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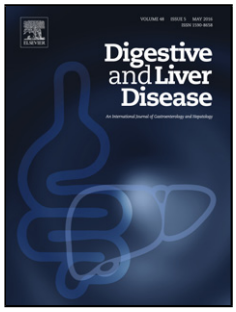

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Genetic polymorphism of sterol transporters in children with future gallstones

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Abstract

Background & aims: Gallstone disease is related to hypersecretion of cholesterol in bile, and low serum phytosterol levels. We examined how genetic polymorphisms of sterol transporters affect childhood cholesterol metabolism trait predicting adult gallstone disease.

Patients and methods: In retrospective controlled study, we determined *D19H* polymorphism of *ABCG8* gene, genetic variation at *Niemann-Pick C1-like 1* (*NPC1L1*) gene locus (rs41279633, rs17655652, rs2072183, rs217434 and rs2073548), and serum cholesterol, noncholesterol sterols and lipids in children affected by gallstones decades later (*n*=66) and controls (*n*=126).

Results: In childhood, phytosterols were lower (9.7%-23.4%) in carriers of risk allele *19H* compared to *19D* homozygotes. Lowest campesterol/cholesterol tertile consisted of 1.9-times more future gallstone subjects, and 3.7-times more *19H* carriers than highest one. Campesterol/cholesterol-ratio was highest in *19D* homozygote controls, but ~11% lower in gallstone *19D* homozygotes and ~25% lower among gallstone and control carriers of *19H*. Gallstone subjects with alleles *CC* of rs41279633 and *TT* of rs217434 of *NPC1L1* had ~18% lower campesterol/cholesterol-ratio compared to mutation carriers.

Conclusions: Risk trait of cholesterol metabolism (low phytosterols) in childhood favouring cholesterol gallstone disease later in adulthood is influenced by risk variant *19H* of *ABCG8* and obviously also other factors. *NPC1L1* variants have minor influence on noncholesterol sterols.

Keywords: Cholesterol; noncholesterol sterols; plant sterols; surrogates of cholesterol absorption

1. Introduction

Adult gallstone disease represents a prevalent and costly health problem. The vast majority - over 90% - of gallstones in adults are cholesterol stones in the Western world [1 - 4]. Although the cholesterol gallstone disease has a multifactorial etiology consisting of genetic and environmental factors, the adults predisposed to the gallstone disease share as a common feature increased biliary output of cholesterol [5-7].

Heterodimeric sterol transporter ABCG5/G8 determines biliary secretion of cholesterol and plant sterols from the hepatocyte into the biliary canaliculus and their efflux out of the enterocyte into the intestinal lumen [8, 9]. Niemann-Pick C1-like 1 (NPC1L1) protein has an essential role in intestinal cholesterol up-take into the enterocytes, and, additionally in humans, in the retention of biliary cholesterol by the hepatocytes [10]. Consequently, the biliary secretion of cholesterol and plant sterols is determined by the balance between their efflux across the hepatocyte canalicular membrane governed by these transporters [11]. The adult gallstone disease has been related to *ABCG8* gene polymorphism *D19H* (rs11887534) in several studies [5, 12]. However, Krawczyk and co-workers suggested that *ABCG8* polymorphism did not fully explain the sterol metabolic trait of the gallstone carriers [5]. Theoretically, decreased expression or function of the hepatic NPC1L1 transporter (*e.g.*, by genetic loss-of-function variants), or its increased expression at the brush border of the enterocytes are both likely to increase biliary cholesterol secretion and the propensity for gallstone formation [13]. Overall, results concerning the relationship between the cholesterol gallstone disease and polymorphism of *NPC1L1* have remained controversial [14, 15]. Interestingly, Lauridsen and co-workers showed in their large population-based study that genetic variation in *NPC1L1* that is associated with lowered cholesterol absorption is also associated with a reduced risk of ischaemic vascular disease, and reduction in serum LDL-cholesterol, but with a concomitant rise in the incidence of gallstone disease [15].

Among the serum noncholesterol sterols, cholestanol (a derivative of endogenous cholesterol), and phytosterols campesterol and sitosterol reflect positively the absorption efficiency of cholesterol [16-20]. Opposite to that, the cholesterol precursor sterols, cholestenol, lathosterol, and desmosterol, mirror whole-body cholesterol synthesis [16, 18, 20], and also the activity of hepatic hydroxy-methyl-glutaryl-CoA reductase [21, 22]. Thus, determination of cholestanol, the cholesterol precursor sterols and phytosterols in serum provides a signature of cholesterol metabolism, which evaluated together with the risk mutations of the genes encoding the major canalicular sterol transporters helps to assess the etiopathogenesis of the gallstone disease. Our previous results indicated that cholesterol metabolism trait characterized by low serum levels of surrogate markers of cholesterol absorption precedes adult gallstone disease already in childhood [7]. Recent data from morbidly obese subjects suggest that low serum plant sterols among patients with gallstones indicate potentially inherited alterations in intestinal absorption and biliary transport of sterols [23].

