Linkage of familial combined hyperlipidaemia to chromosome 1q21–q23

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More than half of the patients with angiographically confirmed premature coronary heart disease (CHD) have a familial lipoprotein disorder[1]. Familial combined hyperlipidaemia (FCHL) represents the most common genetic dyslipidemia with a prevalence of 1.0–2.0% (refs 2,3). FCHL is estimated to cause 10–20% of premature CHD (ref. 1) and is characterized by elevated levels of cholesterol, triglycerides, or both[3,4]. Attempts to characterize genes predisposing to FCHL have been hampered by its equivocal phenotype definition, unknown mode of inheritance and genetic heterogeneity. In order to minimize genetic heterogeneity, we chose 31 extended FCHL families from the isolated Finnish population[5] that fulfilled strictly defined criteria for the phenotype status. We performed linkage analyses with markers from ten chromosomal regions that contain lipid-metabolism candidate genes. One marker, *D1S104*, adjacent to the apolipoprotein A-II (*APOA2*) gene on chromosome 1, revealed a lod score of $Z=3.50$ assuming a dominant mode of inheritance. Multipoint analysis combining information from *D1S104* and the neighbouring marker *D1S1677* resulted in a lod score of 5.93. Physical positioning of known genes in the area (*APOA2* and three selectin genes) outside the linked region suggests a novel locus for FCHL on 1q21–q23. A second paper in this issue (Castellani *et al.*) reports the identification of a mouse combined hyperlipidaemia locus in the syntenic region of the mouse genome[6], thus further implicating a gene in this region in the aetiology of FCHL.

In 1973, Goldstein *et al.*[3] proposed a dominant mode of inheritance for FCHL (which is consistent with data from our Finnish families). The genetic basis for FCHL, however, remains unknown. Segregation analyses have implicated genes in FCHL that control triglyceride levels[7], apoB levels[8], and an LDL subclass[9]. Furthermore, a putative locus for small, dense LDL (characteristic for FCHL) has been linked to the LDL-receptor locus[10]. In addition, several studies have examined the roles of obvious candidate genes in FCHL. Contradictory data exist about the *APOA1/APOC3/APOA4* gene cluster on chromosome 11 (refs 11–14) and association studies implicated the lipoprotein lipase gene[14,15] but our families revealed no evidence for linkage between FCHL and lipolytic enzymes[16].

The original diagnosis for FCHL was based on premature CHD and three different lipid phenotypes among first-degree family members[3,4]: elevated cholesterol (phenotype IIA), elevated cholesterol and triglycerides (phenotype IIB, combined hyperlipidaemia) and elevated triglycerides (phenotype IV). In more recent studies, less stringent criteria for FCHL (such as the existence of different elevated lipid phenotypes in two different family members[12]) have been adopted[14]. To ensure that families in our study were affected with 'true' FCHL (as opposed to a simpler trait involving only one elevated lipid class), we selected probands among individuals with early onset CHD and lipid levels of higher than or equal to the age-sex-specific 90th percentile[16]. We also used strict diagnostic criteria for other family members. Our selection strategy for the 31 families studied thus emphasized the importance of combined hyperlipidaemia in FCHL diagnosis. For linkage analysis, phenotypic information was utilized only from affected individuals to minimize the

Table 1 • Two-point lod scores between FCHL and microsatellite markers within candidate genes

Candidate gene encoding	Chromosomal location	Pairwise lod score (θ=0.00)	Maximum lod score (θ)
Apolipoproteins			
Apolipoprotein A-I/ C-III/ A-IV gene cluster	11q23	−11.20	0.005 (0.40)
Apolipoprotein B	2p24–p23	−7.68	0.001 (0.44)
Apolipoprotein A-II	1q21–q23	−7.39	0.04 (0.34)
Apolipoprotein C-II	19q13.2	−9.90	0.00 (0.50)
Cellular adhesion molecules			
P-selectin	1q22–q25	−0.55	1.45 (0.12)
L-selectin	1q23–q25	−6.09	0.00 (0.50)
Receptors			
LDL-receptor	19p13.2	−6.09	0.00 (0.50)
LDL-receptor related protein	12q13–q14	−5.16	0.14 (0.26)
Insulin receptor	19p13.3	−4.15	0.00 (0.50)
Proteins involved in cellular lipid metabolism			
HMGC-CoA reductase	5q13.3–q14	−9.85	0.00 (0.50)
Intestinal fatty acid binding protein	4q28–q31	−7.24	0.10 (0.32)

