

Cholelithiasis in Patients with Gaucher Disease type 1: Risk Factors and the Role of ABCG5/ABCG8 Gene Variants

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ABSTRACT

Background & Aim: Patients with Gaucher disease type 1 (GD1) show an altered lipid profile and a certain degree of insulin resistance, which might contribute to cholelithiasis (CL) and could possibly be associated with ABCG5/ABCG8 gene variants. We aimed to investigate the prevalence of CL in Caucasian adult patients with GD1 and the possible risk factors, including gene variants of the ABCG5/ABCG8 genes.

Methods: 61 Caucasian patients with GD1 (38 female/23male), aged 18-62 years and 61 healthy subjects matched for age, gender and BMI, without CL, for comparison of lipid profiles. Data before start of enzyme replacement therapy (ERT) were recorded: clinical, haematological, severity parameters, splenectomy, genotype. Fasting lipid profiles before ERT, glycemia, insulinaemia, HOMA-IR at the last visit were documented. Genotyping for the gene variants D19H, Y54C, T400K, A632V (ABCG8); Q604E (ABCG5) was performed.

Results: CL occurred in 45.9% of patients. Risk factors were: age, family history of CL, higher BMI values, LDL-cholesterol (LDL-C), disease severity, splenectomy. A specific dyslipidemia was found in patients vs. controls. Total serum cholesterol (TC) and LDL-C were higher in patients with CL than in those without; no obvious influence of insulin-resistance to lithogenesis was found. Patients with the GG genotype of D19H and the CC genotype of T400K (ABCG8 gene) had significantly higher levels of TC and LDL-C.

Conclusion: Patients with GD1 showed an increased prevalence of CL, which was associated with common and disease-specific risk factors. Starting ERT soon after clinical onset and avoiding splenectomy might reduce the risk of CL in GD1.

Key words: cholelithiasis – Gaucher disease – ABCG5/ABCG8 gene variants.

Abbreviations: ABC: ATP-binding cassette; CL: cholelithiasis; ERT: enzyme replacement therapy; GBA1: acid-beta-glucosidase gene; GD1: Gaucher disease type 1; HOMA-IR: homeostasis model-assessment insulin resistance; HDL-C: HDL-cholesterol; LDL-C: LDL-cholesterol; MN: multiples of normal; PCR-RFLP: polymerase chain reaction-restriction fragment length polymorphism; SSI: severity score index; TC: total cholesterol; TG: triglycerides.

INTRODUCTION

Cholelithiasis (CL) has a prevalence of 9.7-15% in the Western countries [1, 2]. It is a multifactorial disease, determined by environmental and genetic factors in the proportion of 75% and 25%, respectively [3, 4]. Risk factors include age, female gender, ethnicity, visceral obesity and high intake of fat and sugars as well as insulin resistance [3]. The genetic predisposition is

polygenic, except for rare mutations in the genes involved in the synthesis of bile acids (CYP7A1) and phospholipids (ABCB4) [5].

Experimental and clinical data suggest that certain gene variants of ABCG5/ABCG8 increase the risk for CL [3, 4, 6, 7]. ATP-binding cassette (ABC) is a superfamily of proteins involved in the active transmembrane transport [8, 9]. ABCG5 and ABCG8 are two genes coding for the synthesis of half-transporters, functioning together as heterodimers and contributing to the clearance of sterols, including cholesterol [10-12]. These transporter proteins are located in the apical membrane of enterocytes, and facilitate the transport of cholesterol back into the intestinal lumen [13]. Further locations are the hepatocytes and the epithelial cells of the gallbladder [14, 15]. The ABCG5/ABCG8 genes control

the plasmatic and the biliary concentration of cholesterol. Cholesterol gallstones contain more than 80% cholesterol, and previous studies found an association of CL with certain gene variants of *ABCG5/ABCG8*: D19H (*ABCG8*) [4,16, 17, 18]; T400K (*ABCG8*) in males [19] and Q604E (*ABCG5*) [6]. Q604E or D19H polymorphisms have also been associated with insulin resistance [16, 17].

