+KK	p	RR	RK+KK	p
No	120	90		75	89		195	179	
Age (years)	55.4 (10.8)	55.1 (11.7)	NS	63.6 (10.3)	66.1 (10.2)	NS	58.5 (11.3)	60.6 (12.3)	NS
BMI (kg/m^2)	27.2 (3.4)	26.9 (3.2)	NS	27.1 (4.4)	27.9 (4.6)	NS	27.2 (3.8)	27.4 (3.9)	NS
TC (mmol/l)	10.9 (2.0)	10.5 (1.7)	NS	11.2 (2.3)	11.1 (1.9)	NS	11.1 (2.1)	10.8 (1.8)	NS
LDL-C (mmol/l)	9.1 (2.0)	8.6 (1.8)	0.04	9.1 (2.4)	9.1 (1.9)	NS	9.1 (2.1)	8.8 (1.9)	NS
TG (mmol/l)	1.53 (0.65)	1.60 (0.81)	NS	1.46 (0.64)	1.38 (0.63)	NS	1.49 (0.64)	1.49 (0.73)	NS
HDL-C (mmol/l)	1.17 (0.32)	1.19 (0.33)	NS	1.42 (0.38)	1.45 (0.41)	NS	1.27 (0.36)	1.32 (0.40)	NS
Lp(a) (µmol/l)	1.93 (1.70)	1.96 (2.12)	NS	2.00 (2.17)	2.07 (2.05)	NS	1.96 (1.90)	2.00 (2.08)	NS
Xanthomas	47.4%	36.4%	NS	34.2%	28.4%	NS	42.3%	32.4%	0.04
Arcus cornealis	57.9%	68.2%	NS	52.8%	53.4%	NS	55.9%	60.8%	NS

Values are mean (SD) for quantitative variables, and percentages for qualitative variables. BMI, body mass index; TC, total cholesterol; LDL-C, low density lipoprotein cholesterol; TG, triglycerides; HDL-C, high density lipoprotein cholesterol; Lp(a), lipoprotein(a); NS, non-significant.

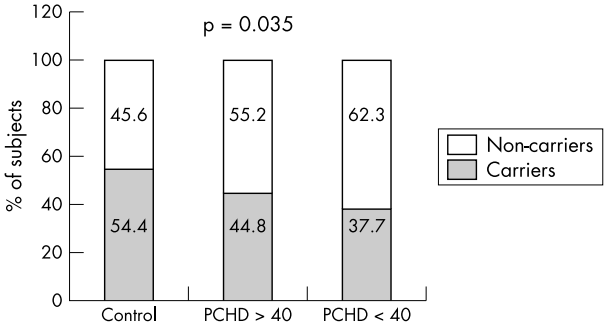

Figure 2 Percentage of non-carriers and carriers of the K allele of the R219K polymorphism in each of the three groups of FH subjects analysed: free of premature coronary heart disease (controls), premature coronary heart disease with first coronary event after 40 years old (PCHD≥40), and premature coronary heart disease with first coronary event before 40 years old (PCHD<40); p indicates the difference between the control and PCHD<40 groups.

CI 0.10 to 1.18, p=0.086). These results would suggest that the protective effect of the K allele of the R219K variant on premature CHD risk is more pronounced in smokers.

DISCUSSION

HDL-C is a major independent factor involved in the development of premature CHD. The anti-atherogenic function of HDL has been attributed to its role in reverse cholesterol transport, where the protein ABCA1 plays a crucial role in its initial step.[20 29] Several studies have reported that mutations in *ABCA1* cause low HDL-C levels, increased triglycerides, depressed levels of cholesterol efflux, and an increased risk of CHD.[5 30 31] Moreover, other studies have shown that some single nucleotide polymorphisms of the *ABCA1* gene influence plasma lipid levels and the severity of CHD. Specifically, the R219K polymorphism has been shown to be associated with decreased TG levels and a decreased severity of CHD, these effects being compatible with a net increase in ABCA1 function and accelerated reverse cholesterol transport.[21] This polymorphism could influence the phenotype of FH in such a way that subjects carrying the protective allele have a slower progression of atherosclerosis and a delayed onset of CHD.