It has remained unexplored (I) how *D19H* of *ABCG8* and polymorphisms at *NPC1L1* locus modify metabolism of cholesterol in childhood, and (II) to what extent are these genetic polymorphisms related to the childhood cholesterol metabolism trait predicting the adult gallstone disease.

To this end, we performed a retrospective controlled study, in which we evaluated how *D19H* polymorphism of the *ABCG8* gene and genetic variation at the *NPC1L1* gene locus affect metabolism of cholesterol, plant sterols and lipids in a pediatric cohort affected by the gallstone disease decades later in adulthood, and compared the results with the age-, gender- and body mass index (BMI) matched control subjects. The study subjects were participants of the prospective Cardiovascular Risk in Young Finns Study [24-26].

2. Subjects and methods

2.1 Subjects and ethics

The Cardiovascular Risk in Young Finns Study is a multicenter follow-up study of Finnish children and adolescents [24-26]. The first cross-sectional survey was conducted in 1980, when 3596 participants, ages 3, 6, 9, 12, 15 and 18 years, were randomly chosen from the five geographic study areas of Finland through the national population register. At the study inclusion, determination of serum lipids, dietary survey and collection of serum samples was performed. In the present study, the participants were followed up based on the unique personal identification codes through the Care Registers for Social Welfare and Health Care until 2012 for the diagnosis of gallstone disease and regular medications. Gallstone disease was defined as a hospital discharge diagnosis that included ICD-10 codes K80.0-K80.8 as either a primary or secondary code. In Finland, the hospital discharge register includes all hospitals. Both primary and secondary codes were used to ensure capture of all gallstone disease patients. For each case, who developed gallstone disease (n=95) two unaffected controls (n=190) were matched for BMI, age and sex recorded at the start of the follow-up in 1980. For the purposes of the present study, the respective BMI-values in year 1983 were registered. The frozen serum samples of the patients and controls obtained at the first survey in 1980 were retrieved for measurement of cholesterol and non-cholesterol sterols, and the preliminary results of these data without knowledge of the genetic data have been reported earlier [7]. Of these cases and controls, genome-wide genotyping data was available in 66 cases and 126 controls. Data of *NPC1L1* gene variants was missing in one of the gallstone patients. All the analyses were repeated after excluding subjects taking lipid-lowering medications at some point during the follow-up (one for the gallstone and two for the control cohort) with essentially similar results.

Consumption of vegetables (excluding potatoes), fruits and juices, meat meals and fish meals had been recorded in year 1980 and classified: 1 = once a day or more frequently, 2 = almost every day, 3 = twice a week, 4 = once a week, 5 = once or twice a month and 6 = seldom or never.

All subjects volunteered to the study and provided their written informed consent. The study followed up the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the human research committee. The Ethics Committee of the Turku University Central Hospital had accepted the study protocol.

2.2. Biochemical determinations and DNA analysis

Standard enzymatic methods were used for determination of serum total cholesterol, HDL cholesterol and serum triglyceride concentrations, and that of LDL cholesterol was calculated. Details of the methods have been described previously [26].

Serum squalene, cholesterol and non-cholesterol sterols were measured by gas-liquid chromatography (GLC) using a 50-m Ultra 2 capillary column (Agilent Technologies, Wilmington, Del) (for detailed method description see ref. 27). The procedure uses 5α-cholestane as internal standard, and it measures the serum concentrations of squalene, cholesterol, cholestanol, cholestenol, desmosterol, lathosterol, campesterol, sitosterol, stigmasterol and avenasterol, in this order of retention time. The serum levels of major lipoproteins, which transport non-cholesterol sterols, particularly LDL, vary with age. For this reason, the values were calculated as ratios to cholesterol measured in the same GLC run, expressed as 100 x mmol/mol of cholesterol, and given in the text as ratios. Serum cholestenol, lathosterol, and desmosterol ratios are called surrogate or relative synthesis markers, and the ratios of cholestanol, campesterol, sitosterol and avenasterol are called surrogate or relative markers of cholesterol absorption efficiency. Calculation of synthesis marker/absorption marker ratios, *e.g.,* lathosterol/sitosterol reflected whole-body cholesterol metabolism [28].

