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ORIGINAL ARTICLES

Common variant p.D19H of the hepatobiliary sterol transporter *ABCG8* increases the risk of gallstones in children

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Abstract

Introduction: Gallstones are increasingly common in children. Genetic analyses of adult cohorts demonstrated that the sterol transporter *ABCG8* p.D19H and Gilbert *UGT1A1*28* variants enhance the odds of developing gallstones. The genetic background of common lithiasis in children remains unknown.

Methods: Overall, 214 children with gallstone disease (1 month–17 years, 107 boys) were inclueded. The control cohorts comprised 214 children (age 6–17 years, 115 boys) and 172 adults (age 40–92 years, 70 men) without gallstones. The *ABCG8* p.D19H and *UGT1A1*28* polymorphisms as well as *ABCB4* (c.504C>T rs1202283, c.711A>T rs2109505) and *NPC1L1* variants (p.V1296V rs217434, c.–18C>A rs41279633) were genotyped using TaqMan assays. Serum concentrations of plant sterols and cholesterol precursors were measured by gas chromatography/mass spectrometry.

Results: The *ABCG8* risk allele was associated with an increased risk of stones (OR = 1.82, p = .03). Children carrying the p.19H allele presented with lower serum

Abbreviations: ABCB4, hepatobiliary phospholipid transporter; ABCG8, hepatobliary cholesterol hemitransporter; CI, confidence interval; G, guanine; GD, gallstone disease; HWE, Hardy–Weinberg equilibrium; NPC1L1, Niemann-Pick C1-Like 1; OR, odds ratio; PAF, population attributable fraction; SNP, single-nucleotide polymorphism; T, UGT1A1, UDP glucuronosyltransferase 1 family, polypeptide A1.

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concentrations of surrogate markers of intestinal cholesterol absorption and decreased ratios of phytosterols to the cholesterol precursor desmosterol. Carriers of the common *NPC1L1* rs217434 allele had an increased gallstone risk compared with stone-free adults (OR 1.90, $p < .01$). This variant also affected the ratio of phytosterols to cholesterol precursors ($p = .03$). Other tested variants were not associated with gallstone risk.

Conclusions: The p.D19H *ABCG8* and, to a lesser extent, *NPC1L1* rs217434 variants increase the risk of early-onset gallstone formation. These results point to the presence of a common lithogenic pathway in children and adults.

KEYWORDS
cholesterol, gallstone disease, Gilbert syndrome, sterols

1 | INTRODUCTION

Gallstone disease represents one of the most common hepatobiliary conditions world-wide.[1] Especially adults often have gallstones[2] but in recent decades, the incidence of gallstone disease in children has been increasing rapidly as well.[3,4] This is attributed to the changes in lifestyle. Indeed, the latest analyses demonstrate that an unhealthy lifestyle resulting in obesity[5,6] is responsible for increased gallstone risk in children and adolescents. It can be speculated that genetic predisposition might also play a role in the development of gallstones in children, so far, however, common variants predisposing to gallstone disease in children have not been detected.

