# Polymorphisms at the apoB, apoA-I, and cholesteryl ester transfer protein gene loci in patients with gallbladder disease

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**Abstract** Alterations in lipoprotein levels are reported to be related to an increased risk of gallstones. Plasma lipid metabolism is regulated by a number of proteins that are polymorphic in the population. The present research was designed to investigate the association between the polymorphisms of these proteins and the presence of various gallbladder diseases. Restriction fragment length polymorphisms (RFLPs) of apolipoprotein B (XbaI, EcoRI), apolipoprotein A-I (PstI, MspI), and cholesteryl ester transfer protein (CETP) (EcoNI, TaqIA, TaqIB) genes were examined in a series of 210 cholecystectomy patients operated on for symptomatic gallbladder disease and in 92 healthy controls. The patients were categorized into four groups according to the type of gallstones and the presence or absence of cholesterolosis. The distribution of CETP TaqIB polymorphism in the patients with cholesterol gallstones differed significantly from that in the controls, with the B1B1 jects (39.7%) ($P$ = 0.036). The patients with both cholesterol and non-cholesterol stones had lower high density lipoprotein (HDL)-cholesterol levels than the control subjects. However, the most distinct difference was found in the gallstone patients with the B2B2 genotype ($P$ = 0.006). The frequency of the X1X1 genotype of the apolipoprotein B XbaI polymorphism was markedly higher in the patients with acalculous cholesterolosis (48.9%) or cholesterolosis with stones (58.1%) than in the gallstone patients with cholesterol stones (27.2%) or with non-cholesterol stones (34.1%) ($P$ = 0.002). The present data suggest that CETP gene polymorphism may be associated with cholesterol gallstone disease, probably in combination with some additional factor that reduces the plasma HDL cholesterol concentration, especially in TaqIB B2B2 genotype. Changes in the apolipoprotein B structure possibly associated with the XbaI polymorphism may be involved in the accumulation of neutral lipids during the development of cholesterolosis of the gallbladder.—**Juvonen, T., M. J. Savolainen, M. I. Kairaluoma, L. H. J. Lajunen, S. E. Humphries, and Y. A. Kesäniemi.** Polymorphisms at the apoB, apoA-I, and cholesteryl ester transfer protein gene loci in patients with gallbladder disease. *J. Lipid Res.* 1995. **36:** 804–812.

**Supplementary key words** gallstones • cholesterolosis • lipids • lipoproteins • cholesterol • genetics

A number of epidemiological surveys have shown an association between altered plasma lipoprotein levels and gallstone disease, especially decreased levels of plasma high density lipoprotein (HDL) cholesterol (1, 2), while increased levels of low density lipoprotein (LDL) cholesterol (3) and triglycerides (4) are related to an enhanced risk of gallstones. Plasma lipid and lipoprotein metabolism is controlled by the activity of a number of enzymes and by the regulation of the levels and function of the apolipoproteins that are the structural components of the lipoproteins (5). Apolipoprotein B (apoB) is the sole apolipoprotein of LDL and binds to specific receptors at the surface of liver cells (6). The activity of the hepatic LDL-apoB receptors is a major determinant of hepatic cholesterol uptake and also plasma cholesterol levels (7, 8). Apolipoprotein A-I (apoA-I) is an important structural protein of HDL particles (9), while cholesteryl ester transfer protein (CETP) transfers cholesteryl esters between lipoproteins (10, 11). Recent research has shown that plasma CETP activity plays an important role in the regulation of plasma HDL concentration, and CETP may also participate in the transport of cholesteryl esters from HDL to the liver (11, 12).

Numerous investigations have documented extensive genetic polymorphism in some of these important proteins of lipid metabolism. The XbaI restriction fragment length polymorphism (RFLP) of the apoB gene, for example, is associated with differences in plasma LDL cholesterol levels (13), and the TaqIB polymorphism of the

Abbreviations: apoA-I, apolipoprotein A-I; apoB, apolipoprotein B; apoE, apolipoprotein E; CETP, cholesteryl ester transfer protein; HDL, high density lipoprotein; LDL, low density lipoprotein; RFLP, restriction fragment length polymorphism; VLDL, very low density lipoprotein.

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CETP gene with plasma HDL cholesterol concentrations (12, 14). We have recently shown that the polymorphism of apolipoprotein E (apoE) strongly affects gallstone cholesterol content and the nucleation activity of the gallbladder bile in cholecystectomy patients (15). It is therefore conceivable that genetic variation among other proteins of lipid metabolism could influence gallstone formation.

The present case-control cohort study was designed to investigate the association between polymorphisms of proteins involved in lipid metabolism and gallstone disease. Specifically, we investigated the genotype distribution and allele frequencies of the restriction fragment length polymorphisms (RFLPs) of the apoA-I, apoB, and CETP genes in patients with various gallbladder diseases.

