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# Decreased cellular cholesterol efflux is a common cause of familial hypoalphalipoproteinemia: role of the ABCA1 gene mutations

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**Abstract**

*Background*. High density lipoproteins (HDL) are complex lipoprotein particles involved in reverse cholesterol (C) transport and are negatively associated with the risk for coronary artery disease (CAD). We have described a disorder of familial HDL deficiency (FHD) due to abnormal cellular cholesterol efflux. In the present study, we investigated cellular cholesterol efflux on skin fibroblast from 15 probands with moderate to severe hypoalphalipoproteinemia, including one subject with Tangier disease (TD). We performed family studies on eight of these probands (269 individuals) with familial hypoalphalipoproteinemia (defined as a HDL-C $<$ 5th%, and with no known cause of HDL deficiency). We have previously shown that four of our FHD patients and patients with TD have mutations at the ABC1 gene, demonstrating that FHD is a heterozygous form of TD. *Methods*. On each subject, we carried out detailed biochemical analysis and determined apoA-I-mediated cellular cholesterol efflux using $^{3}$H-cholesterol labeled skin fibroblasts from study subjects compared with controls. TD has also been associated with abnormal cellular cholesterol efflux. Cell fusion experiments with polyethylene glycol (PEG) were carried out with fibroblasts from a subject with TD and one with FHD in order to determine whether the Tangier cells can complement the FHD defect. In all subjects with a reduced cellular cholesterol efflux, exons of the ABCA1 gene were sequenced. Results. Familial forms of HDL deficiency, defined as HDL-C levels $<$ 5th percentile, are a heterogeneous group of lipoprotein disorders. A reduced cellular cholesterol efflux has been identified in eight subjects from seven kindred (7/14 or 50% of probands tested), being reduced by a mean 59% of controls (range 49–63%). In four of these subjects, a mutation at the ABCA1 gene locus was identified. In three other subjects an efflux defect was idenfified but no critical mutation at the ABCA1 gene locus has been identified. In the remaining subjects, (7/14), no efflux defect was identified. Complementation studies reveal that the FHD defect is not corrected by Tangier cells, confirming that FHD and TD represent a spectrum of the same genetic defect. *Conclusion*. Familial hypoalphalipoproteinemia syndromes are phenotypically heterogeneous; one form is associated with abnormal cellular cholesterol efflux caused by heterozygous mutations at the ABCA1 gene, that defines familial HDL Deficiency while homozygous mutations or compound heterozygocity causes TD. Other forms are primary hypoalphalipoproteinemia of unknown cause, while the remaining cases are associated with hypertriglyceridemia with or without elevated apoB levels. We conclude that a cellular cholesterol defect is a relatively frequent cause of familial HDL deficiency and that a mutation at the ABCA1 gene can be identified in half of these patients. 

*Keywords:* High density lipoproteins (HDL); Familial HDL deficiency; Cholesterol

## 1. Introduction

High density lipoproteins (HDL) play a major role in the lipoprotein transport system and in vascular function [1–3]. The main role of HDL, is thought to be the transport of cholesterol (C) to the liver (the reverse

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cholesterol transport system). In this system, HDL mobilizes peripheral cholesterol (including from arterial tissues) and transports it for subsequent elimination by the liver (as bile salts) and kidneys or uptake by other tissues (steroid hormone producing). This concept is strengthened by epidemiological studies that have shown a strong and graded inverse relationship between plasma levels of HDL-C and the presence and development of coronary artery disease (CAD) [4–8]. Isolated cases of HDL deficiency however, have not been consistently associated with premature CAD [3]. Several primary forms of reduced HDL-C levels are not associated with CAD as seen for instance with apoA-$I_{Milano}$ [9,10]. In most cases of mild to moderate hypoalphalipoproteinemia encountered clinically, a low HDL-C is associated with multiple metabolic abnormalities, including abdominal obesity [11], elevated triglycerides [7,12], elevated plasma apoB levels [13], hyperglycemia and insulin resistance that form part of the 'metabolic syndrome'. Tangier disease (TD) [41] and familial HDL deficiency (FHD) [15] are characterized by a marked deficiency of plasma levels of HDL-C and apoA-I. Both have been shown to result from hypercatabolism of plasma apoA-I by metabolic kinetic studies [16,17]. Studies performed on fibroblasts from TD and FHD patients have shown a marked decrease in cellular cholesterol transport and efflux [18–20]. This results in cholesterol-depleted apoA-I containing lipoproteins that are rapidly catabolized. The genetic defect in Tangier disease and in FHD has been recently shown to be caused by mutations at the ATP binding cassette (ABCA1)-1 gene. Tangier disease subjects have homozygous or compound heterozygous mutations at the ABCA1 gene while FHD subjects are heterozygous for these mutations at this locus [22–24]. We have recently reported that mutations at the ABCA1 gene locus are the cause of familial HDL deficiency [25]. In the present study, we have examined 15 probands with familial hypoalphalipoproteinemia (including one subject with classical TD). We report that a defect in cellular cholesterol efflux is a frequent cause of moderate to severe hypoalphalipoproteinemia and that mutations at the ABCA1 gene underlie most cases of FHD.