Five single nucleotide polymorphisms (SNPs) in the *NPC1L1* locus were genotyped in this study because they are related to serum cholesterol levels: -18C>A (rs41279633), -133A>G (rs17655652), 1679C>G or L272L (rs2072183), 27621T>C or V1296V (rs217434) and g-762T>C (rs2073548).

Genotyping of all SNPs was performed using Illumina Human 670K BeadChip. Genotypes were called using the Illumina clustering algorithm [29] and imputation was performed using SHAPEIT v1 [30] and IMPUTE2 [31] software and the 1000G Phase I Integrated Release Version 3 as a reference panel [32]. Two of the candidate SNPs analyzed in this study were directly genotyped (rs17655652 and rs217434), the other four were imputed. Both genotyped SNPs were in Hardy-Weinberg equilibrium (p>0.05). Since the values of imputed genotypes account for the uncertainty of imputation and are not integer values, the key assumptions of traditional Hardy-Weinberg equilibrium test are not met. Instead, we used post-imputation metric "info score" to measure the quality of imputation for these SNPs. For all four SNPs, the info score was > 0.9 indicating high quality imputations. In addition, the allele frequencies for all six SNPs were very similar to those reported for European and Finnish populations by the 1000 Genomes Project [32].

2.3. Statistical analyses

The data were analyzed for significance and normality with the Number Crunching Statistical Software™ (NCSS™, Statistical Solutions Ltd., 2006, Kaysville, Utah). Most of the variables were normally distributed, but logarithmic transformations were performed in skewed distributions. Multiple comparisons among subgroups (gallstone subjects and control subjects with or without variants) were performed with analysis of variance (ANOVA) applying BMI, age, and consumption of vegetables as covariates. If the *P* value of ANOVA was below 0.05, comparisons between two groups (cases *versus* controls and subjects with *versus* without gene variants) were performed using two-sided unpaired *t*-test. Dietary data were analyzed with Kruskall-Wallis one-way ANOVA with

Bonferroni adjustment and Mann-Whitney *U* test. Statistical analysis for non-continuous variables was performed by using Fisher's exact test. The amount of subjects needed for the study was evaluated according to the results of Gylling and co-workers [33], which revealed that serum campesterol/cholesterol ratio is 32.6% lower in the subjects with risk variant *19H* than in the subjects with *19D*. The type I error rate and the power of the study were considered to be 5% and 80%, respectively. Consequently, at least 12 subjects in the subgroup with the risk variant *19H* of *ABCG8* would have been required for the study using a two-sided test. To identify which variables in childhood predicted the risk of development of gallstones in adulthood, a multivariate stepwise linear regression model was performed. The independent variables were gender, BMI in 1980, change in BMI between 1980 – 1983, serum values (in 1980) of LDL- and HDL-cholesterol and triglycerides, the serum ratios to cholesterol of lathosterol and campesterol, and the *ABCG5/8* and *NPC1L1* genotypes. *P*-value <0.05 was considered significant. The results are expressed as mean ± SEM.

3. Results

3.1 Characteristics, food consumption and serum lipids of the subjects evaluated according to D19H variant of ABCG8

The baseline demographics, essential dietary habits and serum lipids of the study subjects (*N*=192) were evaluated in subgroups: gallstone patients (*N*=66) *versus* control subjects (*N*=126), and subjects homozygous for the *19D* allele in the *ABCG8* (*N*=153) *versus* subjects with *19H* allele (*N*=39) (Table 1).

The mean patient age was 11.8 years at inclusion in 1980 (Table 1). Comparison between the subgroups revealed comparable values in demographics (age, sex, weight and height), BMI, and serum and lipoprotein lipids (Table 1). The control subjects had reported to consume more frequently vegetables than the subjects in the gallstone cohort, but other dietary habits were similar between the subgroups in 1980 (Table 1). The mean follow-up time to the diagnosis of gallstone disease was 22.6±0.8 years. At the time of diagnosis of the gallstone disease, the mean age was 33 years (range, 31.0 – 37.9 years).

3.2. Cholesterol, ratios to cholesterol of squalene and non-cholesterol sterols in serum of the subjects evaluated according to D19H variant of ABCG8

The gallstone cohort had ~11% lower (p=0.023) campesterol/cholesterol, but ~12% higher (p=0.036) lathosterol/campesterol than the controls (Table 2).