## PATIENTS AND METHODS

### Patients

The series comprises 37 patients operated on for acalculous cholesterolosis in Oulu University Hospital during the period 1985–1989 and 193 consecutive cholecystectomy patients operated on for symptomatic gallbladder disease in the same hospital between August 1989 and February 1990. Of these 193 patients, 169 had gallstone disease, with the remaining 24 undergoing cholecystectomy as treatment for acalculous gallbladder disease. Of this latter group, 16 had neither gallstones nor cholesterolosis, while DNA extraction failed in 4 out of the 169 gallstone cases. All these were excluded from the eventual series. All the stones were collected after cholecystectomy, dried in a desiccator, and stored at room temperature for analysis. Biopsy specimens obtained from the gallbladder wall of each patient were carefully evaluated routinely by staff pathologists. The histological diagnosis of cholesterolosis was based on the finding of foam cells in the lamina propria (16). The patients were divided into four categories (Table 1): 8 out of 193 patients with acalculous cholesterolosis together with 37 operated on for acalculous, histologically confirmed cholesterolosis in the same hospital during the period 1985–1989, formed an acalculous cholesterolosis group (Group 1, n = 45); 31 patients in the present consecutive series with a histological finding of cholesterolosis and having cholesterol stones in the gallbladder (Group 2, n = 31); patients with a gallstone cholesterol content less than 50% and no evidence of cholesterolosis, formed a non-cholesterol stone group (Group 3, n = 41); and patients with a gallstone cholesterol content of 50% or more and no evidence of cholesterolosis, formed a cholesterol stone group (Group 4, n = 93).

The control group consisted of 92 subjects from a random population cohort (23 male and 69 female, mean age 55 years, range 40–61 years). These subjects were matched in sex and age distribution with the total gallbladder patient group.

All subjects were interviewed concerning the amount of beer, wine, and strong alcoholic beverages consumed. Special attention was paid to the consumption during the previous 14 days. The mean alcohol intake was calculated and expressed in grams of pure alcohol per day. All subjects were also questioned about their cigarette smoking, past medical history, and medication. Body mass index (BMI) was calculated as body weight (kg)/height$^2$(m$^2$).

All the subjects volunteered for the investigation, which was approved by the Ethical Committee of the University of Oulu.

### Chemical analysis of gallstones

The cholesterol content of the gallstones was determined by a modified infrared spectrometric method (17) as previously reported (15). In short, the whole gallstone was used for the analysis of solitary stones and a pooled sample of 2–12 stones for that of multiple gallstones, depending on the size and number present. Each gallstone sample was pulverized and dried at about 50°C for 3 days. The dry stone powder was then re-ground to a finer grain size in an agate mortar, and aliquots of 10–20 mg were weighed out and dissolved in 1.0 ml $CCl_4$. A series of standards were prepared by dissolving known amounts of cholesterol (cholest-5-en-3$\beta$-ol; Merck AG) in 1.0 ml $CCl_4$, and calibration graphs for cholesterol were evolved using peaks at 3631 1/cm (OH-stretching) and 2940 1/cm ($CH_4$-stretchings). The linear regression coefficients of the calibration graphs were greater than 0.99 for both peaks. The error in the method was calculated to be about 5–10% on the basis of duplicate determinations, equal results being obtained for both peaks. The cholesterol content of the gallstones was expressed as a percentage of dry weight. A Mattson Instruments 6020 Galaxy Series FT-IR spectrometer was used for the measurements.

### Analysis of lipids and lipoproteins

Venous blood samples were taken into EDTA tubes on the morning of the operation day after an overnight fast and the plasma was separated by centrifugation at 800 *g* for 10 min (4°C) within 30 min. Using the method of Lindgren et al. (18), VLDL was isolated by centrifugation of the plasma in a TFT rotor (Kontron Instruments) at 105,000 *g* for 18 h (15°C) and removed by tube slicing. LDL was then precipitated with heparin–manganese according to the Lipid Research Clinics Program (19) to determine HDL cholesterol. Cholesterol and triglyceride concentrations were analyzed enzymatically (20, 21) using a Gilford IMPACT 400E Clinical Chemistry Analyzer.

### Analysis of restriction fragment length polymorphisms

Blood samples were collected in EDTA tubes and stored at −20°C until analyzed. DNA was prepared from these specimens by the salting-out method described by Miller, Dykes, and Polesky (22). Five $\mu$g of DNA was digested with 20 U of restriction enzyme as recommended by the manufacturer (New England Biolabs, Beverly, MA). After electrophoresis on a 0.75% agarose gel, the DNA was transferred to a Hybond-N filter (Amersham, Bucks,