Gaucher disease (GD) is an inborn metabolic disorder with autosomal-recessive inheritance. It is the most frequent lysosomal storage disorder. Gaucher disease is caused by a deficiency of beta-glucocerebrosidase due to mutations in the acid-beta-glucosidase gene (*GBA1*) [20], leading to the accumulation of glucocerebrosides in the macrophageal lysosomes of the liver and spleen, bone and bone marrow [21]. The majority of patients suffer from GD type 1 (GD1; 92.4%) [22] and present with hepato-splenomegaly, bone disease, anaemia and thrombocytopenia. The prevalence of GD varies between 1/40,000 [23] and 1/100,000 [24], with the highest incidence of 1/855 in Ashkenazi Jews [25].

There are few studies regarding CL in patients with GD; only two case reports [26, 27] and three retrospective studies including only or mainly Ashkenazi Jewish patients, suggesting an increased prevalence of CL (20-32%) compared to the general population [28-30]. The prevalence of CL among Caucasian patients with GD is not yet known.

Furthermore, patients with GD show an altered lipid profile [30-33] and a certain degree of insulin resistance [34-36], which might contribute to CL and could possibly be associated with *ABCG5* or *ABCG8* gene variants. Only one study reported the lipid profile in GD patients with CL [30]. There are no previous data on the impact of *ABCG5/ABCG8* gene variants in patients with GD1, with or without CL.

We aimed to investigate the prevalence of CL in a cohort of Caucasian adult patients with GD1, the role of common and disease specific factors, the impact of metabolic factors (lipid profile, insulin resistance), the frequency of gene variants of *ABCG5* (Q604E) and *ABCG8* (D19H, Y54C, T400K, A632V), and possible associations between these gene variants and metabolic factors.

PATIENTS AND METHODS

This was a mono-center cross-sectional observational study. We included 61 adult Caucasian patients (38 females; 23 males) aged 18-72 years, representing all living patients with GD1 diagnosed at the time of the study in Romania, and who are followed up in the Center for Genetic Diseases of the Children's Emergency Hospital, Cluj, Romania. The inclusion criteria were: diagnosis of GD1 confirmed by measurement of glucocerebrosidase activity in peripheral leucocytes and by genotyping the *GBA1* gene, and age > 18 years. Cholelithiasis had been diagnosed by ultrasonography or by documentation of the patient's history with cholecystectomy for symptomatic CL.

For analysis of dyslipidemia, patients with GD1 were compared to a control group including 61 healthy subjects without personal or familial history of CL or diabetes mellitus, matched for age, gender and BMI. Presence of gallstones in controls was ruled out by ultrasonography. Patients and

controls were included after written informed consent and the Local Ethics Committee of the University of Medicine and Pharmacy Cluj approved the study.

The following data were registered from the patients' medical records: gender, age at the last visit, age at the diagnosis of GD1, genotype of *GBA1*, splenectomy status (no/yes; age at splenectomy), enzyme replacement therapy (ERT: no/yes; age at ERT start), family history of CL. The following parameters were recorded at the time of diagnosis: BMI, haemoglobin (g/dl), thrombocytes (/ml³), hepatic and splenic volume measured by ultrasonography [37] and expressed as multiples of upper normal values [38], chitotriosidase (nmoli/ml/h) [39] and the severity score (SSI) [40]. The fasting serum lipid profile before the start of ERT was also documented: total cholesterol (TC), LDL-cholesterol (LDL-C), HDL-cholesterol (HDL-C) and triglycerides (TG).

The fasting values for glycaemia and insulin before ERT were available only in 12 subjects and were analysed in a previous prospective study, regarding changes in insulin sensitivity under ERT [36]. Values for fasting glycaemia and insulin were documented under ERT, at the last visit (after 3.9±3.7 years ERT in patients with CL and after 5.0±3.7 years ERT in those without CL). HOMA-IR (homeostasis model-assessment insulin resistance) was calculated according to the formula: fasting insulinemia (μU/ml) x fasting glicemia (mg/dl)/405 [41]. In healthy controls, the fasting lipid profile was determined.

Genotyping of the *ABCG5/ABCG8* genes

During the last visit, an additional 2 ml of EDTA blood was collected from 60 patients for genotyping of the *ABCG5/ABCG8* gene variants: D19H, Y54C, T400K, A632V in *ABCG8*, and Q604E in *ABCG5*.