Genetic analyses[7,8] of adults with gallstones showed that individuals carrying polymorphisms in the hepatobiliary cholesterol transporter *ABCG8* p.D19H[9,10] as well as the *UGT1A1*28* Gilbert variant[11,12] develop gallstones more frequently. These associations have been replicated in several cohorts coming from different countries and continents,[13] rendering the *ABCG8* and *UGT1A1* polymorphisms ubiquitous determinants of increased gallstone risk in adults. In brief, ABCG8 functions together with ABCG5 as a heterodimeric transporter[14] expressed at the canalicular membrane of hepatocytes and intestinal enterocytes where it actively transports sterols.[15-17] Mutations in this transporter causing loss of its function lead to sitosterolaemia. On the other hand, the lithogenic *ABCG8* variant p.D19H is thought to be a 'gain-of-function' mutation leading to an increased function of this transporter and hence increased biliary cholesterol levels in carriers of the risk allele [p.19H]. This notion is in line with the analyses of serum markers of cholesterol synthesis (cholesterol precursors, eg lathosterol) and cholesterol transport (plant sterols, eg sitosterol and campesterol) in paediatric and adult patients with stones which showed that gallstone patients have lower serum concentrations of markers of cholesterol absorption but higher serum markers of cholesterol synthesis,[18,19] as compared to gallstone-free controls. The *UGT1A1* Gilbert variant, in turn, is known to reduce the activity of the UDP-glucoronyltransferase. This results in increased levels of unconjugated bilirubin. It is believed that bilirubin nidus might represent the starting point of stone formation, which might be the reason for the increased gallstone risk in patients with Gilbert syndrome.[20] Analysis of overall risk conferred by the *ABCG8* and *UGT1A1* variants demonstrates that they might be responsible for 20% of the total gallstone risk in adults.[12] Of note, studies[21] in Swedish twin pairs with gallstone disease demonstrated that inherited predisposition contributes, in average, to 25% of the total gallstone risk. Hence, the *ABCG8* and *UGT1A1* polymorphisms seem to confer most of the predicted risk in non-paediatric patients. Of note, variants in the hepatobiliary phospholipid transporter *ABCB4* have previously been implicated in the development of cholestatic disorders.[22]

In the current study, we investigate, the association between the *ABCG8*, *UGT1A1* and *ABCB4* polymorphisms and gallstone disease in a large cohort of children with gallstone disease. Since Niemann-Pick C1-Like 1 (NPC1L1) mediates sterol uptake in hepatocytes and enterocytes,[23] we also included two common *NPC1L1* variants in the analysis. We also investigate if the presence of the risk polymorphism might affect cholesterol homeostasis in children with stones as reflected by serum sterol levels.

2 | MATERIALS AND METHODS

2.1 | Study cohorts

For this study, we recruited a cohort of 214 children with gallstones (age at inclusion 6.7 ± 5.5 years, 107 males) at three university centres in Poland (Warsaw, Katowice and Bialystok). Gallstone disease was confirmed by either abdominal sonography in patients with gallbladders in situ, or by the history of cholecystectomy for symptomatic gallbladder stones. Children undergoing total parenteral nutrition and with congenital cholestatic liver diseases (progressive familial intrahepatic cholestasis) were excluded from the analysis. Body mass index (BMI) was calculated as the ratio of weight in kilograms and the square of height in meters. Age and sex-specific point estimates of the prevalence of overweight (in this paper, the term overweight always includes obesity) and obesity were calculated with 95% confidence interval (CI) of the estimates based on the International Obesity Task Force (IOTF) cut-offs.[24] Blood samples

were drawn from fasted subjects, and liver function tests were determined by standard clinical-chemical assays in the central laboratory of our centre.

Two separated cohorts of controls were included in the analysis: children and adults without gallstones, both presented in Table 1. The paediatric control cohort was composed of 214 Polish children (age 6–17 years, 115 boys). To exclude a possible bias that children without gallstones might still develop stones in later life, we also used a group of 172 gallstone-free adults (age 40–92 years, 70 males) as controls. All cases and controls were European Caucasians. In both cases and controls blood samples with EDTA were obtained for DNA isolation. Among controls, the presence of gallstones was excluded using abdominal sonography. Informed consent was obtained from all patients and controls, and the study protocol follows the ethical guidelines of the declaration of Helsinki as reflected in an a priori approval by the Ethic Committee of the Children's Memorial Health Institute, Warsaw, Poland (Approval number: 39/KBE/2012).