U.K.) as described by Kessling et al. (23), except that instead of the UV irradiation, the filter was baked at 80°C for 2 h. The gene probe was a unique genomic EcoRI fragment of the apoB gene (pAB3.5c) for the XbaI polymorphism and a pBH2.0 probe for the EcoRI polymorphism (24). The CETP 11 probe (25) was used for the EcoNI and TaqI polymorphisms of the cholesteryl ester transfer protein gene. The probes were labeled with [$^{32}$P]dCTP (3000 Ci/mmol; Amersham) using the random primer method (Random Prime Kit, Boehringer, Mannheim, Germany). The hybridization was carried out for 16 to 20 h at 65°C in a Hoefer hybridization chamber (Hoefer Scientific Instruments, San Francisco, CA). The hybridization solutions and washing solutions were those recommended by the manufacturer. Autoradiographs were exposed at −70°C for 1–7 days using Hyperfilm-MP films (Amersham, UK) and intensifying screens. The common allele in the control group was designated as allele 1. For apoB with XbaI the allele resulting in the formation of an 8.5 kb XbaI fragment was designated as X1 and that generating a 3.5 kb fragment X2. Probing EcoRI digests with pBH2.0 showed two hybridizing fragments of 10.5 kb and 12.5 kb (designated as R1 and R2, respectively).

A primer pair (PS1, nucleotides 2238–2258, 5'GAGCG CTCTCGAGGAGTACAC3'; PS2, nucleotides 2956–2976, 5'GACTGGCTTCCACTGCTGTGC3') was used to amplify a 739 base-pair product containing the PstI polymorphic site located 3' to the apoA-I gene. Another pair of primers (MS1, nucleotides 1021–1043, 5'GTGCTCAAA GACAGCGGCAGAGA3'; MS2, nucleotides 1306–1329, 5'CTTGCTACACTTGCAGGCACAATG3') was used for the amplification of a 309 base-pair product containing the MspI polymorphism at the apoA-I gene. DNA amplification was carried out in a final volume of 50 $\mu$l using 0.25 $\mu$g genomic DNA and 20 nmol of each of the primers. The four dNTPs were present at a final concentration of 200 $\mu$mol/l. The reaction buffer was that recommended by the manufacturer. The amplification reaction was started by the addition of 0.5 units of Taq polymerase (Dynazyme, Finnzymes OY, Espoo, Finland). Annealing, extension, and denaturing were carried out at three temperatures using an automatic thermal cycler (Perkin-Elmer Cetus, CT). After a first cycle of 5 min at 95°C, 1 min at 60°C, and 2 min at 72°C, 39 cycles of 1 min at 95°C, 1 min at 60°C, and 1 min at 72°C were carried out for each reaction.

The PCR products were digested at 37°C for 1 h using 25 $\mu$l of each PCR-amplified product with 10 units of the restriction enzyme (New England Biolabs, Inc., Beverly, MA). The digestion products were electrophoresed at 75 V for 1.5 h through a 1.5% agarose gel stained with ethidium bromide and visualized by ultraviolet light.

### Statistical methods

The data storage and statistical analysis was performed with the SAS program (SAS Institute Inc., Cary, NC). The results are given as means ± standard error of mean (SEM). The differences in lipid values among the groups were calculated with ANOVA when more than two groups were present. When two groups were compared, Student's *t*-test was used. As multiple comparisons were performed, probability values need to be interpreted with caution. Therefore, only group differences with $P < 0.01$ were considered as major findings and taken as a basis for discussion. Unadjusted lipid values are presented in the tables. To exclude the effect of covariates on lipid values, the statistical analyses were also performed after adjustment by linear regression for age, sex, BMI, smoking, and alcohol consumption (data in Fig. 1). Differences in genotype frequencies between controls and patients with gallbladder disease were evaluated for statistical significance by the $\chi^2$ test. The Mantel-Haenszel summary odds ratio (with 95% confidence interval) was used to estimate the risk of gallbladder disease in patients with various genotypes.

## RESULTS

### Plasma lipids and lipoproteins

The clinical characteristics and plasma total, LDL, and HDL-cholesterol and triglyceride concentrations for each group are presented in **Table 1.** Plasma LDL-cholesterol levels did not differ among the groups, but HDL-cholesterol levels were significantly lower in the gallstone patients (groups 3 and 4) (1.31 ± 0.03 mmol/l) than in the controls (1.43 ± 0.04 mmol/l); $P = 0.009$. Total cholesterol levels also tended to be lower in the gallstone patients (5.4 ± 0.1 mmol/l) than in the controls (5.7 ± 0.1 mmol/l); $P = 0.05$.

### ApoB gene

The genotype distribution and allele frequencies of the XbaI and EcoRI polymorphisms of the apoB gene are presented in **Table 2.** The distribution of XbaI genotypes in the control group was not significantly different from the expected distribution for a population in Hardy-Weinberg equilibrium. Overall, the distribution of XbaI genotypes in the gallstone patients did not differ significantly from that of the controls. The X1X1 genotype, however, was enriched in the cholesterolosis patients, being 48.9% in those without stones and 58.1% in those with stones compared with 38.0% in the control subjects. Among all the patients with cholesterolosis (both with or without stones in their gallbladder, groups 1 and 2) the distribution of XbaI polymorphism [X1X1 = 40 (52.6%), X1X2 = 27 (35.5%), X2X2 = 9 (11.8%)]