Genomic DNA was extracted from 300 μl of peripheral whole blood, using a commercial kit (Wizard Genomic DNA purification kit, Promega, MA, USA). Genotyping for the *ABCG5/ABCG8* gene variants was performed by the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique, using specific primers and PCR conditions as previously described [42] with some minor modifications. The PCR mixture (25 μl) consisted of: 12.5 μl 2xPCR Master Mix (ThermoFisherScientific, USA*); 1 μl bovine serum albumin (BSA, ThermoFisherScientific, USA*) solution 5 mg/ml; 8 pM of each forward and reverse primer (Eurogentec, Belgium*), and DNase/RNase free water to complete the 25 μl reaction volume. The specific amplification products were digested with the appropriate restriction enzymes (ThermoFisherScientific, USA*). The digestion products were resolved in 3% high resolution agarose gel electrophoresis (MetaPhor, Lonza, Switzerland) and stained with ethidium bromide.

Statistical analysis

The categorical data were expressed as counts and percentages, continuous data were presented as means and standard deviations for data following a normal distribution, or median and quartiles for skewed data. The associations between categorical variables were assessed with the Fisher's exact test. Differences between two groups of continuous data

were assessed with the Student's *t*-test for independent samples, for data following normal distribution, or with Wilcoxon rank-sum test for skewed data. The two-tailed *p* value was used with a 0.05 level of significance.

To assess the relation between CL and different GD1-related characteristics and genes related to cholesterol metabolism, we further used logistic regression for each of the investigated variables, adjusted for known characteristics related to CL. All the models were checked for multicollinearity and with Hosmer and Lemeshow goodness of fit test, Stukel tests and Osius-Rojek tests. Odds ratios along with the 95% confidence interval (CI) were presented for the unadjusted and adjusted regressions.

All statistical analyses were carried out with the R Environment for Statistical Computing and Graphics, Version 3.2.2.

RESULTS

The characteristics of patients with GD1 and CL are presented in comparison to the patients without CL (Table I).

Cholelithiasis had been diagnosed before the start of ERT in 17/28 patients (66.7%) and shortly thereafter in 11/28 (median 1.33 years, range 0.5- 2.1). Cholecystectomy was performed in 9/28 patients due to symptomatic CL.

Regarding demographic factors, patients with CL were older at the time of their last visit and at the time of diagnosis, compared to patients without CL (*p*<0.001 and 0.002,

respectively) and had higher mean BMI-values before ERT (*p*=0.0018). Overweight and obesity were encountered in 39.3% of patients with CL, compared to 18.2% of patients without CL, and obesity was more frequent in patients with CL (*p*=0.039). Cholelithiasis was diagnosed in most patients at the age of 40-50 years. Family history of CL was positive in 14/28 patients with CL, compared to 3/33 patients without CL (*p*<0.001) (Table I).

Regarding the specific characteristics for GD, we observed in patients with CL a higher number of splenectomies (16/28 vs. 5/33, *p*<0.001), and a higher prevalence of severe bone disease (avascular necrosis, arthroplasties, fractures) (42.9% vs 21.2%) as compared to patients without CL. The mean chitotriosidase values and SSI-values were also higher in patients with CL. Patients with CL were older at the time of splenectomy, and at the start of ERT. ERT was performed in 82.1% of patients with CL and 84.8% of patients without CL.

In order to evaluate the effect of ERT on patients' weight, we compared BMI values under ERT at the last visit with the values before ERT (see Methods). We observed an increase in BMI both in patients with CL (from mean 24.4±4.6 kg/m² to 26.3±4.2 kg/m²) and in those without CL (from mean 20.7±3.4 kg/m² to 22.9±3.2 kg/m²), with significant differences between the two groups before ERT (*p*=0.0018) and under ERT (*p*=0.002). The percentage of overweight patients increased among those with CL from 25% (7/28) to 32.1% (9/28), as compared to 18.2% (6/33) to 30.3% (10/33) in patients

Table I. Characteristics of patients with GD1 and CL compared to patients with GD1 without CL