The cohort with *19H* allele had 9.7% - 23.4% lower surrogate markers of cholesterol absorption (ratios to cholesterol of cholestanol, campesterol, sitosterol, stigmasterol and avenasterol) (p-range

<0.001 – 0.009) than the cohort without the variant. Respectively, lathosterol/campesterol was 36.2% higher (p=0.008) among the *19H* carriers (Table 2).

Relative amounts of subjects carrying the *ABCG8 19H* allele among the gallstone and control cohorts were 22.7% and 19.0%, respectively (p = 0.573) (Table 3). Analysis between the subgroups revealed that cholesterol and ratios to cholesterol of squalene, lathosterol, desmosterol and cholestenol in serum were comparable (Table 3). The gallstone and the control subgroups with the *19H* allele had 10.2% (p=0.038) and 14.7% (p=0.0006) lower cholestanol/cholesterol than the respective subgroups without the *19H* allele (=19D homozygotes) (Table 3).

In the subgroup of gallstone patients with the *19H* allele, ratios to cholesterol of campesterol, sitosterol and avenasterol were 14.7% - 16.8% lower than in the gallstone subgroup without the *19H* allele (p-range 0.007-0.055). Parallel to those, the respective values in the *19H* carrier controls were 18.9% - 27.2% lower than in the subgroups without the *19H* allele (Table 3). In the gallstone subgroup without *19H* allele, campesterol/cholesterol, sitosterol/cholesterol and avenasterol/cholesterol were 11.3% (p=0.062), 11.3% (p=0.034) and 8.7% (p=0.750) lower than respectively in the control subgroup without *19H* allele (Table 3). The lathosterol/campesterol ratio was 14.1% - 30.4% lower in the control subgroup without *19H* allele than in the other groups (p-range: 0.008-0.035) (Table 3).

Subdivision of the study subjects into tertiles (n=64 in each) according to serum campesterol/cholesterol (in 1980) revealed that adult gallstone patients belonged mainly to the lowest (39%, n=26) and the middle tertiles (39%, n=26) (p=0.024 compared to the highest one, n=14). The proportion of the risk allele *19H* carriers was higher in the lowest tertile compared to the middle and the highest campesterol/cholesterol tertile (p-range 0.001 – 0.020, Figure 1).

The frequency of the *ABCG8 19H* carriers did not differ significantly between subjects with future gallstones (non-carriers 77% and carriers 23%) and controls (non-carriers 81% and carriers 19%) (p=0.573).

3.3. Cholesterol, ratios to cholesterol of squalene, and non-cholesterol sterols in serum of the subjects evaluated according to polymorphism of NPC1L1 locus

The analysis of the data according to the genetic variants 1679C>G (rs2072183) and -133A>G (rs17655652) in *NPC1L1* revealed no differences in demographics, in serum cholesterol concentrations or in ratios to cholesterol of squalene and non-cholesterol sterols between the subgroups (patients and controls with or without variants) (data not shown). Evaluation of the data for the variants -18C>A (rs41279633) and V1296V T>C (rs217434) in *NPC1L1* showed that solely campesterol/cholesterol was ~18% lower in the subgroup of future gallstones and with rs41279633 C/C and rs217434 T/T genotype than in the other subgroups (p-range 0.012-0.015) (Figure 2). Evaluation of the data for the variant g-762T>C (rs2073548) in *NPC1L1* revealed ~25% lower lathosterol/campesterol –ratio among the mutation carriers of the gallstone group compared to the controls having T/T genotype (p=0.009).

3.4. Predictors of gallstone disease

In the multivariate stepwise linear regression model with the risk of development of gallstones as the dependent variable (R^2 = 0.060, p=0.01), change in BMI (1980-1983) (β= -0.176, p=0.028) and serum campesterol to cholesterol ratio (β= 0.169, p=0.034) were the only statistically significant independent predictors of the model.

4. Discussion

The present study represents several new findings. (I) Already in childhood serum non-cholesterol sterol surrogate markers of cholesterol absorption were consistently low in the subjects carrying the *ABCG8* risk allele. (II) These markers were low among the children with future gallstone disease, but less consistently than among those having the risk allele. (III) Low relative cholesterol absorption was not exclusively limited to the children with future gallstone disease, but was also present in the healthy controls carrying the *19H* risk allele of *ABCG8*. (IV) Furthermore, serum sitosterol/cholesterol among the children with future gallstones but without the risk allele was ~11% lower than respectively in healthy controls without the risk allele. Altogether, these findings suggest that (V) low relative cholesterol absorption does not solely explain the risk of future gallstone disease in our pediatric gallstone cohort, and the *19H* risk allele does not solely explain the low relative cholesterol absorption markers among the gallstone cohort. This sounds clinically relevant since there are several pathogenic factors having influence on formation of cholesterol gallstones including other genetic factors, gallbladder hypomotility, intestinal factors, hepatic hypersecretion of biliary lipids and accelerated phase transitions of cholesterol [3, 4]. (VI) The variants of *NPC1L1* studied here were not related to the gallstone disease.