TABLE 1. Clinical characteristics and plasma lipid and lipoprotein concentrations in control subjects and in patients with various gallbladder disease

| Group | 1. Acalculous Cholesterolosis | 2. Cholesterolosis with Stones | 3. Non-Cholesterol Stones | 4. Cholesterol Stones | 5. Controls |
|---|---|---|---|---|---|
| N | 45 | 31 | 41 | 93 | 92 |
| Sex (male/female) | 6/39 | 4/27 | 14/27 | 17/76 | 23/69 |
| Age (years) | 50 ± 2 | 51 ± 3 | 60 ± 2 | 56 ± 1 | 55 ± 1 |
| BMI | 26.2 ± 0.7 | 26.6 ± 0.7 | 25.8 ± 0.8 | 27.7 ± 0.45 | 26.43 ± 0.45 |
| Plasma cholesterol (mmol/l) | 5.61 ± 0.18 | 5.29 ± 0.22 | 5.59 ± 0.20 | 5.32 ± 0.12[a] | 5.71 ± 0.12 |
| LDL cholesterol (mmol/l) | 3.30 ± 0.17 | 2.92 ± 0.17 | 3.01 ± 0.18 | 2.99 ± 0.10 | 3.41 ± 0.25 |
| HDL cholesterol (mmol/l) | 1.41 ± 0.06 | 1.49 ± 0.09 | 1.33 ± 0.06 | 1.30 ± 0.03[b] | 1.43 ± 0.04 |
| Plasma triglycerides (mmol/l) | 1.45 ± 0.13 | 1.40 ± 0.22 | 1.48 ± 0.11 | 1.39 ± 0.07 | 1.30 ± 0.06 |
| Gallstone cholesterol content (% by weight) | | 80 ± 5 (50–100) | 13 ± 3 (0–48) | 89 ± 5 (54–100) | |

Values given as mean ± SEM (range); BMI, body mass index (kg/m²).
[a] $P = 0.02$ for difference between patients (group 4) and controls (group 5).
[b] $P = 0.006$ for difference between patients (group 4) and controls (group 5).

tended to differ from that in the controls [X1X1 = 35 (38.0%), X1X2 = 42 (45.7%), X2X2 = 15 (16.3%)] and differed significantly from that found in the gallstone patients without cholesterolosis [X1X1 = 39 (29.3%), X1X2 = 78 (58.6%), X2X2 = 16 (12.0%)] ($P = 0.002$). The likelihood of cholesterolosis was significantly greater for patients with X1X1 genotype than in those with X1X2 or X2X2 genotype (odds ratio 2.68, 95% confidence interval, 1.49 to 4.81). The allele frequencies and the distribution of the observed genotypes for EcoRI polymorphism did not differ between the patients and controls.

### CETP gene

The patients with cholesterolosis (with or without stones, groups 1 and 2) had a different distribution of TaqIA genotypes [A1A1 = 66 (73.7%), A1A2 = 20 (26.3%), A2A2 = 0 (0%)] from the controls [A1A1 = 82 (89.1%), A1A2 = 7 (7.6%), A2A2 = 3 (3.3%)] ($P = 0.015$, odds ratio 0.341, 95% confidence interval, 0.149 to 0.784) (**Table 3**), with the A1 allele less prevalent. The distribution of the CETP TaqIB polymorphism differed significantly between the patients with cholesterol stones and the controls ($P = 0.012$), but the patients with acalculous cholesterolosis had a distribution similar to the controls. When the cholesterol stone patients in the cholesterolosis group were included in the cholesterol stone group, the difference became more evident [cholesterol stone patients B1B1 = 23 (18.9%), B1B2 = 71 (58.2%), B2B2 = 28 (23.0%) and controls B1B1 = 34 (39.7%), B1B2 = 43 (46.7%), B2B2 = 15 (16.3%) ($P = 0.012$). The likelihood of cholesterol gallstones was significantly smaller for patients with B1B1 genotype than in those with B1B2 or

TABLE 2. Apolipoprotein B gene polymorphism in patients with various gallbladder disease and in the controls