θ, Recombination fraction. A dominant mode of inheritance is assumed.

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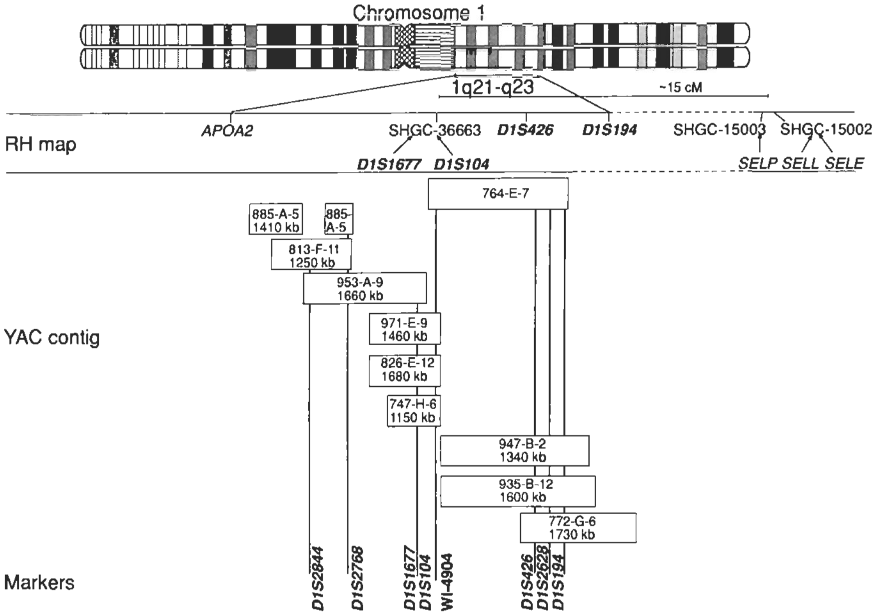

Fig. 1 The critical FCHL area on 1q21–23. The known sizes of the YACs are indicated in the boxes. The STS WI-4904 is included to show the continuity of the reconstructed contig from YACs 971-E-9, 826-E-12 and 747-H-6 to YAC 764-E-7 (according to the database, this STS is detected as an unambiguous hit in YACs 971-E-9, 826-E-12 and 764-E-7 and as a disambiguated hit in the YAC 747-H-6). The SHGC frame-work markers that best link to *D1S104* and *D1S1677* as well as to the *SELL*, *SELP* and *SELE* are indicated on the RH map.

effects of incomplete penetrance. In addition to the FCHL trait, we analysed cholesterol-, triglyceride- and apoB-levels as separate independent phenotypes.

We studied intragenic and flanking markers for 11 candidate genes encoding proteins essential for lipid metabolism[14]. With the exception of chromosome 1, none of the markers resulted in a lod score of greater than 1.00 (Table 1). The results of affected sib-pair analysis were also not significant for any other chromosome. Analysis of *D1S104*, a marker flanking *APOA2* on 1q21–23, resulted in a two-point lod score of Z=3.50 (θ=0.02) under a dominant FCHL model with no evidence of heterogeneity. The intragenic *APOA2* marker (heterozygosity=0.70) resulted in a lod score of Z=0.04 (θ=0.34) for the FCHL trait (Table 2) and Z=0.67 (θ=0.18) for the triglyceride trait with a dominant mode of inheritance (Table 3).