Our earlier study showed that the cholesterol metabolism trait characterized by low serum levels of non-cholesterol sterol surrogate markers of cholesterol absorption precede the development of adult gallstone disease by decades and is present already in childhood [7]. Consequently, low serum cholestanol and plant sterol ratios during normal western diet might serve as predictive biomarkers for the gallstone disease [7]. These earlier results further support the view that low absorption and enhanced biliary secretion of cholesterol and plant sterols are the primary changes in the pathogenesis of gallstones [7].

The findings of the present study concerning the role of *ABCG8* risk variant are parallel to those of Krawczyk and co-workers, who showed in their case-control -study that *ABCG8* variants did not fully

explain the sterol metabolic trait of gallstone disease [5]. Furthermore, the results of the stepwise linear regression analysis of the present study indicated that the surrogate of cholesterol absorption (campesterol/cholesterol) and the change in BMI, but not *D19H* of *ABCG8* or polymorphisms at *NPC1L1* locus, predicted the development of gallstones. Results of an earlier study in patients with orthotopic liver transplantation indicated that there are alternative pathways for the hepatobiliary transport of cholesterol, which are not under regulation of *ABCG8* [34]. Further studies are needed to evaluate whether *ABCG8*-independent pathways are essential in regulation biliary secretion of cholesterol and plant sterols also in humans, as recently documented in mice [35], and what the role of these putative pathways is in the gallstone disease.

Whether genetic polymorphism of the NPC1L1 sterol transporter affects the risk of the gallstone disease has remained controversial. In principle, inhibition of this transporter at the level of the hepatocytes (*e.g.*, by loss of function mutations or ezetimibe, an inhibitor of NPC1L1) may lead to enhanced biliary cholesterol secretion, thus, facilitating the formation of gallstones. On the other hand, increased expression or activity of NPC1L1 transporter at the level of the enterocyte leads to enhanced up-take of cholesterol, and, consequently, to elevated levels of cholesterol in serum and bile facilitating the gallstone formation. Wang and colleagues observed in their intervention study that ezetimibe can significantly reduce cholesterol concentrations and values of cholesterol saturation indexes of gallbladder bile in patients with gallstones, mostly attributable to its inhibitory effect on intestinal cholesterol absorption [36]. Five common variants of *NPC1L1* (*i.e.*, rs17655652, rs41279633, rs2072183, rs217434 and rs2073548) have been linked to changes in cholesterol metabolism in humans [37-41]. A population based study revealed that genetic variation in *NPC1L1* associated with decreased levels of plasma LDL cholesterol protects against ischaemic heart disease, but increases the risk of symptomatic gallstone disease [15]. A controlled Chinese cohort study supported the view that the variant rs2072183 of *NPC1L1* might be a positive marker for the risk of gallstone formation [41]. New findings of the present study were that the above mentioned variants

of *NPC1L1* have minor influence on serum non-cholesterol sterols in childhood. Furthermore, these five variants of *NPC1L1* were distributed in equal amounts in the subgroups of the present study. Our results suggested that children, who develop gallstone disease in the future and without carriers of mutated alleles rs41279633 and rs217434 had low serum campesterol/cholesterol levels, but the clinical relevance of this finding remains unclear.

One of the limitations of this study was that the pigment stones were not identified from gallstones. A careful analysis of the composition of gallstone sterols in 165 consecutively cholecystectomized adult patients revealed that only 9% of adult gallstones are pigment stones [1]. On this basis, it can quite safely be assumed that exclusion of subjects having pigment stones from the present study population would not have changed the results of the study. The second limitation is that the possible symptoms caused by the gallstones were not recorded. However, this study was focused on resolving the etiopathogenesis of incoming gallstone disease in adulthood from the point of view of cholesterol metabolism early in childhood, and, thus, it can be considered that the clinical picture of symptoms is of secondary importance in this study setting. The third limitation is the relatively low number of the study subjects, which prevents sex specific sub-analysis and combination analysis of genetic variants in *ABCG8* and *NPC1L1*. The subjects studied here had reached middle-age at the end of the follow-up. Some of the study participants, also in the control cohort, may obtain the gallstone disease later in life. However, the present study setting allows to reveal the crucial issues in cholesterol metabolism in this context.