## 2. Methods

### 2.1. Patient selection

Study subjects were selected from the Cardiology Clinic of the Clinical Research Institute of Montréal. The main criterion was a HDL-C level $<$ 5th percentile for age and gender, with a plasma concentration of triglycerides $<$ 95th percentile [21] in the proband and at least one first-degree relative with the same lipid abnormality. In addition the patients did not have diabetes. The apoA-I level was determined by nephelometry and its molecular weight verified by polyacrylamide gradient gel electrophoresis (PAGGE); the possibility of an abnormal form of apoA-I causing a change in electrophoeretic mobility was further excluded by isoelectrofocusing (IEF) [15]. Upon fulfilling these criteria, the family of the proband was screened and a skin biopsy was taken in the proband and other affected kindred members for fibroblast culture. Subjects in whom a family study was not performed but who had a primary form of severe HDL deficiency were also recruited. We defined a low HDL-C ($<$ 5th percentile for age and gender-matched subjects) as hypoalphalipoproteinemia, if at least one first degree relative exhibited the same anomaly, we defined this entity as familial hypoalphalipoproteinemia. Familial combined hyperlipidemia and familial hypertriglyceridemia, with hypoalphalipoproteinemia have been previously defined [26]. We based the definition of familial HDL deficiency (FHD) on an abnormal cellular cholesterol efflux performed on skin fibroblasts ($<$ 60% of control values). Tangier disease is defined on the basis of very low HDL-C levels, and evidence of lymphoid tissue infiltration by cholesteryl ester laden macrophages. The protocol has been reviewed and accepted by the Ethics Committee of the Clinical Research Institute of Montréal. All subjects signed separate informed consent forms for plasma sampling and storage, DNA isolation and storage, and skin biopsies. For comparison purposes, we included experiments performed on cells from control subjects, FHD probands and one patient with TD previously reported [19].

### 2.2. Family studies

Family members were contacted by a research nurse after having previously been contacted by the proband. After obtaining informed consent, blood was withdrawn in EDTA-containing tubes for plasma lipid, lipoprotein cholesterol, and apolipoprotein analyses, as well as for storage at $-80$°C. Leukocytes were isolated from the buffy coat for DNA extraction. Plasma levels of apoA-I and B were measured by ELISA as previously described [15,21], and the apoE phenotype was determined by IEF. The family studies were performed in accordance with the guidelines issued by the Ethics Committee of the Clinical Research Institute of Montréal. Fibroblasts from the TD subject were a kind gift from Dr. John Kastelein and are described elsewhere [19].

Lipid and apolipoprotein measurement was performed on fresh plasma as described elsewhere [27]. The laboratory participates and fulfills the criteria of the Center for Disease Control (Atlanta, GA) lipid standardization program for precision and accuracy. Lipoprotein cholesterol and triglyceride levels were determined in total plasma, plasma at density $d < 1.006$ g/ml obtained after preparative ultracentrifugation, be-

fore, and after precipitation with dextran manganese [15]. Apolipoprotein measurement was performed by nephelometry for apoB and apoA-I.

### 2.3. Cell culture

Skin fibroblast cultures were established from 3.0 mm punch biopsies of the forearm of FHD patients and healthy control subjects. Primary cultures were grown in Dulbecco's modified eagle medium (DMEM), supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), 0.1% non-essential amino acids and 20% fetal bovine serum (FBS, all Gibco-BRL) and maintained at 37°C in a humidified incubator (with 5% $CO_2$) in 25 $cm^2$ flasks. After subsequent passages, cells were incubated with DMEM containing 10% FBS (DMEM-FBS) in 75 $cm^2$ stock flasks for 5–15 passages. After the cells were cultured under defined experimental conditions; trypsin (0.05%) in 0.53 mM. EDTA-4Na (Gibco BRL) was used to separate the cells from the flask. Depending on the experiments, $5 \times 10^4$ or $5 \times 10^5$ cells were seeded in 35 mm petri dishes containing 2 ml of DMEM-FBS. Fibroblasts from 23 subjects were used for these experiments: five normolipidemic controls, one subject with TD (cell line TDI as previously described [19]), and 16 subjects with hypoalphalipoproteinemia (Table 1), including one subject with very low HDL-C level but with associated mild hypertriglyceridemia, mild hyperglycemia and elevated apoB level (24292-301), and three subjects from the same kindred (24430-301, 309 and 313). A fourth subject from kindred 24430, subject 24430-307, is normolipidemic and was also included in the analysis.

### 2.4. Cellular cholesterol labeling and loading

The protocol for cellular cholesterol efflux experiments was described in detail elsewhere [28]. The cells were $^3$H-cholesterol labeled during growth and free cholesterol loaded in growth arrest. We seeded $5 \times 10^4$ cells in 35 mm cell culture dishes; at $\approx 50\%$ confluence, 0.2 μCi/ml $^3$H-cholesterol was added. When the cells reached confluence, they were washed in PBS containing 1 mg/ml BSA and the medium was replaced by DMEM without serum (growth arrest) containing 2 mg/ml BSA and 20 μg/ml free cholesterol for 24 h. Cellular cholesterol pools were allowed to equilibrate for another 24 h in DMEM containing 1 mg /ml BSA and efflux studies (0–24 h) were then carried out using 10 μg/ml purified apoA-I. We used apoA-I rather than $HDL_3$ because differences in cholesterol efflux between control and FHD cells were more pronounced with apoA-I [21,28].

Table 1
Characteristics of the probands[a]