The chromosomal region 1q21–q23 was further examined using a denser set of markers covering an 8-cM area. When we combined information from the two most informative markers,

Table 2 • Two-point linkage analysis of the FCHL trait

Genetic distance	Locus	**Dom**	DomNuc	**Rec**	RecNuc	ASP
	APOA2	**0.04 (0.34)**	0.18 (0.26)	**0.06 (0.34)**	0.20 (0.28)	0.11
<1.0 cM						
	D1S2768	**0.26 (0.16)**	0.12 (0.26)	**0.28 (0.22)**	0.45 (0.20)	0.05
2.4 cM						
	D1S2844	**0.23 (0.22)**	0.31 (0.20)	**0.21 (0.28)**	0.29 (0.26)	0.23
1.0 cM						
	D1S1677	**0.91 (0.10)**	1.02 (0.10)	**0.47 (0.24)**	0.51 (0.24)	0.53
0.4–0.5 cM	**multipoint lod score**	**MLINK 5.93 (0.02)**				
	D1S104	**3.50 (0.02)**	2.46 (0.02)	**1.16 (0.16)**	1.14 (0.16)	0.85
1.5 cM	**multipoint lod score**	**MLINK 2.51 (0.04)**				
	D1S426	**0.89 (0.02)**	0.69 (0.08)	**0.39 (0.22)**	0.43 (0.22)	0.23
0.0 cM						
	D1S2628	**0.08 (0.30)**	0.05 (0.34)	**0.25 (0.24)**	0.34 (0.22)	0.01
2.0 cM						
	D1S194	**0.00 (0.50)**	0.00 (0.50)	**0.01 (0.38)**	0.06 (0.32)	0.00
~12.0 cM						
	SELP	**1.45 (0.12)**	1.48 (0.08)	**0.96 (0.18)**	1.00 (0.20)	0.74
0.3 cM	**multipoint lod score**	**MLINK 0.54 (0.18)**				
	SELL	**0.00 (0.50)**	0.00 (0.50)	**0.00 (0.50)**	0.00 (0.50)	0.00

Markers in 8-cM region on chromosome 1 from extended pedigrees were analysed using a dominant (Dom) and recessive (Rec) inheritance model. Lod scores of the pedigrees divided into nuclear families (DomNuc, RecNuc), the best multipoint lod scores under a dominant inheritance model, as well as the lod scores of the affected sib-pair analysis (ASP) are shown. The pairwise and multipoint lod scores for the two *SELP* and *SELL* intragenic markers are also shown. The recombination fractions (θ) of the maximum lod scores are given in parentheses. There was no evidence for heterogeneity in these analyses. The corresponding maximum lod scores of the extended pedigrees using elevated apoB as a segregating trait were 1.62 (θ=0.10) for *D1S104* and 0.28 (θ=0.26) for *D1S1677*. When increased total cholesterol was used as a trait, the lod scores were 0.73 (θ=0.12) and 0.38 (θ=0.14), respectively.

Table 3 • Two-point linkage analysis of the triglyceride trait

Genetic distance	Locus	Dom	DomNuc	Rec	RecNuc	ASP
	APOA2	**0.67 (0.18)**	1.87 (0.04)	**0.62 (0.20)**	1.51 (0.12)	2.03
<1.0 cM						
	D1S2768	**0.31 (0.20)**	0.70 (0.06)	**0.14 (0.28)**	0.30 (0.20)	0.54
2.4 cM						
	D1S2844	**1.21 (0.10)**	2.22 (0.00)	**1.00 (0.16)**	1.28 (0.12)	1.51
1.0 cM						
	D1S1677	**0.46 (0.18)**	0.95 (0.08)	**0.62 (0.20)**	0.54 (0.22)	0.82
0.4–0.5 cM	**multipoint lod score**	**MLINK 3.25 (0.06)**				
	D1S104	**2.47 (0.04)**	2.74 (0.00)	**2.00 (0.10)**	1.98 (0.10)	2.54
1.5 cM	**multipoint lod score**	**MLINK 2.02 (0.07)**				
	D1S426	**0.64 (0.12)**	1.74 (0.00)	**1.15 (0.10)**	1.31 (0.10)	1.34
0.0 cM						
	D1S2628	**0.83 (0.16)**	0.99 (0.10)	**0.52 (0.20)**	0.60 (0.20)	0.84
2.0 cM						
	D1S194	**0.23 (0.20)**	0.17 (0.22)	**0.08 (0.30)**	0.12 (0.28)	0.17
~12.0 cM						
	SELP	**2.89 (0.02)**	2.45 (0.02)	**1.38 (0.14)**	1.74 (0.10)	1.61
0.3 cM	**multipoint lod score**	**MLINK 1.49 (0.10)**				
	SELL	**0.00 (0.50)**	0.00 (0.50)	**0.00 (0.50)**	0.00 (0.50)	0.00