2.2 | Measurements of sterol concentrations

In gallstone patients recruited in Warsaw in addition to EDTA-anticoagulated blood, fasting serum specimens, each containing butylated hydroxytoluene, were collected at the time of inclusion and stored at −70°C. The concentrations of cholesterol precursors, plant sterols and stanols were measured by gas chromatography/mass spectrometry (GC/MS).[25]

2.3 | Genotyping

Membrane-based QIAamp DNA extraction protocol (Qiagen, Hilden, Germany) was used to isolate genomic DNA. All individuals were genotyped for the *ABCG8* p.D19H (rs11887534), *ABCB4* c.504C>T (rs1202283) and c.711A>T (rs2109505) as well *NPC1L1* p.V1296V (rs217434) and c. − 18C > A (rs41279633) variants using PCR-based assays with 5′-nuclease and fluorescence detection (TaqMan®, Life Technologies).[18] The Gilbert *UGT1A1*28* variant was also genotyped by TaqMan® assays as described.[12,26] To ensure genotyping quality, we included negative controls and DNA samples with known *UGT1A1* genotypes as internal controls.

2.4 | Statistics

All tests were performed with GraphPad Prism 11.0 (GraphPad Software) or SPSS 26.0 (SPSS). For all tests, two-sided $p < .05$ were regarded as significant. Phenotypic quantitative data were expressed as means ± SD or medians and ranges. Kolmogorov–Smirnov tests were used to determine whether data were normally distributed. Normally distributed continuous variables were compared between groups using Student's *t*-tests or ANOVA. Non-normally distributed traits were compared using Mann–Whitney rank sum tests or non-parametric ANOVA. Categorial variables were tested in contingency tables. To investigate whether carriers of either of the studied variants develop gallstones more often than carriers of the wild-type genotypes, we performed case-control association analyses in contingency tables (alleles: χ^2 test; genotypes: Armitage's trend test). Hardy–Weinberg equilibrium (HWE) was checked with exact tests. The population attributable fraction (PAF) was calculated using PARC software (http://www.miner.rochester.edu/cpm/education/match/productspubs.html).

3 | RESULTS

3.1 | Characteristics of children with gallstones and gallstone-free adults

Table 1 summarizes the clinical characteristics of paediatric patients and gallstone-free controls. All controls had gallbladder in situ, none underwent retrograde cholangio-pancreatography (ERCP), and none received ursodeoxycholic acid (UDCA). Among 214 children with gallstones, 47 underwent cholecystectomy. Acute pancreatitis was diagnosed in 14 children, and ERCP was required in a total of 10 patients. In four patients in addition to abdominal sonography, opaque stones could be visualized by abdominal X-ray. In total, 37% of children were overweight. A total of 123 cases had a positive family history of gallstones.

TABLE 1 Baseline characteristics of patients with gallstones and controls

	Cases	Paediatric controls	Adult controls
N (female/male)	214 (107/107)	214 (99/115)	172 (102/70)
Age (years)	1/12–17	6–17	40–92
Symptomatic stones (*n*)	138 (64.5%)	0	0
Cholecystectomy (*n*)	47 (21.9%)	0	0
ERCP (*n*)	10 (4.8%)	0	0
Therapy with UDCA (*n*)	126 (58.9%)	0	0

Abbreviations: ERCP, retrograde cholangio-pancreatography; UDCA, ursodeoxycholic acid.

Among included gallstone patients, a total of 126 received UDCA as gallstone therapy. This therapy lasted for a mean period 10.2 ± 15.7 months, and during this time the mean size of stones decreased from 6.8 ± 3.9 mm to 6.0 ± 4.0 mm ($p = .23$).

3.2 | The *ABCG8* variant p.D19H is associated with increased gallstone risk in children