| | # FAM | AGE | SEX | CHD | TCHOL | TRIG | HDL-C | VLDL-C | LDL-C | DIAG | Cholesterol efflux[b] |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Controls ($n=5$) | | 33.8 | 3M+2F | 0 | 4.60 | 4.73 | 1.37 | 0.55 | 2.68 | Normal. | Normal |
| TD[c] | 1 | 44 | M | | 2.30 | 1.96 | <0.1 | 0.81 | 1.39 | TD | Abnormal |
| 24430-301[c] | 37 | 48 | M | 1 | 3.68 | 1.48 | 0.18 | 0.46 | 3.04 | FHD | Abnormal |
| 27359-301 | 21 | 54 | M | 1 | 4.78 | 4.89 | 0.39 | 1.83 | 1.14 | FHD | Abnormal |
| 28127-301 | 25 | 52 | M | 0 | 6.40 | 2.22 | 0.18 | 1.00 | 5.20 | FHD | Abnormal |
| 24723-301 | 16 | 30 | F | 0 | 4.61 | 2.61 | 0.27 | 1.09 | 3.25 | FHD | Abnormal |
| 24430-309 | 37 | 54 | M | 0 | 6.46 | 3.93 | 0.50 | 1.80 | 4.44 | FHD | Abnormal |
| 24430-313 | 37 | 39 | M | 0 | 3.53 | 2.65 | 0.13 | 1.19 | 2.21 | FHD | Abnormal |
| 28214-301 | 1 | 49 | M | 0 | 4.51 | 1.49 | 0.53 | 0.55 | 3.43 | HA | Equivocal |
| 28037-301 | 1 | 41 | M | 0 | 4.28 | 1.64 | 0.68 | 0.75 | 2.85 | TgHA | Equivocal |
| 24842-301 | 23 | 53 | M | 1 | 6.02 | 3.63 | 0.38 | 1.77 | 3.87 | HAB | Equivocal |
| 27587-301 | 38 | 56 | M | 0 | 2.43 | 2.29 | 0.37 | 1.04 | 1 03 | FHA | Normal |
| 24430-307 | 37 | 53 | M | 0 | 4.30 | 1.10 | 1.02 | 0.62 | 2.66 | Normal | Normal |
| 27413-301 | 1 | 51 | M | 0 | 4.68 | 2.76 | 0.65 | 1.26 | 2.76 | HA | Normal |
| 24292-301 | 71 | 42 | M | 1 | 4.72 | 1.85 | 0.70 | 0.84 | 3.18 | FHA | Normal |
| 27730-301 | 1 | 40 | M | 1 | 5.85 | 2.04 | 0.28 | 0.93 | 4.68 | HA | Normal |
| 27558-301 | 1 | 62 | F | 1 | 3.83 | 2.18 | 0.34 | 1.05 | 2.44 | HA | Normal |
| 23855-301 | 1 | 45 | M | 1 | 3.50 | 1.12 | 0.68 | 0.51 | 2.31 | HA | Normal |

[a] CHD refers to coronary heart disease, TCHOL to total cholesterol, TRIG to triglyceride levels, expressed in mmol/L, as are levels of HDL-C, VLDL-C and LDL-C; # FAM refers to the number of subjects examined in each family. DIAG refers to the lipoprotein phenotype identified in the proband. FHD: familial HDL deficiency; TgHA: hypertriglyceridermia with low HDL; TD: Tangier disease; HAB: hyperapob; HA: hypoalphalipoproteinemia.

[b] $^3$H-cholesterol efflux at 24 h.

[c] Subjects used in the cell fusion studies.

### 2.5. Preparation of $HDL_3$ and apoA-I

$HDL_3$ was freshly prepared from a plasma pool of normolipidemic donors. Lipoproteins were isolated by standard sequential ultracentrifugation with density ($d$) adjusted with KBr ($HDL_3$ $d = 1.125–1.210$ g/ml). The preparation was extensively dialyzed in PBS (NaCl, 138 mM; KCl, 2.7 mM; NaOH, 51.7 mM; $KH_2PO_4$, 0.575 mM; EDTA, 0.385 mM; pH 7.4) and stored at 4°C for up to 1 month. Protein concentration was determined by the method of Lowry. ApoA-I was isolated by gel permeation chromatography as described [15] after isolation from whole blood of total HDL particles by ultracentrifugation. The HDL preparation was delipidated in acetone:ethanol (1:1) and diethyl ether. HDL proteins were then evaporated to dryness under a stream of $N_2$ and resuspended in 50 mM glycine, 4 mM NaOH, 0.5 M NaCl and 6 M urea (pH 8.8) at the concentration of 20–30 mg/ml. HDL proteins were fractionated at 4°C on two Sephacryl S-200 (Pharmacia) column (2.6 × 100 cm) equilibrated and eluted with the same buffer (45 ml/h). Fractions containing the apoA-1 peak were extensively dialyzed in 0.01 M $NH_4HCO_3$, then lyophilized and resuspended in PBS at concentration of 1 mg/ml. Protein purity on each apoA-I fraction was assayed on PAGGE and appropriate fractions pooled, dialyzed in PBS and Iyophilized before being stored at $-70$°C.

### 2.6. Cholesterol efflux studies

Efflux studies were carried out from 0 to 24 h in the presence of purified apoA-I (10 μg protein/ml medium). Efflux was determined as percent of $^3H$ cholesterol in the medium after the cells were incubated for specified periods of time. All experiments were performed in triplicate, in the presence of cells from one control subject and the cells from the study subjects to be examined; some experiments included cells from one subject with TD. All results were confirmed at least twice. We performed experiments on the kinetics of cholesterol efflux in the presence of apoA-I at times 0, 4, 6, 12 and 24 h (data not shown). The most significant difference in efflux was observed at the 24-h time point. Because of the biological variability in efflux in control subjects, we considered a $>40\%$ reduction from the mean of all controls (set at 100%) as a significant reduced efflux.

### 2.7. Cell fusion studies

The $^3H$-cholesterol labeled cells were treated with trypsin (0.05% trypsin, 0.53 mM EDTA-4Na) to detach cells from the flasks. The cells were counted and 200 000 cells of each cell type (400 000 cells in total) were plated together in 35 mm Petri dishes and grown overnight until confluence in 2 ml DMEM-FBS. The cells were washed once with PBS and then incubated for exactly 57 s with 0.7 ml of a 40% polyethylene glycol 1000 (J.T. Baker) solution in PBS. After extensive washes with freshly prepared DMEM, the cells were incubated in 1 mL of DMEM-FBS and this medium was changed after 1 h. The loading with free cholesterol (20 μg/ml) was performed 8 h after the cell fusion. The efflux protocol was then followed as described above. All fusion experiments were carried out at times 0 and 24 h. As a control, all experiments were carried out in the absence or the presence of PEG treated cells. As a control, we also included a fusion of FHD with FHD and TD with TD cells. Cellular proteins were determined in order to ascertain that cell loss did not occur when incubated in the presence of PEG. However, as PEG is highly cytotoxic, a certain amount of cell loss is inevitable. In such experiments, a large percentage of cells will not become fused; furthermore, cells from one kind could fuse with other cells of the same kind. Thus, the percentage of TD + FHD fused cells was expected to be relatively small. In the TD + FHD cells without PEG, the cholesterol efflux is expected to be the mean level of efflux seen with the FHD cells and TD cells individually. In the presence of PEG, this TD + FHD fusion would be expected to increase efflux above this level, if the cells complement each other. However, a lack of increase in cellular cholesterol efflux would suggest that complementation did not occur.