Markers in 8-cM region on chromosome 1 from extended pedigrees were analysed using a dominant (Dom) and recessive (Rec) inheritance model. Lod scores of the pedigrees divided into nuclear families (DomNuc, RecNuc), the best multipoint lod scores under a dominant inheritance model, as well as the lod scores of the affected sib-pair analysis (ASP), are shown. The pairwise and multipoint lod scores for the two *SELP* and *SELL* intragenic markers are also shown. The recombination fractions (θ) of the maximum lod scores are given in parentheses. There was no evidence for heterogeneity in these analyses.

D1S104 and *D1S1677* (located 0.4–0.5 cM apart), we obtained a lod score of $Z=5.93$ ($\theta=0.02$) with multipoint MLINK assuming dominant mode of inheritance for FCHL (see Methods). The dominant model yielded the highest two-point lod scores for the chromosome-1 markers for both the FCHL and triglyceride traits (Tables 2,3). The complete linkage data for all traits and markers under both dominant and recessive inheritance models are available on our web site (http://www.ktl.fi/molbio/fchl).

To control for the possibility of bilineal transmission, aetiological heterogeneity and influence of diagnostic classifications, we also analysed the component nuclear pedigrees as if they were independent, by both affected sib-pair analysis (which disregards parental phenotype information) and parametric lod score analysis (which includes parental phenotype information). The results of these analyses (Tables 2,3) are consistent with our lod scores from the extended pedigrees, although somewhat lower because of the loss of phase information in assuming that the component nuclear pedigrees are independent. There was no evidence for genetic heterogeneity in the analyses between component nuclear pedigrees, which is consistent with the presence of a major genetic component of FCHL in this region of chromosome 1.

We searched for potential linkage disequilibrium (LD) by using the haplotype–relative-risk approach[17] but found no evidence for it. There are several explanations for the lack of LD despite the evidence for linkage. For common complex diseases like FCHL, more than one founder mutation probably exists even in isolated populations. Our FCHL families were collected from geographically different parts of Finland and therefore do not represent any subisolate. The population expansion rate has been shown to interfere with the detection of LD where common disease alleles of complex diseases are concerned[18]. In recently expanded populations like the Finns, common disease alleles may be very old and consequently display very little LD (ref. 18). Furthermore, when taking the effects of nongenetic aetiological factors as well as allelelic and non-allelic heterogeneity into account, a very dense marker map would be needed to detect LD (which is likely to be restricted to a small region around genes mutated in common diseases[19]).

In addition to *APOA2*, the region on chromosome 1 linked to FCHL harbours other potential susceptibility genes for atherosclerosis: *SELL*, *SELP* and *SELE*, encoding L-selectin, P-selectin and E-selectin, respectively. Selectins could be involved in atherogenesis by causing circulating monocytes and lymphocytes to adhere to the endothelium. The genes are known to be clustered within 300 kb somewhere in the 1q22–q25 interval. The intragenic *SELP* marker (heterozygosity=0.76) gave a lod score of $Z=1.45$ ($\theta=0.12$) under the dominant FCHL model (Table 2) and $Z=2.89$ ($\theta=0.02$) under the dominant triglyceride trait (Table 3). The *SELL* marker, with a lower heterozygosity (0.33), revealed no positive lod scores under either model. Multipoint analysis of these selectin markers using a dominant FCHL or triglyceride model, resulted in lod scores of $Z=0.54$ ($\theta=0.18$) and $Z=1.49$ ($\theta=0.10$), respectively.