The distributions of all variants were within Hardy-Weinberg equilibrium in cases and controls (all $p > .05$). Table 2 present the frequencies of the *ABCG8* genotypes. The lithogenic *ABCG8* allele p.D19H was present in 14.9% of children with gallstones and was three times more frequent in this cohort as compared to paediatric controls (frequency 5.2%) and twice as frequent when compared to gallstone-free adults (frequency 7.5%). The lithogenic genotype was associated with an increased risk of stones (common OR = 4.04, $p < .01$ as compared to paediatric controls; common OR = 1.82, $p = .03$ as compared to adult controls). Notably, even carriers of one copy of the lithogenic p.D19H allele demonstrated increased gallstone prevalence (OR = 2.18, 95%CI 1.08–4.40, $p = .02$). PAF was 3.7% of the total gallstone risk in children; in other words, 3.7% of the entire gallstone risk was attributed solely to the presence of the *ABCG5/8* polymorphism. In terms of clinical characteristics of gallstone patients, as presented in Table S1, we did not detect any association of this variant with age at diagnosis of gallstones, serum ALT, ALT of GGT activities (all $p > .05$), nor with the frequency of overweight or the change in stone size under therapy with UDCA ($p = .45$). Interestingly, there was a trend ($p = .053$) for an older age at diagnosis of gallstones in carriers of the [p.19H] allele as compared to patients presenting with the genotype [p.19DD] (9.0 ± 5.8 vs. 6.6 ± 5.4 years respectively).

Since *ABCG8* transports sterols into bile, we aimed to further characterize the effects of the *ACBG8* p.D19H variant on the gallstone trait by comparing serum sterol concentrations in children with gallstones ($n = 52$) in relation to the *ABCG8* genotype. For these analyses we matched, based on age and sex, 15 carriers of the *ABCG8* [p.19H] allele with 37 carriers of the common *ABCG8* [p.19DD] genotype. As demonstrated in Figure 1 gallstone patients positive for the lithogenic allele had significantly lower serum levels of the natural phytosterol sitosterol (panel Figure 1A) as well as phytostanols campestanol and sitostanol (panels Figure 1B,C respectively). In line with these results, the *ABCG8* variant was associated with decreased ratios of phytosterols to cholesterol precursors (panels Figure 1D,E). Taken together, these results demonstrate that the lithogenic variant is associated with increased cholesterol transport (or decreased absorption), possibly coupled with increased cholesterol synthesis, which might promote stone formation in carriers of the risk variant.

Among other tested variants, we detected an increased risk of developing gallstones in the carriers of the common allele of the *NPC1L1* rs217434 polymorphism but only when comparing gallstone patients with gallstone-free adults (Table 3; common OR 1.90, $p < .01$). This variant also affected the ratios of phytosterols to cholesterol precursors, and carriers of the risk genotype showed a significantly ($p = .027$) lower campesterol:desmosterol ratio as compared to carriers of the minor allele.

Other tested variants were not significantly associated with the odds of developing gallstones (all $p > .05$; genotype distribution and association tests are presented in Material S1). In contrast to data known for adult patients, we did not detect a difference in the distribution of the *UGT1A1* genotype between cases and controls ($p > .05$, Table S2), albeit carriers of the *UGT1A1* variant had increased serum bilirubin concentrations ($p < .01$). Table S3 demonstrates that the *UGT1A1* polymorphism did not affect the clinical characteristics of our patients. Since previous studies demonstrated that the effects of this variant on gallstone incidence are sex-specific, we performed analyses separately for males and females. Tables S4A,B show that we did not detect effects of this variant on gallstone risk in either of the genders (all $p > .05$).

4 | DISCUSSION

In our study, we combined the analysis of the common *ABCG8*, *ABCB4*, *NPC1L1* and *UGT1A1* genetic variants and serum sterol

TABLE 2 Distribution of *ABCG8* alleles and genotypes in children with gallstones and in controls

ABCG8 p.D19H alleles/genotypes	Cases	Count of alleles/genotypes	
		Paediatric controls	Adult controls
[D]	394 (92.0)	417 (97.4)	330 (95.9)
[H]	34 (8.0)	11 (2.6)	14 (4.1)
[DD]	182 (85.1)	203 (94.8)	159 (92.5)
[DH]	30 (14.0)	11 (5.2)	12 (6.9)
[HH]	2 (.9)	0 (.0)	1 (.6)
Armitage's trend test		OR (*p*-value) 4.04 (<.01)	OR (*p*-value) 1.82 (.03)

Note: Genotype frequency differences were assessed by Armitage's trend test (https://ihg.gsf.de/cgi-bin/hw/hwa1.pl).