| | Genotypes | | | | | | Allele Frequency | |
|---|---|---|---|---|---|---|---|---|
| | n | % | n | % | n | % | | |
| XbaI | X1X1 | | X1X2 | | X2X2 | | X1 | X2 |
| 1. Acalculous cholesterolosis (n = 45) | 22 | (48.9) | 18 | (40.0) | 5 | (11.1) | 0.689 | 0.311[a] |
| 2. Cholesterolosis with stones (n = 31) | 18 | (58.1) | 9 | (29.0) | 4 | (12.9) | 0.725 | 0.274[a] |
| 3. Non-cholesterol stones (n = 41) | 14 | (34.1) | 21 | (51.2) | 6 | (14.6) | 0.598 | 0.402 |
| 4. Cholesterol stones (n = 92) | 25 | (27.2) | 57 | (62.0) | 10 | (10.9) | 0.582 | 0.418 |
| 5. Controls (n = 92) | 35 | (38.0) | 42 | (45.7) | 15 | (16.3) | 0.609 | 0.391 |
| EcoRI | R1R1 | | R1R2 | | R2R2 | | R1 | R2 |
| 1. Acalculous cholesterolosis (n = 45) | 27 | (60.0) | 15 | (33.3) | 3 | (6.7) | 0.766 | 0.233 |
| 2. Cholesterolosis with stones (n = 31) | 15 | (48.4) | 12 | (38.7) | 4 | (12.9) | 0.677 | 0.323 |
| 3. Non-cholesterol stones (n = 41) | 25 | (61.0) | 14 | (34.1) | 2 | (4.9) | 0.780 | 0.220 |
| 4. Cholesterol stones (n = 92) | 55 | (59.8) | 32 | (34.8) | 5 | (5.4) | 0.772 | 0.228 |
| 5. Controls (n = 92) | 55 | (59.8) | 33 | (35.9) | 4 | (4.3) | 0.777 | 0.223 |

The XbaI and EcoRI genotypes could not be determined unequivocally in one patient.
[a] $P = 0.002$ (chi-square = 12.099) for difference in genotype distribution between cholesterolosis patients (groups 1 and 2) and gallstone patients (groups 3 and 4). $P = 0.009$ (chi-square = 13.415) for difference between cholesterolosis patients (groups 1 and 2), gallstone patients (groups 3 and 4), and controls (group 5).

TABLE 3. Cholesteryl transfer protein (CETP) gene polymorphisms in patients with gallbladder disease and in controls

| | Genotypes | | | | | | Allele Frequency | |
|---|---|---|---|---|---|---|---|---|
| | n | % | n | % | n | % | | |
| TaqIA | A1A1 | | A1A2 | | A2A2 | | A1 | A2 |
| 1. Acalculous cholesterolosis (n = 45) | 34 | (75.6) | 11 | (24.4) | | | 0.878 | 0.122[a] |
| 2. Cholesterolosis with stones (n = 31) | 22 | (71.0) | 9 | (29.0) | | | 0.855 | 0.145[a] |
| 3. Non-cholesterol stones (n = 40) | 34 | (85.0) | 6 | (15.0) | | | 0.925 | 0.075 |
| 4. Cholesterol stones (n = 93) | 74 | (79.6) | 19 | (20.4) | | | 0.898 | 0.102 |
| 5. Controls (n = 92) | 82 | (89.1) | 7 | (7.6) | 3 | (3.3) | 0.929 | 0.071 |
| TaqIB | B1B1 | | B1B2 | | B2B2 | | B1 | B2 |
| 1. Acalculous cholesterolosis (n = 45) | 14 | (31.1) | 21 | (46.7) | 10 | (22.2) | 0.544 | 0.456 |
| 2. Cholesterolosis with stones (n = 31) | 5 | (16.1) | 17 | (54.8) | 9 | (29.0) | 0.435 | 0.565[b] |
| 3. Non-cholesterol stones (n = 39) | 12 | (30.8) | 18 | (46.2) | 9 | (23.1) | 0.538 | 0.462 |
| 4. Cholesterol stones (n = 91) | 18 | (19.8) | 54 | (59.3) | 19 | (20.9) | 0.495 | 0.505[b] |
| 5. Controls (n = 92) | 34 | (39.7) | 43 | (46.7) | 15 | (16.3) | 0.603 | 0.397 |
| EcoNI | N1N1 | | N1N2 | | N2N2 | | N1 | N2 |
| 1. Acalculous cholesterolosis (n = 45) | 10 | (22.2) | 23 | (51.1) | 12 | (26.7) | 0.478 | 0.522 |
| 2. Cholesterolosis with stones (n = 31) | 8 | (26.7) | 17 | (56.7) | 5 | (16.7) | 0.532 | 0.468 |
| 3. Non-cholesterol stones (n = 41) | 9 | (22.0) | 20 | (48.8) | 12 | (29.3) | 0.463 | 0.537 |
| 4. Cholesterol stones (n = 92) | 29 | (31.5) | 47 | (51.1) | 16 | (17.4) | 0.571 | 0.429 |
| 5. Controls (n = 92) | 34 | (37.0) | 43 | (46.7) | 15 | (16.3) | 0.603 | 0.397 |

The TaqIA, TaqIB, and EcoN genotypes could not be determined unequivocally in one, four, and one patient, respectively.

[a] $P = 0.013$ (chi-square = 8.644) for difference in genotype distribution between acalculous cholesterolosis patients (group 1) and controls (group 5); $P = 0.006$ (chi-square = 10.097) for difference between patients with cholesterolosis and stones (group 2) and controls (group 5); $P = 0.002$ (chi-square = 12.750) for difference between patients with cholesterolosis (groups 1 and 2) and controls (group 5).