To clarify the positional relationship between *APOA2*, the selectin genes and the markers linked to FCHL, we analysed the physical map over the critical DNA region. Radiation hybrid (RH) mapping indicated that the markers *D1S104* and *D1S1677* are located close to each other (0.4–0.5 cM) and lie about 5 cM away from *APOA2* and more than 15 cM away from the selectin gene cluster (Fig. 1). Furthermore, PCR analysis of 20 human YAC clones from the WC1.16 contig which form a 5–6 Mb contig flanking the critical DNA-region (Fig. 1) proved that neither *APOA2* nor the selectin genes are present in these clones, consistent with their distant genetic map location. The markers providing the best evidence for linkage, therefore, map at a significant distance from both *APOA2* and the selectin genes. However, because any linkage study makes some incorrect assumptions regarding the mode of inheritance of a complex disease like FCHL, the final exclusion of any gene in this wide chromosomal region will require sequence analysis.

Recent data from mouse models support the existence of an FCHL locus in this region of chromosome 1. First, a major gene (*Ath1*) participating in the development of early atherosclerotic lesions in response to diet was linked near, but not within, the *APOA2* locus on mouse chromosome 1 (ref. 20). Second, a mouse locus contributing to combined hyperlipidaemia in the region syntenic to human 1q21–q23 has been recently identified, and is also published in this issue[6].

The current Finnish population originated from a limited number of founder ancestors[5] and genetic, environmental and cultural homogeneity render the Finns attractive for genetic studies. Even in this isolated population, however, the existence of founder effects in complex diseases with common disease alleles is very unlikely. Despite this, the study of Finns may help to solve the complexity of traits like FCHL as the expected variation in the environment, culture and polygenic background is smaller in Finland than in more heterogeneous populations. Our data indicate a novel FCHL locus on 1q21–23 and should stimulate characterization of potential new genes on this chromosomal region. The identification of gene(s) associated with this common genetic disorder may help to develop new intervention strategies to inhibit or reverse the atherosclerotic process.

Methods

Pedigrees, diagnosis and biochemical analysis. Individuals from 31 Finnish FCHL families were recruited in the Helsinki (16 families), Turku (9 families) and Kuopio (6 families) University Hospitals as a part of the EUFAM study[16]. Male probands were between 30 and 55 yr and female probands between 30 and 65 yr. Probands suffered from either premature CHD, confirmed by angiography (greater than 50% stenosis in one or more coronary arteries; 27 probands), or myocardial infarction (4 probands[21]), and all had total serum cholesterol and/or serum triglyceride levels higher than or equal to the age-sex specific 90th percentile. Eight probands had phenotype IIA, fifteen had IIB and eight had phenotype IV. Individuals with type 1 diabetes, hypothyreosis or renal disease were excluded as probands. The details of the family collection are reported elsewhere[16]. We selected families in which, besides premature CHD, the proband or a first-degree relative had the combined phenotype IIB. Family members were scored as affected if they had the combined phenotype IIB or if they had high cholesterol (phenotype IIA) or high triglycerides (phenotype IV), both above the age-sex specific 90th percentiles. In addition (also in extended pedigrees), each individual with only elevated cholesterol or triglycerides was required to have a first-degree relative (parent, child or sibling) with the combined phenotype IIB to be scored as affected in this analysis. Thus, five subjects with only one lipid class elevated and without a first-degree relative with the combined phenotype IIB were initially coded as unknown for the analyses. This strategy results in the ascertainment of a combined phenotype of FCHL because elevated levels of one lipid class alone can be caused by environmental as well as genetic causes other than FCHL.