[H] represents the gallstone risk allele.

Abbreviations: *ABCG8*, hepatobiliary cholesterol hemitransporter; OR, odds ratio.

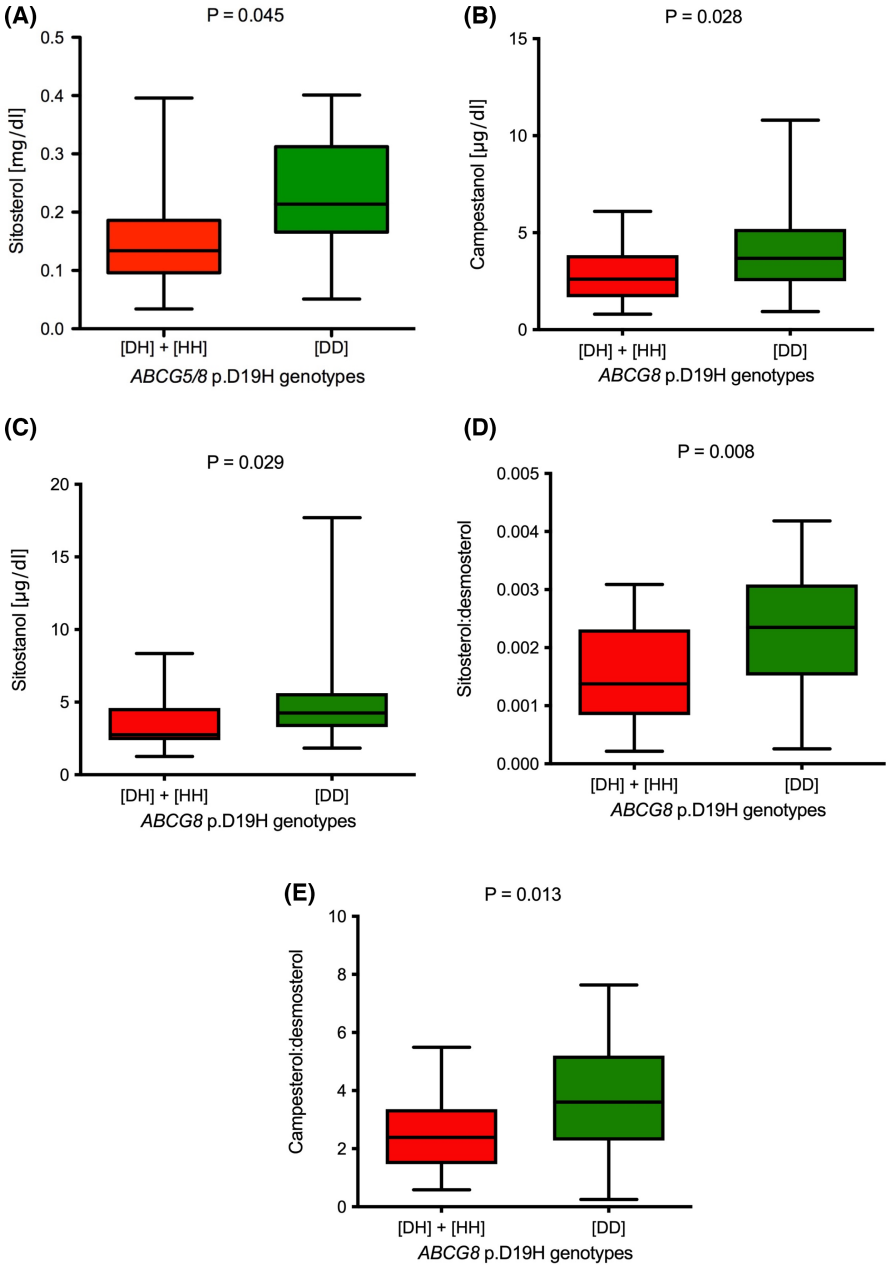

FIGURE 1 Sterol levels in patients with gallstones in relation to the *ABCG8* p.D19H genotype. Individuals carrying the lithogenic allele present with lower phytosterol (panel a) and phytostanol (panels b and c) levels. At the same time, they display lower ratios of phytosterols to cholesterol precursors (panels d and e). Carriers of genotypes were matched based on age and sex; [DD] + [DH] $n = 15$, [HH] $n = 37$; all values were compared using either Student's *t*-test or Mann-Whitney U test

TABLE 3 Distribution of *NPC1L1* rs217434 alleles and genotypes in children with gallstones and in controls

NPC1L1 rs217434 alleles/genotypes	Cases	Count of alleles/genotypes	
		Paediatric controls	Adult controls
[A]	353 (82.5)	332 (77.6)	253 (73.5)
[G]	75 (17.5)	96 (22.4)	91 (26.5)
[AA]	142 (66.4)	127 (59.3)	90 (52.3)
[AG]	69 (33.2)	78 (36.5)	73 (42.4)
[GG]	3 (1.4)	9 (4.2)	9 (5.3)
Armitage's trend test		OR (*p*-value) 1.50 (.06)[a]	OR (*p*-value) 1.90 (<.01)[a]

Note: Genotype frequency differences were assessed by Armitage's trend test (https://ihg.gsf.de/cgi-bin/hw/hwa1.pl).

Abbreviations: NPC1L1, Niemann-Pick C1-Like 1.

[a]Risk conferred by the common allele.

measurements in a cohort of paediatric patients. Here we present data that the *ABCG8* and *NPC1L1* variant increase the risk of gallstone disease already at a young age. In line with the genetic predisposition, analysis of serum sterols indicates that their presence might modulate the cholesterol homeostasis in children towards a more lithogenic bile composition. Overall, our results underscore the existence of a pro-lithogenic pathway which is present already at young age.

Although the association between *ABCG8* p.D19H and gallstone disease in adults has been known for more than a decade,[9,10] we are still lacking a clear mechanistic explanation of the effects of this variant on the function of the protein. The idea that the *ABCG8* p.19H is a 'gain-of-function' mutation of the hepatobiliary cholesterol transporter is the most plausible, but the data to support this hypothesis is rather scarce. Acalovschi et al.[27] demonstrated that carriers of the *ABCG8* [p.19H] allele have lower serum cholesterol levels. This observation is in line with analyses of transgenic mice overexpressing *ABCG8* showing increased biliary sterol concentrations in these animals.[28] Von Kampen et al.[29] extended on these observations by analysis of the HEK293 cells transiently coexpressing *ABCG8* p.19H allele and demonstrated a 3.2 increased transport efficacy of the transporter. Further insights on the function of the [p.19H] variant and on the overall gallstone risk in relation to the cholesterol homeostasis were provided by the analyses of serum sterols using GC/MS. The ABCG5/8 are expressed both in the hepatocytes and in enterocytes. In enterocytes, the ABCG5/G8 seems to pump out more phytosterols than cholesterol, while the heterodimer transporter in the hepatocyte pumps out both, cholesterol and phytosterol (or xenosterols). Hence, liver remains the main organ to keep low levels of xenosterols in humans.