### 2.8. Sequencing of the ABCA1 gene

All the exons of the ABCA1 gene were sequenced as previously described [22,25].

## 3. Results

The clinical and biochemical characteristics of the probands are listed in Table 1. We studied 14 probands with unexplained hypoalphalipoproteinemia, one patient with classical TD (previously described in reference [19]) and completed families on eight kindred. In one kindred (24430), we report data on the affected proband (301), two affected brothers (309 and 313) and one normolipidemic, non-affected brother (307) (Fig. 1). Thus, we present data on 15 separate probands and three additional family members from one kindred. Subjects were selected on the basis of an unexplained decrease in HDL-C the absence of diabetes and severe hypertriglyceridemia, a normal pattern of migration of apoA-I on IEF and the lack of clinical features seen in TD or LCAT deficiency [15]; specifically, clinical examination of the probands did not reveal corneal opacities, the presence of enlarged, orange tonsils,

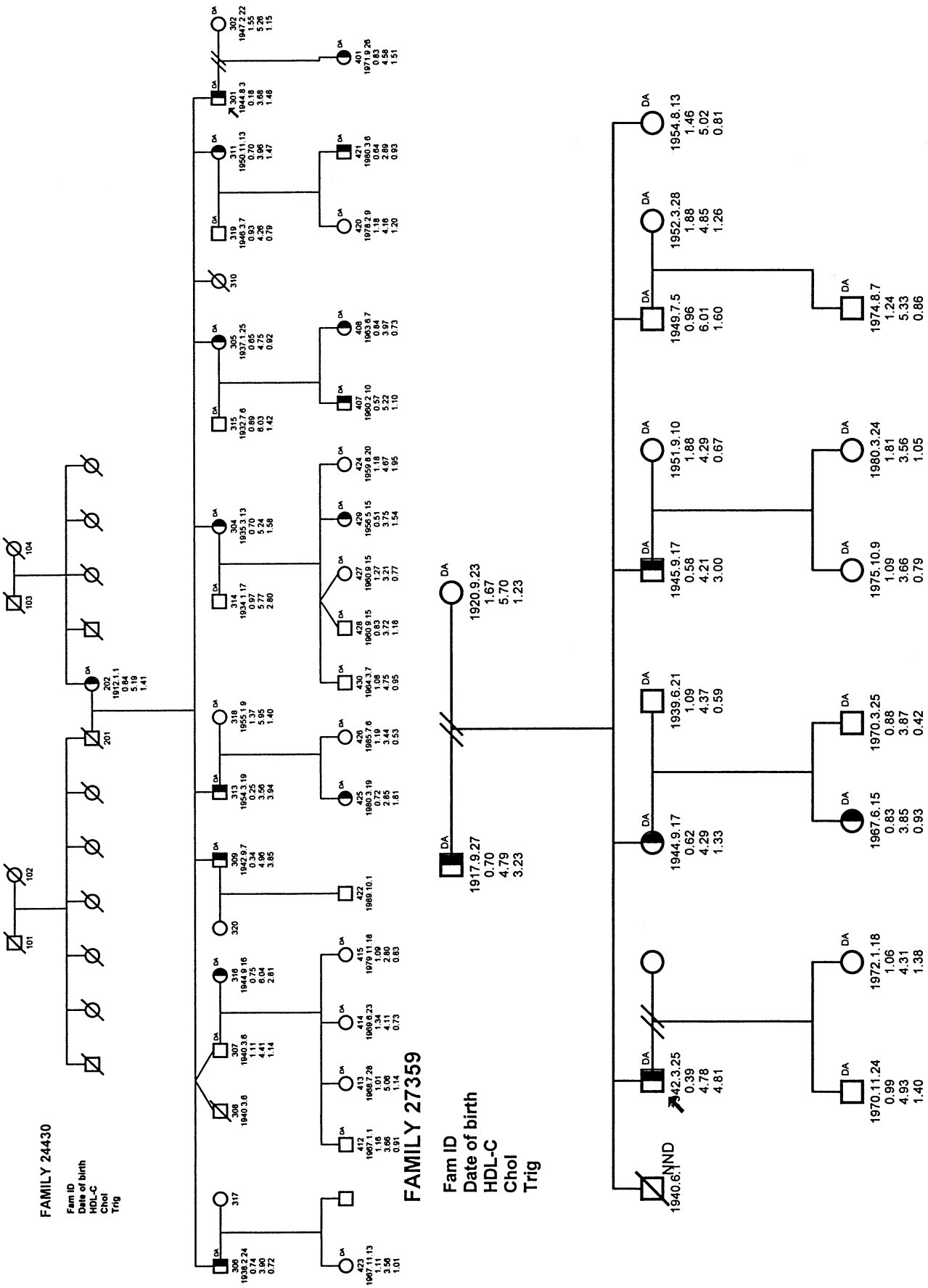


Fig. 1. The four kindred with FHD are shown in panels A–D. The proband is referred to as 301. HDL-C levels are shown below each subject, followed by total cholesterol and triglyceride levels (all in mmol/l). Affected individuals are identified.