We can conclude that the risk allele *19H* of *ABCG8* is clearly related to low surrogate sterol markers of cholesterol absorption already in childhood. Our results confirm the earlier findings [7] indicating that individuals, who develop gallstone disease in adulthood are characterized by low serum surrogates of cholesterol absorption decades earlier already in childhood. In addition, our new results support the view that there are also other factors behind this cholesterol gallstone facilitating trait of

cholesterol metabolism than just the risk allele *19H*. The variants of *NPC1L1* studied here had minor influence on serum noncholesterol sterols and cholesterol metabolism.

I confirm that there are no conflicts of interest in the manuscript "**Genetic polymorphism of sterol transporters in children with future gallstones. The Cardiovascular Risk in Young Finns Study**" by investigators Markku J. Nissinen, Niina Pitkänen, Piia Simonen, Helena Gylling, Jorma Viikari, Olli Raitakari, Terho Lehtimäki, Markus Juonala and Mikko P. Pakarinen.

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Markku Nissinen

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Figure legends

Figure 1. Distributions of *D19H* allele genotypes W (=*19D* allele) and w (=risk allele *19H*) of ABCG8 in serum campesterol/cholesterol tertiles of the whole study population (*n*=192).

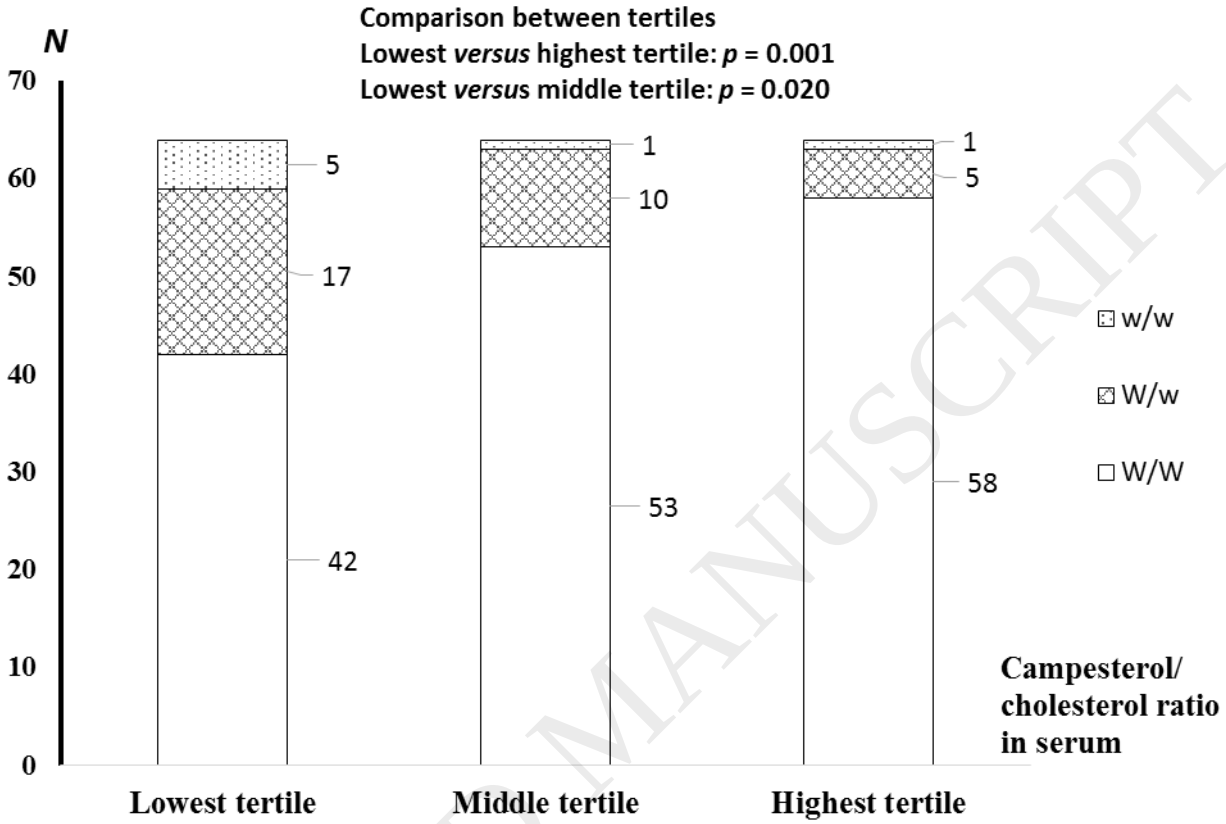

Figure 2. Evaluation of the data according to the variants -18C>A (rs41279633) (A) and V1296V T>C (rs217434) (B) of *NPC1L1* showed that campesterol/cholesterol ratio in serum was ~18% lower in the groups of the gallstone patients homozygous for C (rs41279633) (WW) and homozygous for T (rs217434) (WW) than in the other subgroups (*p*-range 0.012-0.015). Ww/ww: heterozygous or homozygous carriers of the allele A of rs41279633 or allele C of rs217434. GS: group of future gallstone subjects.