Patients' characteristics	Patients with CL (n=28; 45.9%) [#]	Patients without CL (n=33; 54.1%) [#]	<i>p</i> value
Age at last visit (years)	46.6 (40.8-54.2)	32.1 (25.3-39.5)	<0.001
Age at diagnosis	38 (32.9-49.5)	25.6 (18-36.2)	0.002
Gender (n;%)			
F	20 (71.4)	18 (54.6)	0.196
M	8 (28.6)	15 (45.5)	
BMI kg/m ² *	24.4±4.6	20.7±3.4	0.0018
≤25	17(60.7)	27(81.8)	0.088
>25; ≤30	7(25)	6(18.2)	0.547
>30	4(14.3)	0(0)	0.039
Splenectomy n(%)	16 (57.1)	5 (17.8)	<0.001
Age at splenectomy	25.1 (11-32)	5 (2.5-5)	0.002
Splenic volume (MN) *	14.2 (7-15.1)	8.9 (7-13.3)	0.53
Liver volume (MN) *	1.27 (1.1-1.5)	1.23 (1.1- 1.5)	0.94
Hemoglobin (g/dl) *	12.1 (11.1-12.9)	11.5 (10.7-12.6)	0.45
Platelets (x1,000/mm ³) *	118.5 (68.5-219.5)	88 (55-120)	0.14
Chitotriosidase * (nmol/ml/h)	44,000 (14,500-87,500)	25,500 (16,750-42,500)	0.03
SSI (Zimran) *	14.8±6.9	11.7±3.9	0.008
Genotype			
N370S/N370S	8 (28.5%)	9 (27.2%)	1
N370S/L444P	6 (2.15%)	9 (27.2%)	0.766
N370S/ other alleles	14(59%)	15 (45.5%)	0.799
ERT (n; %)	23 (82.1)	28 (84.8)	1
Age at start of ERT	41.1 (34.7-46.9)	26.4 (18.4-38)	0.003
Family history of CL			<0.001
Grade 1/ Grade 2/Total	12 (42.9) / 2 (7.1) / 14(50)	3 (9.1) / 0/3 (9.1) / 3 (9.1)	

[#] matched regarding age, gender and BMI with an equal number of controls. GD1: Gaucher disease type 1; CL: cholelithiasis; MN: multiples of normal; ERT: enzyme replacement therapy; SSI: severity score index; CI: confidence interval; * before ERT.

Table II. Logistic regression assessing associations between CL and different GD1-related characteristics and genes related to cholesterol metabolism. For each variable the unadjusted regression as well as the adjusted regression coefficients for gender, age at last visit, BMI before ERT, LDL- cholesterol, and family history of CL are given.

	OR unadjusted	(95% CI)	p	OR Adjusted	(95% CI)	p
Gender (female/male)	2.08	(0.73 - 6.28)	0.178			
Age at last visit (years)	1.07	(1.03 - 1.12)	0.003			
BMI before ERT (kg/m ²)	1.19	(1.06 - 1.37)	0.009			
LDL-Cholesterol (mg/dL)	1.03	(1 - 1.05)	0.021			
Family history of cholelithiasis	10	(2.75 - 48.72)	0.001			
Chitotriosidase (nmol/mL/h)	1.000019	(1.000002 - 1.000004)	0.039	1.000041	(1.00001- 1.00008)	0.019
Splenectomy (yes vs. no)	7.47	(2.35 - 27.36)	0.001	8.2	(1.61-55.77)	0.017
Bone disease severity (severe vs. less severe)	2.79	(0.93 - 8.92)	0.073	3.97	(0.83-21)	0.089
SSI (severity score index) *	1.15	(1.02 - 1.34)	0.038	1.45	(1.17-1.93)	0.003
Y54C (AA vs. AG)	0.67	(0.23 - 1.91)	0.453	0.44	(0.09-1.89)	0.28
Y54C (GG vs. AG)	0.5	(0.02 - 5.73)	0.587	0.14	(0-6.26)	0.306
T400K (AA vs. CC)	2.71	(0.24 - 61.08)	0.432	1.39	(0.07-45.54)	0.835
T400K (CA vs. CC)	1.2	(0.38 - 3.79)	0.751	1.54	(0.34-7.46)	0.579
Q604E (CG vs. CC)	1.86	(0.59 - 6.09)	0.294	2.08	(0.35-12.69)	0.411
D19H (GG vs. GC)	2.6	(0.31 - 54.33)	0.42	2.68	(0.05-18.01)	0.779

* For the multiple logistic regression model that included the SSI (severity score index), in the adjusted variables list, the family history of cholelithiasis was removed due to multicollinearity BMI: body mass index; CL: cholelithiasis; ERT: enzyme replacement therapy; GD: Gaucher disease.

without CL (0.678). Obesity was encountered in 14.3% (4/28) of patients with CL before ERT and in 25% (7/28) of patients with CL under ERT compared to no patients without CL before or under ERT (before ERT, $p=0.039$; under ERT, $p=0.002$).