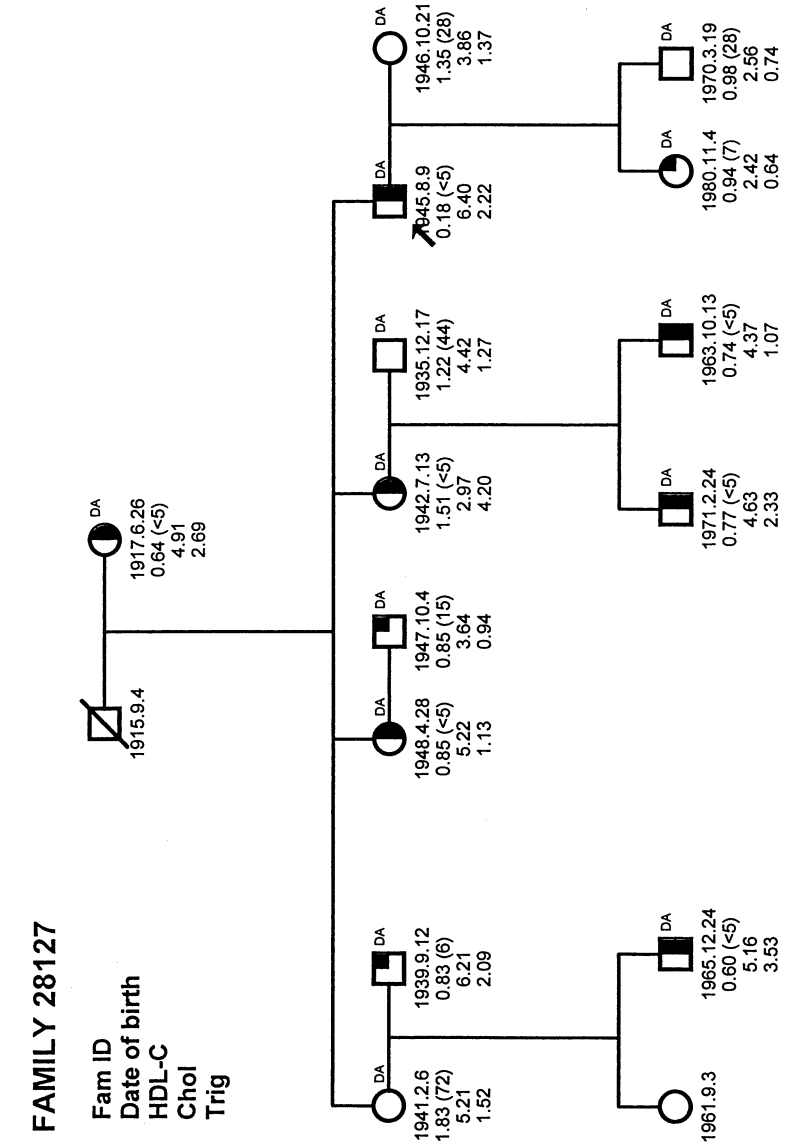


Fig. 1. (*Continued*)

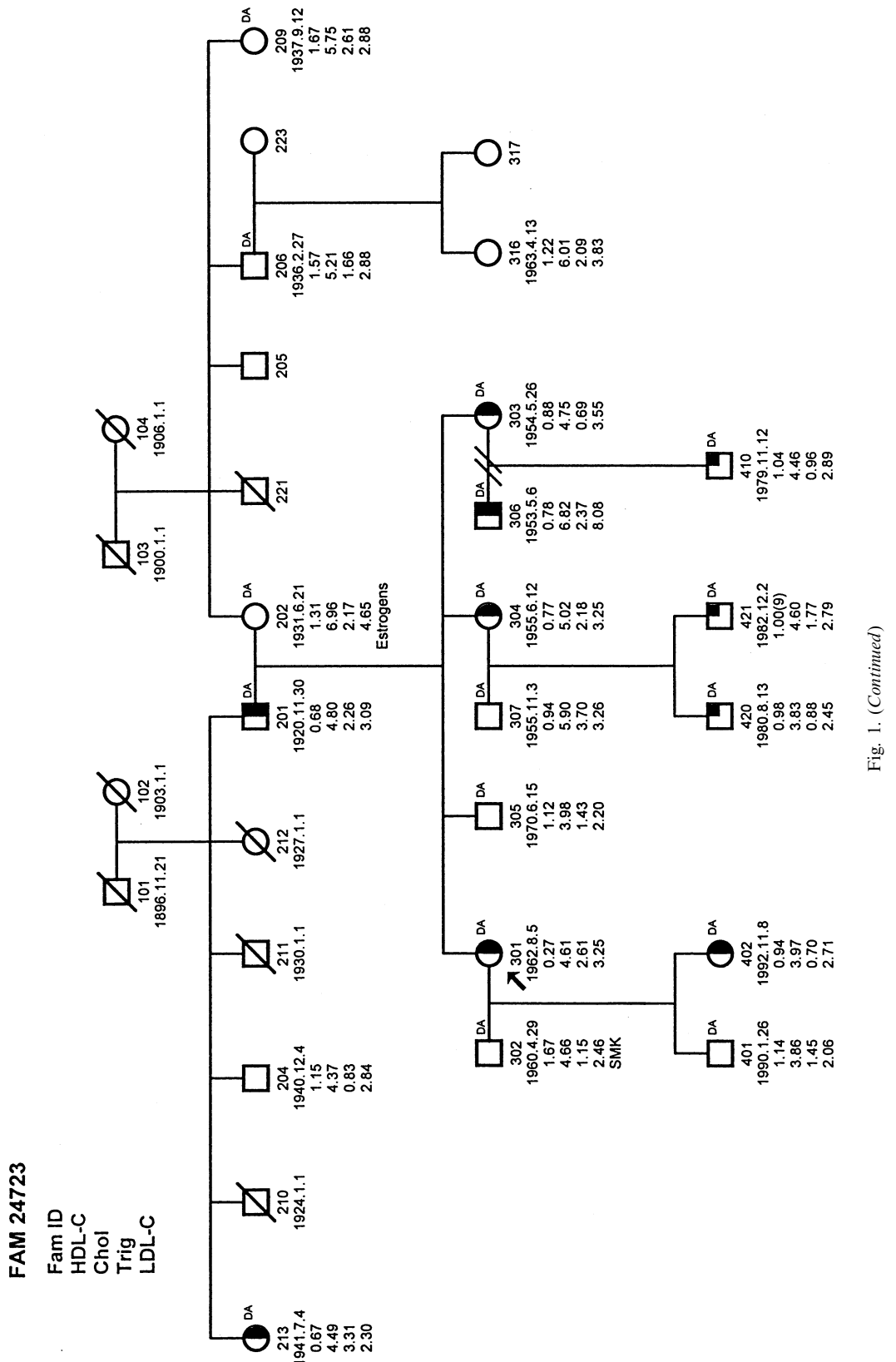


Fig. 1. (*Continued*)