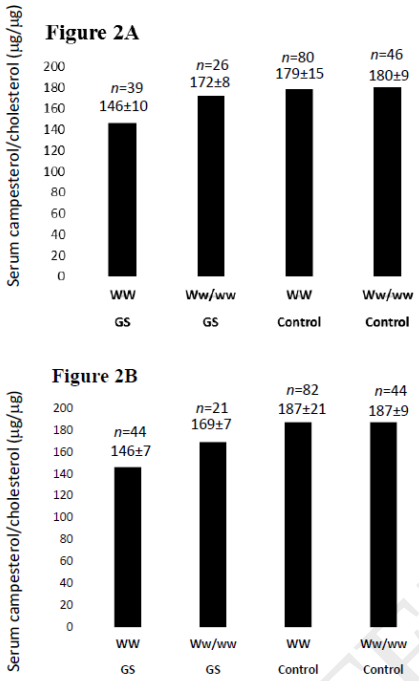

Table 1. Demographics, food consumption and lipids and lipoprotein lipids in serum of study subjects subdivided into gallstone and control cohorts, and according to variants of *D19H* of *ABCG8* gene in year 1980.

Variables	Gallstone	Controls	*P*	*19H*	*19D*	*P*
				carriers	homozygotes	
N	*n*= 66	*n*= 126		*n=39*	*n*= 153	
Age (years)	11.8±0.6	11.4±0.4	*0.540*	11.2±0.7	11.6±0.4	*0.627*
Sex (F/M)	50/16	104/22	*0.340*	32/7	122/31	*0.087*
Weight (kg)	44.6±2.3	42.0±1.6	*0.367*	42.9±1.5	42.9±2.9	*0.962*
Height (cm)	148±3	145±2	*0.307*	146±2	146±3	*0.910*
BMI(kg/m^2)	19.2±0.4	19.0±0.3	*0.726*	19.0±0.6	19.1±0.3	*0.883*
BMI(kg/m^2)†	20.8±0.5	19.8±0.4	*0.104*	20.6±0.7	20.0±0.3	*0.499*
Vegetables††	2.5±0.1	2.0±0.1	*0.002*	2.1±0.2	2.1±0.1	*0.881*
Fruits and						
juices††	2.0±0.1	1.9±0.1	*0.469*	1.8±0.2	1.9±0.1	*0.550*
Meat meals††	2.6±0.1	2.6±0.1	*0.925*	2.5±0.1	2.6±0.1	*0.455*
Fish meals††	4.4±0.1	4.3±0.1	*0.293*	4.4±0.1	4.3±0.1	*0.848*
Cholesterol	5.2±0.1	5.3±0.1	*0.171*	5.2±0.1	5.3±0.1	*0.401*
LDL-cholesterol	3.3±0.1	3.4±0.1	*0.372*	3.2±0.1	3.4±0.1	*0.101*
HDL-cholesterol	1.6±0.0	1.6±0.0	*0.851*	1.6±0.0	1.5±0.0	*0.146*
Triglycerides	0.7±0.0	0.7±0.0	*0.987*	0.8±0.1	0.7±0.0	*0.334*

Values are mean ± SEM. Lipids and lipoprotein lipids are mmol/l. †in year1983, ††Consumption: 1 = once a day or more frequently, 2 = almost every day, 3 = twice a week, 4 = once a week, 5 = once or twice a month and 6 = seldom or never.

BMI: body mass index

Table 2. Cholesterol and ratios to cholesterol of squalene and noncholesterol sterols in serum of study subjects (*n*=192) subdivided into gallstone and control cohorts, and according to variants of *D19H* of *ABCG8* gene in 1980.