In order to analyze the relation between CL and different GD-related characteristics and genes related to cholesterol metabolism, we used logistic regression for each of the explored variables, adjusted for known characteristics related to CL: gender, age, BMI, LDL cholesterol, and family history of CL. Statistically significant relationships between the characteristics linked to the severity of GD1 and presence of CL were found for chitotriosidase, severity score index, splenectomy, both in unadjusted and adjusted regressions for known factors associated with CL. The odds of CL were higher in those with higher values of chitotriosidase, in those with higher severity score index, and in those who underwent splenectomy. Within the same characteristics linked to GD severity, bone disease severity was not statistically significant ($p=0.089$). None of the genetic variants of the *ABCG5/ABCG8* genes were found to be directly associated to CL, either in regression with or without adjustment (Table II).

The fasting lipid profiles before ERT in patients with GD1 showed lower mean HDL-C values compared to healthy controls (Table III). Furthermore, we observed lower concentrations of LDL-C and higher values of TG among patients (with and without CL) when compared to the corresponding control groups (Table III).

Table IV shows the fasting values of glycaemia and insulinaemia as well as HOMA-IR under ERT in patients with and without CL. There were no significant differences between patients with and without CL concerning these parameters.

The distribution of the gene variants of *ABCG5/ABCG8* genes was not different according to CL status (Table V). When comparing patients with CL and positive family history (subgroup 1, $n=13$) with those with CL without family history of CL (subgroup 2, $n=14$), the following differences were observed: patients in subgroup 1 presented more frequently the homozygous and heterozygous genotypes for the minor allele of the Y54C gene variants in the *ABCG8* gene (GG and AG) compared to subgroup 2 (11/2 vs. 6/8; $p=0.046$). Furthermore, the patients in subgroup 1 had more frequently

Table III. Fasting lipid profiles in patients with GD1 with and without CL before ERT

Lipids	1. Patients with CL (n=28)	M1. Controls for 1.	p(1,M1)	2. Patients without CL (n=33)	M2. Controls for 2	p(2,M2)	p(1,2)
TC (mg/dl)	141.8+-35.6	188+-35.9	<0.001	125 (100-143)	100 (143-196)	<0.001	0.03
HDL-C (mg/dl)	29.5 (23.5-37.5)	59 (51-65)	<0.001	25 (22-31)	52 (43-64)	<0.001	0.16
LDL-C (mg/dl)	83.5 (63.3-107.3)	112 (93-120)	0.003	73 (54-91)	94 (75-104)	0.003	0.03
LDL/HDL	2.85 (2.4-3.3)	1.9 (1.58-2.4)	<0.001	2.9 (1.9-3.3)	1.9 (1.1-2.5)	<0.001	0.37
TG (mg/dl)	119 (99.5-149.5)	94.5 (68-132.25)	0.01	116 (102-154)	78 (67-98)	<0.001	0.99

TC: total cholesterol; HDL-C: HDL cholesterol; LDL-C: LDL cholesterol; TG: triglycerides CL: cholelithiasis; ERT: enzyme replacement therapy; GD: Gaucher disease.

Table IV. Parameters of insulin resistance and BMI in patients with GD1 with and without cholelithiasis, at the last visit*

Parameters	Patients with CL (n= 28)	Patients without CL (n = 33)	p value
Glycaemia (mg/dl)	96.6+-8.6	98+-10.1	0.567
Insulinaemia (µU/ml)	9.2+-5.1	9.6+-6.7	0.779
HOMA-IR	2.18+-3.4	2.34+-1.98	0.721
BMI (kg/m ²)	26.3+/-4.6	22.9+-3.4	0.002
Correlation (r)HOMA-IR/BMI	r=0.658, p<0.001	r=0.5212, p=0.001	

* after 5.29 (0.33-8.75) years of enzyme replacement therapy (ERT)

the heterozygous genotype for the minor allele of the Q604E variant in the *ABCG5* gene (CG) compared to subgroup 2 (8/5 vs. 2/12; p=0.018). No further differences in genotype distribution could be found for the analysed genes between patients with CL, with respect to family history of CL.