[b] $P = 0.067$ (chi-square = 5.409) for difference between patients with cholesterolosis and cholesterol stones (group 2) and controls (group 5); $P = 0.036$ (chi-square = 6.636) for difference between patients with cholesterol stones (group 4) and controls (group 5); $P = 0.012$ (chi-square = 8.900) for difference between all patients with cholesterol stones (groups 2 and 4) and controls (group 5).

B2B2 genotype (odds ratio 0.396, 95% confidence interval, 0.215 to 0.730). The distribution of the CETP TaqIB genotypes in the patients with non-cholesterol stones [B1B1 = 12 (30.8%), B1B2 = 18 (46.2%), B2B2 = 9 (23.1%)] did not differ from that in the controls.

A somewhat high frequency of the N2 allele of the CETP EcoNI polymorphism was observed in the patients with acalculous cholesterolosis and non-cholesterol stones, but these differences were not statistically significant.

Plasma HDL cholesterol values varied according to the TaqIB genotype. Those in the patients with acalculous cholesterolosis of different genotypes did not differ from the respective values in the control subjects, but the patients with both cholesterol and non-cholesterol stones had lower HDL cholesterol levels than the control subjects (**Table 4**). The most distinct difference among gallstone patients was in those with the TaqI B2B2 genotype ($P = 0.006$). This difference remained constant also after adjustment for age, sex, BMI, smoking, and alcohol consumption (**Fig. 1**).

### ApoA-I gene

The PstI and MspI polymorphisms of apoA-I gene are presented in **Table 5.** A slightly increased frequency of the P2 allele of apoA-I PstI polymorphism was found in the patients with cholesterolosis and cholesterol stones, but this difference was not statistically significant.

### Cholesterol content of the gallstones

The cholesterol content of the gallstones (n = 164) ranged from 0 to 99.9% (15) and was not related to the apoB, apoA-I, or CETP polymorphisms (data not shown).

## DISCUSSION

The mechanisms by which an altered plasma lipoprotein profile (1, 2, 4) increases the risk of gallbladder disease are largely unknown. We have previously approached this question by determining apoE phenotypes in patients with gallstones (15) and have shown that the cholesterol content of the gallstones is related to apoE polymorphism. The effect of apoE seems to be mediated through differences in the formation of cholesterol crystals, the E4 isoform promoting nucleation compared with the other isoforms (15). The present work extends the search for possible regulatory factors of bile lithogenicity to other proteins involved in lipoprotein metabolism by investigating the occurrence of gallbladder disease in subjects with different genotypes of apolipoprotein gene DNA polymorphism.

TABLE 4. Clinical characteristics and plasma lipid and lipoprotein levels in patients with cholesterol gallstones or non-cholesterol gallstones, and in controls according to TaqIB polymorphism of the CETP gene

| | Controls | | | Non-Cholesterol Gallstones | | | Cholesterol Gallstones | | |
|---|---|---|---|---|---|---|---|---|---|
| TaqIB genotype | B1B1 | B1B2 | B2B2 | B1B1 | B1B2 | B2B2 | B1B1 | B1B2 | B2B2 |
| N | 34 | 43 | 14 | 12 | 18 | 9 | 18 | 54 | 19 |
| Sex (male/female) | 10/24 | 11/32 | 2/12 | 8/4 | 4/14 | 2/7 | 4/14 | 8/46 | 5/14 |
| Age (years) | 54.3 ± 1.3 | 55.1 ± 1.0 | 55.2 ± 1.8 | 56.8 ± 4.1 | 62.5 ± 3.2 | 65.1 ± 3.4 | 57.7 ± 3.1 | 52.6 ± 1.8 | 61.3 ± 3.1 |
| BMI | 26.3 ± 0.7 | 27.1 ± 0.7 | 25.3 ± 0.7 | 26.7 ± 1.1 | 25.2 ± 1.4 | 26.1 ± 1.6 | 29.3 ± 1.2 | 27.4 ± 0.6 | 27.0 ± 0.9 |
| Smokers (%) | 23.4 | 37.2 | 35.7 | 33.3 | 21.4 | 25.0 | 22.2 | 27.8 | 10.5 |
| Alcohol usage (teetotalers/occasional[a]/ moderate or heavy drinkers[b]) | 7/22/5 | 12/39/3 | 2/9/3 | 6/5/1 | 14/2/2 | 6/3/- | 12/5/1 | 29/19/6 | 12/6/1 |
| Plasma cholesterol (mmol/1) | 5.67 ± 0.23 | 5.78 ± 0.16 | 5.65 ± 0.23 | 5.78 ± 0.52 | 5.67 ± 0.23 | 5.39 ± 0.44 | 4.92 ± 0.31 | 5.28 ± 0.13 | 5.75 ± 0.36 |
| LDL cholesterol (mmol/1) | 3.81 ± 0.23 | 3.70 ± 0.13 | 3.44 ± 0.21 | 3.32 ± 0.39 | 2.95 ± 0.26 | 2.90 ± 0.31 | 2.64 ± 0.26 | 3.00 ± 0.10 | 3.19 ± 0.28 |
| HDL cholesterol (mmol/1) | 1.27 ± 0.05 | 1.48 ± 0.13 | 1.66 ± 0.21 | 1.27 ± 0.13 | 1.39 ± 0.13 | 1.24 ± 0.13 | 1.14 ± 0.05 | 1.37 ± 0.05 | 1.27 ± 0.08 |
| Plasma triglycerides (mmol/1) | 1.36 ± 0.14 | 1.28 ± 0.07 | 1.21 ± 0.08 | 1.40 ± 0.23 | 1.57 ± 0.18 | 1.55 ± 0.20 | 1.49 ± 0.15 | 1.34 ± 0.10 | 1.44 ± 0.15 |