We studied 250 individuals, of which 115 were affected with FCHL. DNA samples were available from 111 of the affected individuals. In addition, 70 unaffected individuals were genotyped to increase the marker locus genotype information needed in the analysis. The remaining 65 individuals (dead grandparents, grandparents not able or willing to participate, dead spouses or spouses working abroad) were used to construct the pedigrees (which are presented on our web site http://www.ktl.fi/molbio/fchl). The number of all possible affected sib-pairs was 141, and the number of independent sib-pairs was 71. The study was approved by the ethical committees of the participating centres and all samples were collected in accordance with the Helsinki declaration. Lipid-lowering medication was interrupted for four weeks before blood sampling. The lipid measurements were performed in the laboratories of the Helsinki and Kuopio University Hospitals. Serum total cholesterol and triglycerides were determined enzymatically (Boehringer, Hoffman-La Roche). Serum apoB was measured by immunoturbidimetric methods (Orion Diagnostica, Kone Instruments). The lipid criteria used for classification of study subjects aged 25–60 yr were derived from FINMONICA (ref. 22). The percentile values for those of 60–65 yr were applied to all individuals over 60 yr. The cutoff for the upper 90th percentiles of each phenotype for subjects under the age of 25 was derived from the 'Cardiovascular Risk Factors in Young Finns' study follow-up samples of 1986 (n=2236; ref. 23). Differences in lipid assays between centres were estimated in small subsamples (n=50–215) with linear regression analyses, and levels were unified accordingly. The Pearson correlations between the centres were all greater than or equal to 0.99. Familial hypercholesterolaemia was excluded from each pedigree by determining the LDL-receptor status of the proband from lymphocyte cultures[24]. Furthermore, in clinical examination, none of the participants had tendon xanthomas. For the classification, the highest lipid values from two measurements were used; from 31 of the 181 genotyped individuals only one lipid measurement was available.

Genotyping. The fluorescently labelled PCR products were electrophoretically separated either on an automated laser fluorescence (ALF) DNA sequencer (Pharmacia Biotech) using 6% Hydrolink gels or with an ABI 377 sequencer (Perkin Elmer) with Genescan v.2.1 peak calling software. The alleles were identified by using the Fragment Manager (Pharmacia Biotech) program and the Automate Linkage Pre-processor (ALP) software (A. Brown) if the gels were run with the ALF sequencer, or by the Genotyper v.2.0 program (Perkin Elmer) when the ABI 377 sequencer was used.

Microsatellite marker primers for chromosome 1 and the intragenic and flanking candidate gene markers for other chromosomal regions were selected using the most recent Généthon marker map (http://www.genethon.fr), the GDB6.0 database (http://gdbwww.gdb.org), the CHLC database (http://www.chlc.org) and the Location DataBase (http://cedar.genetics.soton.ac.uk/public_html/index.html). Our strategy was to genotype an intragenic marker and one or two flanking markers for each candidate gene. The primer sequences and original references for all intragenic markers are available on our web site (http://www.ktl.fi/molbio/fchl). The locations, distances and identification of markers adjacent to *D1S104*, *D1S1677* and *SELL*, *SELP* and *SELE* were determined by radiation hybrid mapping using a G3 RH panel (Research Genetics), the Stanford Human Genome Centre Radiation Hybrid Mapping e-mail Server (http://www-shgc.stanford.edu/Mapping/rh/search.html) and the program RHMAP-package v. 2.01 (M. Boehnke). The marker order and distances from other markers on chromosome 1 were based on sex-averaged genetic maps from Généthon. The single nucleotide polymorphism (T→C, in nucleotide position 668) in *SELL* was characterized with the solid-phase minisequencing method[25]. A 186-bp segment of *SELE* was amplified with the primers 5′-AGTAATAGTCCTCCTCATCATG-3′ and 5′-ACCATCTCAAGTGAAGAAAGAG-3′ and was used in the RH and YAC analysis.