The analysis of lipid parameters before ERT in relation to the *ABCG5/8* gene variants showed a tendency towards higher TG levels in all patients with the GG and AG genotypes of the Y54C gene variant (*ABCG8*), compared to the AA genotype (median 132 vs 111 mg/dl, p=0.07). A similar tendency was seen for TGs concerning the CT genotype of the A632V gene variant (*ABCG8*), compared to the CC genotype (median 121 vs. 110 mg/dl; p=0.08) (data not shown).

Regarding the lipid profiles of patients before ERT, in relation with *ABCG5/ABCG8* genotype and CL status, we observed the following differences (Table VI): patients with CL and the homozygous genotype for the common allele of D19H (GG) had higher concentrations of TC and LDL-C compared to patients without CL. Patients with the homozygous genotype for the common allele of T400K (CC) showed significantly higher values for TC and LDL-C compared to patients without CL. Patients with CL and the CG genotype of Q604E had higher values for HDL-C, compared to those without CL.

The parameters of insulin resistance did not differ as related to the *ABCG5/ABCG8* genotype between patients with and without CL (data not shown).

DISCUSSION

To the best of our knowledge, there are only five studies published regarding CL in patients with GD [26-30]. The only study carried out on 67 Caucasian patients with GD, mentioned CL among another nine co-morbidities without presenting the prevalence [43]. Our study is the first to analyse the issue of CL in adult Caucasian patients with GD in relation to genetic variability and to lipid and carbohydrate metabolism.

We found CL in 28/61 (45.9%) of our patients, which is much more frequent than in the general population [2] or in previously reported data from patients with GD. This is possibly due, in part, to a higher impact of the genetic background, since 50% of patients with GD1 had a positive family history for CL, compared to 9.1% in patients without CL (p<0.001).

The comparison between patients with GD1 with and those without CL confirms the known general risk factors for CL, such as age and BMI, and also the impact of GD1 specific factors such as splenectomy (p<0.001) and disease severity (p=0.008), as previously reported [28-30].

The mean BMI values before ERT were below 25 kg/m² in both patients with and without CL, which is in accordance with previous data that pointed to lower BMI values as a metabolic trait of untreated patients with GD [30]. However, BMI was higher in patients with CL (p=0.0018) and obesity was more frequent in patients with CL, compared to none in those without CL.

Furthermore, some particular findings have been observed.

Beyond a certain degree of weight increase under ERT [34], there are certainly other genetic and environmental factors involved. Our data showed that more patients with CL became overweight after a similar duration of ERT, compared to patients without CL. A similar dynamics has been observed in the development of obesity in patients with CL. There were no obese patients in the group without CL.

Splenectomy was frequently performed before both specific diagnosis and ERT were available, and preceded treatment start with a mean of 14.5 (range 9-36.4) years. The number of splenectomies in our patients (57%) was obviously higher

Table V. ABCG5/ABCG8 gene variants in patients with Gaucher disease type 1 (GD1) with and without cholelithiasis (CL).

Genotype ABCG5/8 (n;%)	Patients with CL (n=27)	Patients without CL (n=33)	All genotyped patients with GD1 (n=60)	p (patients with vs without CL)
D19H				
GG	26 (96.3%)	30 (81.8%)	56 (93.3%)	0.62
CC/CG	0/1 = 1 (3.7%)	0/3 = 3 (9.1%)	0/4 = 4 (6.7%)	
Y54C				
AA	10 (37.1%)	15 (44.5%)	25 (41.6%)	0.60
GG/AG	1/16 = 17(62.9%)	2/16 = 18 (54.6%)	3/32 = 35 (58.3%)	
T400K				
CC	17 (62.9%)	23 (69.7%)	40 (66.7%)	0.58
AA/CA	2/8 = 10 (37.1%)	1/9 = 10 (30.3%)	3/17 = 20 (33.3%)	
A632V				
CC	15 (55.6%)	14 (42.4%)	29 (48.3%)	0.31
CT	12 (44.4%)	19 (57.6%)	31 (51.6%)	
Q604E				
CC	18 (66.6%)	26 (78.8%)	44 (73.3%)	0.29
CG	9 (33.3%)	7 (21.2%)	16 (26.6%)	

Table VI. Serum lipid parameters and ABCG5/ABCG8 genotype in patients with and without cholelithiasis (CL)