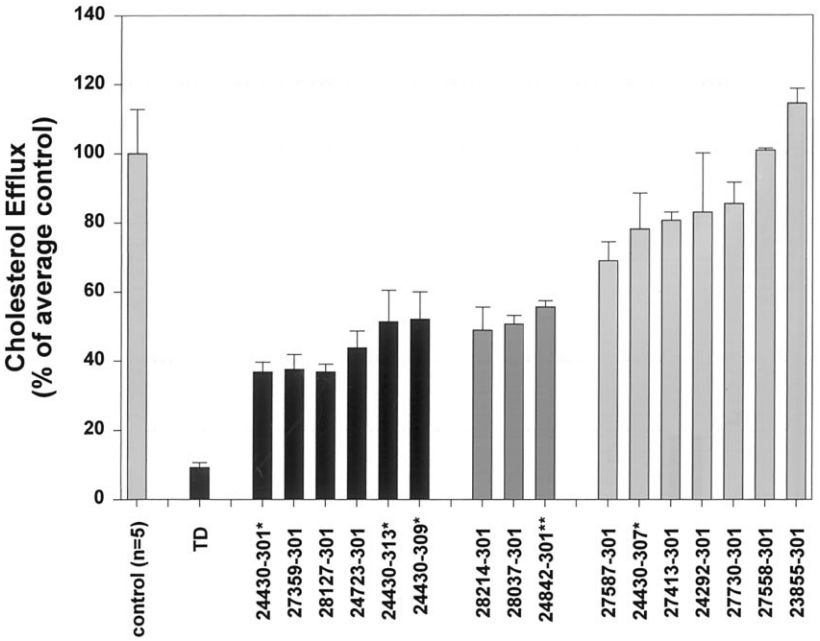


Fig. 2. Cellular cholesterol efflux in control subjects ($n = 5$, normalized to 100%) one subject with TD, six subjects with FHD, three subjects with equivocal results (the lack of family data does not allow unambiguous assignment to FHD), seven subjects with unexplained hypoalphalipoproteinemia and one subject (24430-307) who is the normolipidemic brother of affected sibs 24430-301, 24430-309 and 24430-313 (from Table 1). Cholesterol efflux was measured at 24 h, normalized to a percentage of controls. In a typical experiment, the level of cholesterol efflux in control subjects at 24-h was 8–12% of total cholesterol (medium plus cell $^3$H-cholesterol); that of FHD cells was 4–6% and TD cells 2% of total cholesterol.

hepatomegaly or enlarged spleen or evidence of a peripheral demyelinating neuropathy. Colonoscopy was not performed on any subject to test for the presence of hystiocytic infiltration of the mucosa, a finding considered by some to be pathognomonic of TD [14]. We have previously reported that plasma triglyceride levels were mildly to moderately elevated in FHD; therefore only patients with triglyceride $< 95$th percentile were included. The mean plasma lipid and lipoprotein cholesterol values for the study subjects are shown in Table 1. Reference values for HDL-C were taken from the Lipid Research Clinical data tables [29]. As previously noted, many cases of reduced HDL-C are secondary to elevated triglycerides and (or) increased apoB-containing lipoprotein secretion by the liver. Cellular cholesterol efflux was examined on fibroblasts obtained from each patient. We have previously reported a decrease in cholesterol efflux in cells from subjects 24430-301 and his brother 24430-313, and subject 24723-301 [28]. These results were confirmed in the present study and three new probands and one additional family member were identified: subjects 27359-301, 28127-301 24842-301, and 24330-309 were also found to have decreased cellular cholesterol efflux (Fig. 2 and Table 1). Thus, 7/14 (50%) of the probands studied had a reduced cellular cholesterol efflux defect. Mutations in these affected subjects have been previously reported [25]: subject 24430-301 had a C6370T mutation, leading to a nonsense mutation Arg2084STOP; patient 24723-301 had a Δ2017-19 deletion, leading to a ΔLeu631 deletion; patient 28127-301 had a C2665T mutation, leading to a nonsense mutation Arg849STOP and patient 27359-301 had a 6 bp deletion Δ5618-23, leading to a ΔGlu1833 and ΔAsp 1834 deletion. The patient with TD was heterozygote for two mutations: a T4369C (Cys1417Arg) mutation and a mutation leading to a truncated mRNA species [22]. In the remaining seven probands, no cholesterol efflux defect was identified. These cases, therefore, are still considered to be FHD but possibly caused by a different genetic disorder than mutations at the ABCA1 gene locus. In the other cases, the patients were considered to have primary hypoalphalipoproteinemia (HA) or, on the basis of family studies, as having familial hypoalphalipoproteinemia (FHA) of yet unknown causes. In other families, a pattern of familial hypertriglyceridemia with hypoalphalipoproteinemia (FT-gHA) was identified, as we previously reported [13]. We pooled the results from five control subjects and arbitrarily set this value at 100%. Because of variability in the assay, we consider values that fall within 75–125% of controls as normal cholesterol efflux.

We have defined familial HDL deficiency on the basis of abnormal cellular cholesterol efflux. Three additional subjects, labeled 28214-301, 28037-301 and 24842-301 had a cellular cholesterol efflux approximately 50% of control values. We did not identify a cellular cholesterol efflux defect in the other patients.