Variables	Gallstone	Controls	*P*	*19H*	*19D*	*P*
				carriers	homozygotes	
	n= 66	*n*= 126		*n=39*	*n*= 153	
Cholesterol	183.9±3.8	182.8±3.4	*0.423*	178.6±4.5	184.3±3.0	*0.483*
Cholestanol	142.2±3.2	146.8±2.6	*0.411*	129.5±3.5	149.2±2.3	*<0.001*
Cholestenol	9.1±0.4	9.0±0.3	*0.264*	9.5±0.6	8.9±0.3	*0.195*
Desmosterol	74.9±1.4	75.0±1.2	*0.818*	76.7±2.1	74.5±1.0	*0.717*
Lathosterol	88.8±3.7	84.3±3.3	*0.162*	90.6±5.7	84.6±2.8	*0.344*
Campesterol	158.0±8.4	175.0±5.8	*0.023*	137.2±8.1	177.4±5.4	*<0.001*
Sitosterol	105.1±5.5	116.4±4.2	*0.717*	90.5±5.5	118.1±3.8	*<0.001*
Avenasterol	29.2±1.0	32.1±1.0	*0.153*	26.3±1.4	32.3±0.8	*<0.001*
Stigmasterol	17.5±0.6	17.1±0.4	*0.733*	15.9±0.7	17.6±0.4	*0.009*
Squalene	8.3±0.6	8.8±0.5	*0.592*	7.4±0.4	8.9±0.5	*0.184*
Lathosterol/	0.68±0.05	0.60±0.04	*0.036*	0.79±0.09	0.58±0.03	*0.008*
campesterol						

Values are mean ± SEM. Cholesterol: mg/dL. Noncholesterol sterols and squalene: 100x mmol/mol of cholesterol

Table 3. Serum cholesterol and ratios to cholesterol of squalene and noncholesterol sterols (in 1980) of future gallstone patients (*n*=66) and control subjects (*n*=126) subdivided according to variants of *D19H* of *ABCG8* gene.

Variables	Gallstone	Gallstone	Controls	Controls	*P*
	19D	*19H* carriers	*19D*	*19H* carriers	
	homozygotes		homozygotes		
	n= 51	*n*=15	*n*= 102	*n*= 24	
Cholesterol	185.3±4.2	178.9±8.6	183.8±4.0	178.4±5.2	*0.677*
Cholestanol	145.5±3.6[a,b]	130.7±5.7[c]	151.0±2.9[d]	128.8±4.5	*<0.001*
Cholestenol	8.9±0.3	9.8±1.0	8.9±0.4	9.4±0.7	*0.425*
Desmosterol	74.4±1.5	76.7±3.2	74.6±1.3	76.8±2.8	*0.823*
Lathosterol	87.5±3.9	93.1±9.8	83.2±3.8	89.1±7.1	*0.407*
Campesterol	163.5±9.9[e,f]	139.5±13.9[g]	184.3±6.4[h]	135.7±10.1	*<0.001*
Sitosterol	108.9±6.5[i,j]	92.3±9.2[k]	122.8±4.7[l]	89.4±6.9	*<0.001*
Avenasterol	30.4±1.1[m,n]	25.3±1.9[o]	33.3±1.1[p]	27.0±2.0	*<0.001*
Stigmasterol	18.0±0.8	15.9±1.0	17.4±0.4	15.8±1.0	*0.075*
Squalene	8.7±0.8	7.2±0.6	9.1±0.6	7.5±0.5	*0.566*
Lathosterol/					
campesterol	0.64±0.05[q]	0.79±0.13[r]	0.55±0.04[s]	0.79±0.60	*0.007*

Values are mean ± SEM. Cholesterol: mg/dL. Noncholesterol sterols and squalene: 100x mmol/mol of cholesterol. *P*-value indicates ANOVA comparison between all four groups.

[a]*p*=0.038 compared to Gallstone *19H* carriers, [b]*p*=0.007 compared to Control *19H* carriers,

[c]*p*=0.012 compared to Control *19D* homozygotes, [d]*p*=0.006 compared to Control *19H* carriers.

[e]*p*=0.007 compared to Gallstone *19H* carriers, [f]*p*= 0.043 compared to Control *19H* carriers, [g]*p*=0.008 compared to Control *19D* homozygotes, [h]*p*=0.0003 compared to Control *19H* carriers.

[i]*p*=0.034 compared to Control *19D* homozygotes, [j]*p*=0.032 compared to Control *19H* carriers, [k]*p*=0.012 compared to Control *19D* homozygotes, [l]*p*<0.0002 compared to Control *19H* carriers.

[m]*p*=0.007 compared to Gallstone *19H* carriers, [n]*p*=0.024 compared to Control *19H* carriers, [o]*p*=0.002 compared to Control *19D* homozygotes, [p]*p*=0.003 compared to Control *19H* carriers,

[q]*p*=0.021 compared to Control *19D* homozygotes, [r]*p*=0.035 compared to Control *19D* homozygotes, [s]*p*=0.008 compared to Control *19H* carriers.