Genotype ABCG5/ABCG8	TC (mg/dl)		HDL-C (mg/dl)		LDL-C (mg/dl)		TG (mg/dl)	
	With CL (1)	Without CL (2)	With CL (1)	Without CL (2)	With CL (1)	Without CL (2)	With CL (1)	Without CL (2)
D19H*: GG p1,2	143±35.7 0.019	122±25.3	30.5±10.3 0.130	26.8±7.5	87.4±25.4 0.005	68.2±24.9	132.4±50.9 0.606	126.1±39.4
GC/CC p1,2	109(1pt)	130.1±31.2	21(1pt)	25±10.1	65(1pt)	72.3±29.5	112 (1pt)	145.6±56.2
Y54C*: AA p1,2	142.9±39.9 0.079	118±25.3	31.5±12.8 0.455	28.1±9.4	91.9±9.4 0.154	75.8±28.3	134.9±57.9 0.264	120.1±4.12
AG/GG p1,2	123.3±25.3 0.791	138±34.4	29.2±8.2 0.090	25±4.7	79.6±24.3 0.087	66.4±17	129.3±45.3 0.673	135.6±32.1
T400K*: CC p1,2	142.6±35.01 0.049	123.8±27.3	29.1±8.9 0.291	26.4±7.4	89.2±23.9 0.016	71.4±22.7	127.2±42.2 0.917	128.5±38.9
CA/AA p1,2	139.7±39.4 0.405	125.3±25.8	29.1±14 0.753	27.2±8.6	80.3±29.2 0.593	73.1±25	142.8±68.2 0.5726	126.2±46.8
A632V*: CC p1,2	137.6±34.5 0.125	119.5±28.2	29±10.2 0.789	28±8.6	84.8±26 0.068	67.5±23.6	121.6±42.5 0.873	124.2±45.2
CT p1,2	142.3±35.8 0.233	127.6±25.4	31.8±10.6 0.0903	25.6±6.1	89.1±25.3 0.109	74.1±23.1	145±58 0.4522	130.5±37.6
Q604E***: CC p1,2	143.9±40.2 0.111	126.5±26.9	30±11.9 0.450	27.6±7.7	87.4±26.4 0.063	73±22.4	139.4±54.2 0.331	124.6±42.2
CG p1,2	137.2±24.5 0.109	115.7±25	30.5±6 0.028	23±6.1	85.1±24.4 0.141	64.8±26.5	115.4±37.7 0.192	139.8±33.2

* in ABCG8 gene, **in ABCG5 gene; TC – total cholesterol; HDL-C – HDL cholesterol; LDL-C – LDL cholesterol; TG - triglycerides

compared to the largest published study (24%) [30]. The splenectomies also might explain the apparently discordant results in patients with CL. These patients had significantly higher values of chitotriosidase and SSI at diagnosis, as an expression of more severe disease forms, but showed normalised haematologic parameters when compared to patients without CL. It is not clear how splenectomy can increase the risk of CL. However, Ben-Harosh-Katz et al. speculated that the almost threefold increase of hepatic glucosylceramide, glucosylsphingosine and GM3 after splenectomy could be an explanation, presumably because after removal of the primary reservoir of storage cells, there was a greater demand to alternative routes of deposition and excretion [28].

Our findings also suggested that the severity of GD as measured by chitotriosidase or the severity score index was associated with higher odds for CL. These results were reinforced also by the adjustment made in regression for known factors associated to CL.

These observations, together with the older age of patients with CL both at GD diagnosis and at the start of ERT, might suggest that a rapid start of ERT after GD diagnosis and hence onset of clinical manifestations could reduce the risk of CL development in GD.

Gallstone formation is the result of the bile supersaturation in cholesterol due to an increase in cholesterol and decrease in bile acid content. Patients with GD show a specific serum lipid profile with decreased TC, LDL-C, strongly reduced HDL-C [33, 44, 45] and increased TGs [31, 32], which improve under ERT [33]. The low concentrations of LDL-C in GD are the result of its high metabolism rate [45], due to the excessive synthesis and secretion of apolipoproteins E (apoE) by glucocerebrosides laden macrophages [47]. ApoE

facilitates the reverse-transport of HDL-C to the liver, and the excessive uptake and catabolism of HDL-C leads to reduced plasmatic levels.

The lipid profile in our patients was significantly different from healthy controls, according to the pattern described above (Table III). In the setting of disease-specific hypocholesterolaemia, patients with CL had higher values for TC and LDL-C than those without CL, in line with the single existing study on this subject [30].