We had previously classified subject 24842-301 as having familial combined hyperlipidemial hyperapoB on the basis of elevated cholesterol, triglyceride and apoB levels [15]. However, on repeated testing, cells from this patient had a lesser degree of defective efflux and the cholesteryl ester mobilization was reduced, compared with normal [15]. Using DNA markers spanning the ABC1-CERP genomic region, we were unable to show co-segregation of the low HDL-C trait with the chromosome 9q31 region in this subject. Extensive haplotype analysis of the region at 9q31 in these families fail to show segregation with the low HDL-C trait, although family 28037 is limited in size and, in family 28214, no other subject had and HDL-C $<$ 5th percentile.

There is a significant correlation ($r = 0.545$, $P = 0.029$) between the HDL-C level and cholesterol efflux in our 17 (14 probands plus three additional family members) subjects tested (the TD subject and controls were excluded from this analysis). This suggests that $\sim 30\%$ of the variability in HDL-C levels in our subjects with low HDL-C may be due to cellular cholesterol efflux.

We performed family studies in eight kindred. Three of these families have been reported elsewhere [22–25]. In six subjects from four kindred (indicated in Table 1), a cellular cholesterol efflux defect was identified. The pedigree of the four kindred with a cholesterol efflux defect are shown in Fig. 1a–d. The most consistent pattern of inheritance is Mendelian autosomal co-dominant, homozygous FHD subjects have TD. Subjects with heterozygous TD disease have been reported as being clinically normal but present a low half-normal HDL-C compared with age-matched controls [30].

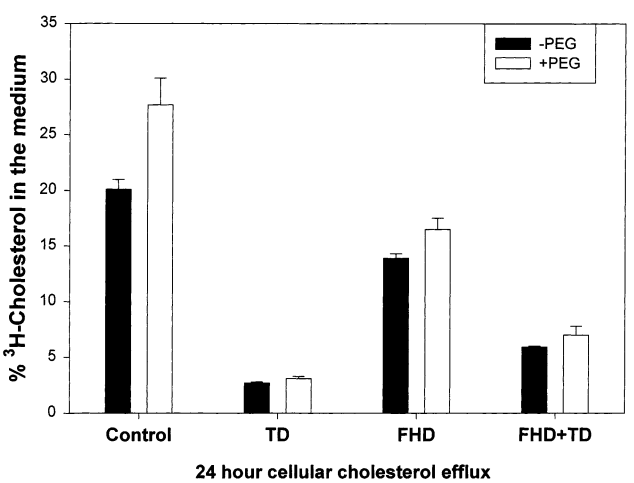


Fig. 3. Cell fusion results. Efflux was determined on cells in the presence (open bars) and in the absence (closed bars) of PEG. As a control, FHD and TD cells were fused with themselves. There was a slight increase in efflux in controls in the PEG fused cells. The FHD + TD efflux in the absence of PEG was approximately halfway between TD and FHD cells alone. The TD + FHD fusion did not result in increased efflux. This experiment was performed four times, with similar results.

We next investigated whether these familial forms of decreased HDL-C were associated with atherosclerotic vascular disease. In 8/18 subjects, there was clinically evident CAD) (as evidenced by strict angiographic criteria, myocardial infarction, or myocardial revascularization) [27]. The association of the low HDL-C trait with CAD must, however, be interpreted with caution. Because of the referral bias of the clinic, many patients have pre-established cardiovascular disease [27]. The prevalence of a reduced HDL-C level in our patients with premature CAD is 50% [27,31] according to the National Cholesterol Education Program (NCEP) criteria (HDL-C $<$ 0.9 mmol/l). The presence of vascular disease in the family was established through direct interview of all family members. The presence of vascular disease in the coronary, aorto-iliac and carotid vascular beds was sought on clinical grounds. In subjects with FHD, no segregation with the presence of a low HDL-C and vascular disease was identified [15].

Cell fusion studies were carried out on fibroblasts from a control subject, a subject with FHD and a subject with TD. We determined the optimal time for PEG fusion to be exactly 57 s; longer incubation times resulted in increased cell death due to the toxicity of PEG, as documented by cell protein content, and shorter incubation times resulted in a lack of fusion (data not shown). Approximately 40% of the cells were found to be diploid (or more) by microscopic examination and flow cytometric analysis of the cells. After staining for DNA, cells revealed a marked increase in polyploidy after PEG fusion. Cellular protein mass after PEG incubation and extensive washes was not significantly different than when the cells were incubated without PEG. The results of one cell fusion experiment are shown in Fig. 3. Results from four separate fusion experiments yielded similar findings. As a control for PEG fusion, cellular cholesterol efflux is not significantly affected by the presence of PEG in control, FHD or TD cells. The TD + FHD fused cells showed a 24-h efflux level intermediate between that observed in individual FHD and TD cells. The fused TD + FHD cells did not exhibit increased efflux, suggesting that TD cells do not complement the genetic defect in FHD. On the basis of these results, we confirm that FHD and TD represent different manifestations of the same genetic defect.

## 4. Discussion

In the present study, we examined 14 probands (and three family members, 17 subjects) with a marked reduction in plasma HDL-C levels. In two of these subjects, a syndrome of familial combined

hyperlipidemia/hyperapoB was identified. In 7/14 (50%) probands (from four kindred), a cellular cholesterol efflux defect was identified, an entity referred to as FHD. We have previously postulated that a reduced cholesterol efflux onto apoA-I containing nascent particles (either pre-β migrating or small $HDL_3$ particles) would lead to small HDL particles that are rapidly catabolized. This was documented in two subjects with FHD from the same family (24430-301 and 24430-313, Table 1) in whom stable isotope kinetic studies were performed. In these subjects, apoA-I catabolism was increased 4–5-fold compared to control subjects [17]. Similar findings have been reported by Emmerich et al. [32] in a subject with unexplained low HDL-C levels. This supports the concept that an intracellular cholesterol transport and efflux defect can lead to hypercatabolism of HDL particles. However, in half of the cases examined (7/14, of which five have a primary form of hypoalphalipoproteinemia and two had a combination of elevated apoB and increased triglyceride levels), no efflux defect was identified with our protocol. These subjects have a primary form of HDL deficiency that remains unexplained.