[30] Major differences in serum sterols between patients with stones and stone-free individuals were demonstrated previously. As shown by others[19,31,32] and us[18] gallstone patients present with a distorted cholesterol transport and homeostasis as reflected by lower serum phytosterol levels and increased cholesterol precursors in comparison to gallstone-free individuals. This lithogenic phenotype seems to be present already before gallstones develop and points to serum sterols as potential markers of the future gallstone disease both in adults[15] and in children.[33] Analysis of 32 paediatric patients with gallstones from Finland (15 with cholesterol stones and 17 with pigment stones) also showed that patients with cholesterol stones had lower serum plant sterols and higher cholesterol precursors what was also mirrored by sterol contents in the stones.[34] Hence, the results of our study are consistent with data available in the literature. We extended, however, on these observations by analysing sterol homeostasis in children with gallstone in relation to the *ABCG8* p.D19H variant. Data presented in Figure 1 imply that the presence of the risk allele might further potentiate the lithogenic predisposition in children affected by gallstone disease. On the other hand, it has to be kept in mind that although the *ABCG8* polymorphism is associated with gallstone risk, its effects on cholesterol transport might be subtle. As demonstrated in Table 2, the lithogenic *ABCG8* allele [p.19H] was present in 7.5% of gallstone-free adults. Hence, as should be expected for the common variants,[35] the presence of the *ABCG8* p.D19H variant increases the odds of developing gallstones but should be seen only as a predisposition and not as a verdict. Indeed, we could estimate that this polymorphism is responsible for almost 4% of gallstone risk in children. In adults in turn the p.D19H accounts for as much 8% of the total gallstone risk.[10] The *NPC1L1* variants were previously analysed as potential modulators of gallstone risk.[33,36] Nissinen[33] demonstrated that carriers of the *NPC1L1* rs217434 polymorphism have lower campesterol:cholesterol ratios. In our cohort carriers of the minor allele of this polymorphism also showed lower campesterol:cholesterol ratios but the difference was not significant. On the other hand, we detected an association between this variant and increased gallstone risk as well as its effects on the ratio of phytosterols to cholesterol precursors in the analysis including adult patients as controls only (Table 3). Altogether it seems plausible to state that among tested variants the *ABCG8* p.D19H represents the pivotal common genetic risk factor for gallstone disease, already in children.