Previous studies have reported a carrier frequency of the R219K polymorphism of 46% in the European population,[21] which is similar (47.8%) to that found in our FH patients. Clee *et al*[21] have previously shown that, in the population studied by them, carriers of the K allele have decreased atherosclerosis and fewer coronary events. In the present work, we have found that the frequency of the K allele of the R219K variant is significantly higher in the group of FH subjects without premature CHD than in the group of FH subjects with premature CHD. The presence of the K allele of the R219K polymorphism seems to be protective against onset of premature CHD in FH subjects. One result of our study is that the K allele of the R219K variant seems to modify CHD risk without important modification of plasma HDL-C concentration. This probably reflects that the anti-atherogenic function of HDL is not only explained by plasma HDL-C levels. Other works based on different polymorphisms in coding and non-coding regions of the *ABCA1* gene have also found differences in the risk of CHD without detectable changes in plasma lipid levels.[22 32 33] However, another common variant in *ABCA1*, I823M, has been reported to be a significant source of variation in plasma HDL-C.[34] These findings of different risk of CHD but no differences in lipid levels would suggest that modification in reverse cholesterol transport may vary the flux of cholesterol towards the liver without necessarily modifying the plasma lipid concentrations. Singaraja *et al*[35] have shown that overexpression of ABCA1 induced the increase of cholesterol efflux from macrophages, the HDL particles being better acceptors of cholesterol, although the increase in plasma HDL-C levels was small. It is possible that the R219K variant increases the activity of ABCA1 in a similar way, although the precise mechanism underlying the functional effect of this variant will require further analyses.

In this series of FH patients studied, we have observed a correlation between the presence of the K allele of the R219K variant and the age of onset of the first coronary event. The younger FH subjects with early proven coronary events are less often carriers of the protective K allele. Comparison of the odds ratio of the control *v* the PCHD<40 group (OR=0.51) with the odds ratio of the control *v* the premature CHD total group (OR=0.63) suggests that younger subjects lacking the K allele of the R219K variant have a higher coronary risk. This observation confirms the protective effect of the K allele, as subjects lacking the K allele of the R219K variant develop CHD at an earlier age than subjects carrying this allele. Another observation of our study is that the R219K variant appears to be more protective for smokers and ex-smokers than non-smokers in the PCHD<40 group. This finding of an interaction of smoking with a determined genotype has already been described for other genes. Therefore, Humphries *et al*[36] have reported that smokers who were carriers of the E4 allele of apo E showed an increased risk of CHD compared with non-smokers. Similarly, in our series of FH patients, smoking increases the risk of CHD in all subjects, but particularly in those subjects lacking the K allele of the R219K polymorphism in *ABCA1*. Smoking increases the rate of oxidation of lipoprotein particles, and it might be possible that this oxidative stress would be alleviated in part through the ABCA1 pathway, since ABCA1 has been reported to mediate the cellular secretion of α tocopherol, the active form of vitamin E with antioxidant properties.[37] Thus, it might be possible that

subjects carrying the K allele of the R219K variant would be more protected against lipoprotein oxidation and subsequent risk of atherosclerosis. Further studies will be necessary to confirm this hypothesis.

In this work we show that the R219K variant of *ABCA1* influences premature CHD frequency in subjects with heterozygous familial hypercholesterolaemia. FH subjects are a very high cardiovascular risk owing to their high LDL-C levels as a consequence of a mutation in the *LDLR* gene, but other genetic and/or environmental factors modify the disease expression[18] and it is possible that one of them could be the variant R219K in *ABCA1*. Our results indicate the importance of considering other loci than *LDLR* in the clinical consequences of FH. Although the *ABCA1* locus, and specifically the polymorphism R219K, influences the early onset of CHD in FH, other different loci are also implicated with small and/or large contributions, the disease expression being the result of the interaction of these loci.[16] Further studies to that effect will help to identify FH subjects at high risk of premature CHD, which will be helpful for better prevention and management of cardiovascular disease in familial hypercholesterolaemia.

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