Patients exhibiting moderate to severe HDL-C deficiency comprise therefore a heterogeneous group. The search for a genetic etiology in such patients has been thwarted by a lack of precise phenotypes. The identification of subjects with FHD (defined here as a defect in cellular cholesterol transport and efflux) has allowed a molecular diagnosis, with mutations at the ABCA1 gene having been identified, segregating with the low HDL trait [22,25].

Investigations of fibroblasts obtained from subjects with Tangier disease have shown that these cells have a profound defect in apoA-I or $HDL_3$-mediated cellular cholesterol efflux [18–20]. Similarly, one report on subjects with severe HDL deficiency but without clinical evidence of TD has also shown a defect in cellular cholesterol efflux [33]. We have reported similar findings on our patients.

The results obtained with the cell fusion experiment confirm that FHD and TD represent different manifestations of a similar genetic defect. Tangier disease results from a homozygous (or compound heterozygous) genetic defect at the ABCA1 gene locus while FHD (defined as a defect in cellular cholesterol efflux) is caused by heterozygous mutations at the ABCA1 gene. Because FHD is transmitted as an autosomal co-dominant trait, the phenotypic defect in FHD is less severe than TD, but far more frequent. Cell fusion experiments suffer severe limitations in interpretation. First, only a percentage of cells are fused with PEG; second, there are no cell markers that allow distinction between fused cells (i.e. fusion between FHD and TD cells) and third, cell fusion methodology is best applied in recessive disorders.

Tangier disease is considered a very rare lipoprotein disorder [14,30]. But a defect in cellular cholesterol transport and efflux is identified in half of the patients we have examined in the present study. It is possible, therefore, that anomalies in cellular processing of cholesterol represent a hitherto unrecognized etiology for moderately severe hypoalphalipoproteinemia. The TD/FHD cellular phenotype therefore might be a relatively common cause of low HDL-C states.

The treatment of subjects with FHD presents a clinical challenge. Reports from Serfaty–Lacrossnière on one subject with Tangier disease show that these patients show little or no response to conventional treatments (despite an increase in apoA-I levels by a factor of two, the clinical significance of raising apoA-I from 2 to 4 mg% is doubtful) [30]. Subject 24430-301 has been treated sequentially for three-month periods with lovastatin 20 mg/d, niacin 3000 mg/d and fenofibrate 300 mg/d with little effect on HDL-C levels (data not shown). Although anecdotal, these results suggest that the cellular defect in TD/FHD might be difficult to overcome with currently available lipid modulating agents.

Cholesterol efflux from the cell follows two processes: first, diffusion of membrane cholesterol onto an accepting particle, and second, the active transport of intracellular cholesterol to the plasma membrane. Efflux of cholesterol from the membrane appears to be mediated by passive desorption of cholesterol from the cell membrane to apoA-I-phospholipid complexes, following a concentration gradient of free cholesterol and acceptor particles [34–36]. The shape, composition and conformation of HDL particles may also influence cholesterol efflux [37,38]. Intracellular cholesterol translocation to the plasma membrane may be mediated through apoA-I-cell interaction via yet uncharacterized specific membrane proteins, the nature of which continue to stir controversy [39]. Subsequent work has suggested that a HDL receptor may act as a signal-transducing protein, which promotes intracellular cholesterol transport to the cell membrane. In this model, the binding of HDL stimulates the activation and movement to the plasma membrane of protein kinase C (PKC), most likely via the phosphoinositol pathway [40]. The PKC isoforms identified in fibroblasts are PKC α, δ, ε and ζ (the $\beta_1$, $\beta_2$, γ and η are not seen in fibroblasts or expressed in very small quantities) [41,42]. $HDL_3$ binding to cells stimulates the phosphorylation of several proteins, the roles of which are not fully known but include ERK-1 and ERK-2, members of the mitogen-activated protein kinases (MAPK) [42,43]. The current concept is that this mechanism translocates cellular cholesterol from various pools to the cell membrane where it will be available for efflux by desorption. $HDL_3$ induces the hydrolysis of phosphatidylcholine by phospholipases C and D. In cells from subjects with TD, this $HDL_3$ and

apoA-I-mediated activity of phospholipase C and D (as assessed by the formation of DAG and phosphatidic acid) was found to be markedly impaired [44]. A similar pattern of abnormal phospholipase C and D activation in TD cells can be reproduced by incubation with pertussis toxin, suggesting the involvement of a G protein in TD [44]. Cellular cholesterol content is regulated by: a) cholesterol synthesis in the smooth endoplasmic reticulum (via HMG CoA reductase); b) receptor-mediated endocytosis of LDL or selective free cholesterol uptake from LDL; c) cholesterol efflux from plasma membrane to cholesterol acceptor particles (predominantly HDL), and d) intracellular cholesterol esterification via the enzyme acyl-CoA:cholesteryl acyltransferase (ACAT) [34,45]. The first two pathways are coordinately regulated at the level of gene transcription. Cells obtain cholesterol from two predominant sources: the *de novo* synthesis involving the HMG CoA reductase pathway or, preferentially, the endocytosis of LDL particles [46]. Surprisingly, relatively little is known about the intracellular trafficking of cholesterol and its transport to the plasma membrane from the various pools of origin [34]. There is accumulating evidence that the trans-Golgi network is a common pathway for transport of cellular cholesterol to the plasma membrane [47].

In the study by Rogler et al. in fibroblasts from a patient with Tangier disease, the efflux defect was reversible by the selective activation of PKC with 1,2-dioctanoylglycerol (DOG) ($10^{-5}$ mol/l) [20]. This strongly suggests that translocation of cellular cholesterol is mediated, at least in part, by a PKC-mediated pathway and can be overcome by activation of the signal transduction pathway.

In conclusion, moderately severe HDL-C states are frequently caused by a defect in cellular cholesterol efflux. Half such patients identified, including those with Tangier disease, have mutations at the ABCA1 gene [22–25]. It remains to be determined whether the cholesterol efflux pathway can be modulated by hormonal mediators or from selective activation of the signaling transduction pathway.

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