Although we did not detect any significant association between the *UGT1A1*28*, *ABCB4* or *NPC1L1* rs41279633 variants and gallstone risk in children, it seems that genetic testing might help to identify children at risk of gallstone disease. It remains an open question which additional lithogenic risk factors are needed to come into play to enhance the gallstone risk in carriers of the *ABCG8* variant. According to data presented in Table S1, overweight did not seem to substantially modulate the effects of the studied *ABCG8*polymorphism. In contrast to adults by whom cholesterol gallstones represent the most type of stones,[37] children are thought to develop cholesterol stones far less frequently. The above-presented results might suggest a switch of this paradigm as they point to a higher prevalence of cholesterol stones in paediatric patients, especially in carriers of the lithogenic *ABCG8* variant. Overall, given the increasing prevalence of lithogenic lifestyle changes which are present already at young age, we should focus on developing strategies that will help to identify gallstone-predisposed individuals. If genetic analyses or measurements of serum sterols might be regarded as such markers needs to be investigated in future clinical studies.

It has to be kept in mind, that our study has some limitations. Firstly, the peak of the gallstone prevalence is estimated to be present in the 7th life decade.[38] Hence, although our cohort included many individuals who were 70–90 years old, we have a few who were younger (40–60 years old) and we cannot exclude that they could still develop stones in the latter stages of their lives. Secondly, because of the design of the study, we were not able to compare sterol levels between children with stones and gallstone-free adults. Thirdly, we do not have data on the composition of stones in operated children, this analysis would definitely help to draw more conclusions as to the effects of the studied variants on bile lithogenecity.

In summary, our study provides evidence that, alike adults, children carrying the frequent *ABCG8* variant p.19H are at-risk of gallstones. Also, the *NPC1L1* polymorphism might increase the gallstone risk in young individuals. This is most likely because of

lithogenic cholesterol homeostasis already at a young age. These results indicate that cholesterol gallstones might be more frequent in paediatric patients than estimated. Finally, they underline the presence of a common lithogenic pathway in adults and children, for which precise therapeutic strategies might be envisioned in the future.

CONFLICT OF INTEREST

We declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data of all patients who consented to share the data with other researchers are available upon request from the corresponding author.

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SUPPORTING INFORMATION

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