Another factor which might contribute to an increased prevalence of CL in GD is insulin resistance. The accumulation of glucosylceramides in specialised cell membrane domains called rafts, and the increase in plasma ganglioside GM3 leads to an impaired insulin signalling [34]. Insulin resistance was suggested to be associated with weight increase under ERT [34]. However, insulin resistance has also been observed in normal weight patients with GD [35]. Our team found previously normal values of fasting glycaemia, insulinaemia and HOMA-IR in the treatment naïve patients, and a tendency towards insulin resistance under ERT, which was independent of the weight gain [36].

We observed in our patients a positive correlation between HOMA-IR and BMI. This was seen in both groups and after a comparable duration of ERT (Table IV). HOMA-IR values above 2.05 are considered predictive for cardiovascular risk in Caucasians [48]. Of note, all HOMA-IR values in our patients under ERT were above this cut-off, irrespective of CL-status, even if no significant differences between patients with and without CL concerning HOMA-IR could be detected.

Literature data suggest a relationship between several variants of the ABCG5/ABCG8 genes and lipid profile [6, 13, 17] or insulin resistance [16, 17], and hence also a possible association with gallstone formation. The distribution of

homozygous genotypes for the common allele, compared to the minor allele (homo- and heterozygotes) did not differ among our patients with and without CL regarding the analysed gene variants. The distribution of gene variants in our patients with CL was close to that reported from another Romanian study investigating CL in the general population [6]: identical for Q604E and comparable for Y54C, T400K and A632V. The distribution of the gene variants in patients without CL was close to that reported for Caucasian patients [11, 13], except the lower frequency of the common variant of A632V.

Regarding the association of these gene variants with plasma lipids, the existing data are scarce and inconclusive. A relationship between the GG genotype of D19H (*ABCG5*) and higher plasmatic concentrations of TC and LDL-C was reported in Caucasians [16], while in Chinese patients these values were lower [17]. One study of 34 Romanian sibling pairs with CL showed that homozygotes for the common alleles of Q604E have higher concentrations of TC and TG, and lower levels of HDL-C; homozygotes for the common allele of D19H had higher values of TC and TG, while homozygotes for the common allele of Y54C show higher TG concentrations [6]. These data suggest a lithogenic plasmatic lipid profile in twins with CL. However, a meta-analysis of 16 studies including 3,364 subjects found only a weak association between the minor allele of A632V and lower LDL-C [49].

The comparison of lipid profiles between GD patients with and without CL in relation to the *ABCG5/ABCG8* gene variants showed that patients with CL had significantly higher levels of TC and LDL-C when presenting with a homozygous genotype for the common allele of the D19H variant (GG), or the common allele of the T400K variant (CC) (Table VI). These observations suggest an indirect contribution via lipid metabolism of these genetic factors to lithogenesis. The finding regarding the D19H gene variant is in line with previous data [16].

Gylling et al. [16], reported an association of insulin resistance with the hetero- and homozygous genotype of the minor allele of Q604E in male Caucasian patients, while Chen et al. [17] found an association with the heterozygous genotype of the minor allele of D19G (GC). No explanation was provided regarding the influence of insulin signalling by a gene implicated in the cholesterol transport. We could not find differences in HOMA-IR values in relation to the *ABCG5/ABCG8* gene variants between GD1 patients with and without CL.

CONCLUSIONS

Our study reports the presence of CL in 45.9% of Caucasian patients with GD1. The risk factors for CL were older age, family history of CL, higher BMI values in the known setting of disease-associated lower BMIs, higher LDL-C levels, increased disease severity, and previous splenectomy.

The start of ERT as soon as possible after clinical onset and avoidance of splenectomy could reduce the risk of CL in GD1.

We found higher concentrations of TC and LDL-C in GD1 patients with CL than in those without, in the setting of the disease-specific hypocholesterolaemia, with no obvious association with insulin resistance.

Our study is the first to evaluate the impact of certain *ABCG5/8* gene variants in patients with GD and CL. Patients with the GG genotype of D19H and the CC genotype of T400K (*ABCG8* gene) had significantly higher levels of TC and LDL-C, suggesting a possible role of these gene variants as risk factors for CL in GD1 patients. Due to the low number of patients, this observation is speculative; further multicenter studies with a larger cohort are needed to confirm this hypothesis.

Conflicts of interest: No conflict to declare.

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