The individual human YAC clones for the contig WC1.16 were selected from the database of Whitehead Institute, MIT Center for Genome Research (htpp://www.genome.wi.mi). The individual human YAC clones were received from the CEPH human YAC library (denis@ceph.cephb.fr). The presence of the chromosome-1 markers in the YACs was tested by PCR amplification.

Linkage analysis. Two-point pairwise linkage analysis was performed using the MLINK program of the linkage package[26] and the version FASTLINK 2.2 (refs 27–29). The MLINK program was also used to perform multipoint analysis combining the information from the adjacent markers to the best marker *D1S104* as follows:

0.4 cM θ
----*D1S1677*--------------*D1S104*--------------Disease
or
1.5 cM θ
----*D1S426*--------------*D1S104*--------------Disease

In each case, parameter θ is estimated. We avoided multipoint analysis with flanking markers (moving the disease across the map) because of its known propensity for false exclusions[30]. However, because the marker *D1S104* is not fully informative, we had to extract additional information about recombinant and non-recombinant meioses[31]. To avoid the negative side-effects of multipoint analysis with flanking markers, we performed an analysis in which the tightly linked markers were placed in a fixed order and the disease locus was allowed to vary outside the marker map. The main purpose was to score meioses uninformative for marker *D1S104* for nearby markers (analogous to the previous studies[31] with the benefit of controlling for the intermarker recombination fractions), and thus allowing all meioses on all pedigrees to be scored in the analysis.

To circumvent problems of incomplete penetrance and ambiguity of the 'unaffected' phenotype, we selected an affecteds-only strategy. The subjects were classified either 'affected' or 'unknown' according to their lipid levels. The 'unknown' family members were genotyped only to maximize the marker locus and phase information. In addition to the FCHL trait, we also analysed elevated cholesterol-, triglyceride- or apoB-levels as separately segregating traits. We used the age-sex specific 90th percentile threshold for the affecteds and, as before, treated individuals with values lower than the 90th percentile as 'unknown'. We performed both dominant and recessive pseudo-marker linkage analysis[32], with gene frequencies of 0.6% for the dominant and 10.95% for the recessive model. To control for the bilineal introduction of the trait (and thus possible genetic heterogeneity), the offspring of an affected individual were only included in the analysis if the spouse of this affected family member did have cholesterol and triglyceride levels lower than the age-sex specific 90th percentile. It has been shown[33] that bilineal pedigrees have little effect on the expected maximum lod score. Therefore, the omission of such pedigrees tends to make mapping more cost-efficient. Furthermore, in none of the selected families were both grandparents in the first generation known to have elevated cholesterol and/or triglycerides. If one of the grandparents in the first generation died from early-onset CHD and the other (still living) grandparent had elevated lipid levels, both the grandparents were initially treated as 'unknown' because it was not clear which (if either) of the them was affected. For each marker, the allele frequencies were estimated from an allele counting on one individual randomly selected from each of the families. The possibility of LD was examined using the HRRLAMB program[17]. Genetic heterogeneity between the families was examined using the HOMOG program[26,34]. The identical-by-descent status of the affected sib-pairs was assessed using allele-sharing analysis on nuclear families with the SIBPAIR program of the ANALYZE program[34].

Acknowledgements

We thank members of Finnish FCHL families participating in the EUFAM study. We thank S. Koskela, L. Pahlama and A. Heine for sample collection, E. Kempas, M. Sipilä, and M. Parkkonen for excellent laboratory technical assistance, P. Kovanen for the LDL-receptor status determinations and K. Kainulainen for her assistance with this work. The study was supported by grants from the Academy of Finland, the University of Helsinki, the Finnish Heart Foundation and the Emil Aaltonen's Foundation. J.D.T. had a Hitchings-Elion Fellowship from the Burroughs-Wellcome Fund. This study is a part of the EUFAM study supported by the European Commission, contract number BMH4-CT96-1678.

Received 23 September, 1997; accepted 4 February, 1998.

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