[a] Alcohol consumption less than 84 g pure alcohol per week.
[b] Alcohol consumption more than 84 g pure alcohol per week.

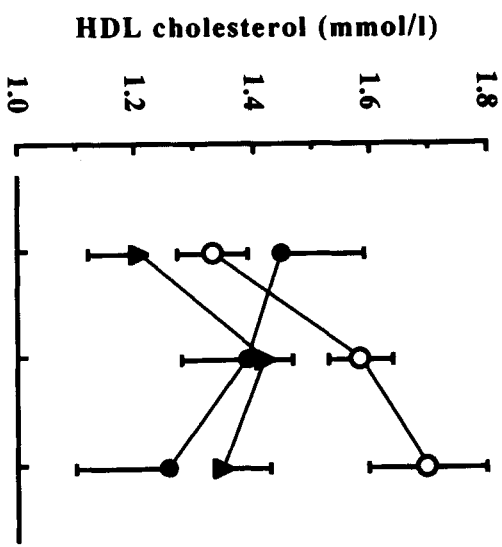


**Fig. 1.** Plasma HDL cholesterol levels in patients with cholesterol stones (▲) (n = 91), non-cholesterol stones (●) (n = 39), and controls (○) (n = 92) according to TaqIB polymorphism of the CETP gene. The data are adjusted for sex, age, BMI, and smoking and are expressed as means ± SEM.

A derangement in cholesterol metabolism may increase the occurrence of gallstones by affecting the cholesterol content and other properties of the bile at several levels. First, an increase in lipoprotein uptake by the liver may enhance the flux of lipoprotein cholesterol secreted into the bile, biliary cholesterol probably originating mainly from free cholesterol in HDL (26). Second, as the sterol content of hepatocytes regulates both LDL uptake and de novo synthesis of cholesterol (27), enhanced flux of LDL cholesterol into the hepatocytes may reduce bile acid synthesis from newly synthesized cholesterol (28). Third, variations in the plasma HDL cholesterol concentration may be associated with variations in the secretion of apolipoprotein A-I into the bile. All these possibilities could be influenced by the genetically determined polymorphisms of CETP, apoB, and apoA-I.

In contrast to our findings on apoE polymorphism (15), the XbaI and EcoRI polymorphisms of apoB were not associated with the differences in the cholesterol content of the gallstones. This argues against a promoter function for apoB in cholesterol crystal nucleation in bile. Moreover, there were no differences in the prevalence of apoB genotypes between the patients with gallstones and the control subjects, which suggests that in spite of the major role of apoB-containing LDL in delivering cholesterol to the hepatocytes (7, 8) and the effects of the differences in apoB structure near the LDL receptor binding site (determined here in terms of the XbaI and EcoRI polymorphisms of the apoB gene) on LDL uptake by the liver (29), apoB polymorphism probably does not markedly alter the

TABLE 5. Apolipoprotein A-I gene polymorphisms in patients and in the controls

| | Genotypes | | | | | | Allele Frequency | |
|---|---|---|---|---|---|---|---|---|
| | n | % | n | % | n | % | | |
| PstI | P1P1 | | P1P2 | | P2P2 | | P1 | P2 |
| 1. Acalculous cholesterolosis (n = 37) | 33 | (89.2) | 4 | (10.8) | | (0) | 0.946 | 0.054 |
| 2. Cholesterolosis with stones (n = 30) | 22 | (73.3) | 8 | (26.7) | | (0) | 0.867 | 0.133 |
| 3. Non-cholesterol stones (n = 34) | 30 | (88.2) | 2 | (5.9) | 2 | (5.9) | 0.912 | 0.088 |
| 4. Cholesterol stones (n = 88) | 72 | (81.8) | 16 | (18.2) | | (0) | 0.909 | 0.091 |
| 5. Controls (n = 92) | 78 | (84.8) | 13 | (14.1) | 1 | (1.1) | 0.918 | 0.082 |
| MspI | M1M1 | | M1M2 | | M2M2 | | M1 | M2 |
| 1. Acalculous cholesterolosis (n = 40) | 33 | (82.5) | 7 | (17.5) | | (0) | 0.912 | 0.088 |
| 2. Cholesterolosis with stones (n = 31) | 27 | (87.1) | 4 | (12.9) | | (0) | 0.935 | 0.065 |
| 3. Non-cholesterol stones (n = 38) | 31 | (81.2) | 6 | (15.8) | 1 | (2.6) | 0.895 | 0.105 |
| 4. Cholesterol stones (n = 89) | 68 | (76.4) | 21 | (23.6) | | (0) | 0.882 | 0.118 |
| 5. Controls (n = 92) | 71 | (77.2) | 18 | (19.6) | 3 | (3.2) | 0.870 | 0.130 |

The PstI and MspI genotypes were not determined in 21 and 11 patients, respectively.

secretion of cholesterol into the bile, or at least it does not have any overall effect on the lithogenicity of the bile.

The low HDL cholesterol concentration reported in gallstone patients (1, 2, 4) led us to search for possible differences in the CETP and apoA-I genes between the patients and the healthy control subjects. Plasma CETP activity is one of the major regulators of plasma HDL cholesterol concentration (30), and the polymorphism of the CETP gene has been reported to be associated with differences in plasma HDL cholesterol levels (12, 14). The present data for the control subjects confirmed this finding, but no association between the TaqIB polymorphism of the CETP gene and HDL cholesterol concentrations could be demonstrated in the gallstone patients, due in part to the lower than usual HDL levels in the patients with genotype B2B2. Whether the low HDL in these patients is secondary to the gallbladder disease or whether it is a genetically determined causative factor in the development of gallstones cannot be determined on the basis of the present results. The low HDL cholesterol level may be due to secondary changes in lipoprotein metabolism caused by the ability of the gallstones themselves to alter the enterohepatic metabolism of cholesterol and bile acids, e.g., by autocholecystectomy (31). This suggestion is supported by the present and previous (4) findings that patients with both cholesterol and non-cholesterol stones have a similar plasma lipoprotein pattern.

Cholesterolosis is a gallbladder wall disease characterized by altered uptake of sterols from the bile, and accumulation of triglycerides and cholesteryl esters in the gallbladder mucosa (32, 33). Cholesteryl esters and triglycerides do not exist in bile but are synthesized in the gallbladder wall (32). In fact, the gallbladder mucosa seems to act somewhat like the intestinal mucosa, in that it absorbs lipids, esterifies sterols, and synthesizes cholesterol and triglycerides (34). Therefore, lipids may be transferred from the gallbladder mucosa by lipoproteins in a manner analogous to the process in the intestinal mucosa. The pathogenesis of cholesterolosis may be linked with this process, and this hypothesis is supported by a recent demonstration of apoB in the gallbladder mucosa (35). It is possible that differences in receptor binding properties between the apoB genotypes could alter the onward transport (from the mucosa to the circulation) of lipoproteins and thus predispose the individual to cholesterolosis. The present results show that XbaI polymorphism of human apoB is related to cholesterolosis of the human gallbladder, with the X1X1 genotype twice as common in the cholesterolosis patients as in the controls, although this polymorphism seems to be independent of the presence of gallstones. Our data thus support previous findings that the pathogenesis of cholesterolosis is unrelated to that of cholesterol stones (33, 36, 37).

The polymorphisms studied here are markers for only a fraction of the possible mutations that may affect the development of gallbladder disease. To our knowledge this is, nevertheless, the first investigation into the mechanisms of gallbladder disease at the DNA level. The DNA approach may be superior to the large array of previously reported lipoprotein studies, because the results are not distorted by confounding factors such as alterations in the enterohepatic cycle secondary to the gallbladder disease or dietary changes due to gallstone symptoms. In addition, the serum levels of many lipoproteins are altered at menopause and with increasing age, so that the lipoprotein profile at the time of symptomatic gallbladder disease may no longer represent the dyslipidemic changes that initially led to its development. As the genes involved remain unchanged throughout life, DNA analysis may be

useful for detecting this type of genetic predisposition. Secondly, DNA tests may be useful if the "quality" of a particular apolipoprotein is more important than its "quantity." This would be the case if single amino acid changes caused a subtle alteration in the function of an apolipoprotein, i.e., its "lithogenic or lithoprotective potential."

We have adopted the "candidate gene approach" to examine the possible involvement of genetic variations in the genes of apoB, apoA-I, and CETP in the alterations in lipoprotein metabolism that may lead to the development of gallbladder disease. As an initial attempt to investigate the possible association of genetic factors with gallbladder disease, seven DNA polymorphisms were studied in cholecystectomy patients. Further investigations using more specific methods such as linkage analysis and sequencing of the genes of patients with gallbladder disease are needed to search for possible mutation(s).

The authors thank Saija Kortetjärvi, Marja-Leena Kytökangas, Sari Liikanen, and Tiina Lapinkari for their skillful technical assistance. This work was supported by grants from the Medical Council of the Academy of Finland, the Finnish Cultural Foundation, the Finnish Foundation for Cardiovascular Research, and the Foundation of Oulu University.

*Manuscript received 7 September 1994 and in revised form 17 November 1994.*

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