Mol Diagn Ther (2015) 19:45–52
DOI 10.1007/s40291-014-0128-x

ORIGINAL RESEARCH ARTICLE

Efficacy of Ezetimibe Is Not Related to *NPC1L1* Gene Polymorphisms in a Pilot Study of Chilean Hypercholesterolemic Subjects

Tomás Zambrano · Nicolás Saavedra · Fernando Lanas · José Caamaño · Luis A. Salazar

Published online: 15 January 2015

Abstract

Background and Objective Niemann-Pick C1 Like 1 (NPC1L1) is a multi-transmembrane transport protein highly expressed in the small intestine. It mediates sterol transfer throughout the brush border membrane of enterocytes, becoming essential for intestinal cholesterol absorption and ensuing whole-body cholesterol homeostasis. This protein is targeted by ezetimibe, a potent cholesterol absorption inhibitor. Single nucleotide polymorphisms (SNPs) in *NPC1L1* have been associated to variation in both plasma low-density lipoprotein (LDL) cholesterol levels and lipid-lowering medication with ezetimibe. However, there are no data evaluating the impact of *NPC1L1* variants on Chilean subjects medicated with ezetimibe monotherapy. Therefore, we assessed the contribution of two unexplored *NPC1L1* variants on plasma lipids and response to ezetimibe in Chilean hypercholesterolemic individuals.

Methods Using PCR-restriction fragment length polymorphism (RFLP), we analyzed the SNP distribution of two common variants; −133A>G (rs17655652) and 1679C>G (rs2072183), and their relation with plasma lipids and lipid-lowering response to ezetimibe in 60 hypercholesterolemic Chilean subjects.

Results Genotype distribution for the rs17655652 variant was AA 57 %, 40 % AG and 3 % GG, whereas for the rs2072183 SNP was 57 % CC, 35 % CG and 8 % GG. Minor allele frequencies (MAFs) were 0.23 and 0.26, respectively. No association was observed between *NPC1L1* SNPs and baseline cholesterol. After therapy, none of the polymorphisms affected ezetimibe response in the studied cohort ($P > 0.05$).

Conclusion Data obtained indicates that polymorphisms rs17655652 and rs2072183 were not related to cholesterol variability. Also, lipid-lowering response to ezetimibe is not impacted by the *NPC1L1* polymorphisms studied in Chilean hypercholesterolemic subjects.

Key Points

Polymorphisms in *NPC1L1* are related to differential response to ezetimibe, but reports documented among different ethnicities are inconsistent.

Response to ezetimibe therapy in Chilean individuals evidenced wide inter-individual variability.

The variants studied are not related to plasma lipids and ezetimibe response in hypercholesterolemic subjects from Chile.

1 Introduction

Although cholesterol is crucial to several functions of mammalian cells, elevated plasma cholesterol levels—or

T. Zambrano · N. Saavedra · F. Lanas · L. A. Salazar
Center of Molecular Biology and Pharmacogenetics, Scientific and Technological Bioresource Nucleus (BIOREN), Universidad de La Frontera, Temuco, Chile

J. Caamaño
Universidad Santo Tomás, Temuco, Chile

L. A. Salazar (✉)
Departamento de CienciasBásicas, Facultad de Medicina, Centro de Biología Molecular and Farmacogenética, Universidad de La Frontera, Av. Francisco Salazar 01145, Casilla 54-D, Temuco, Chile
e-mail: luis.salazar@ufrontera.cl

hypercholesterolemia—is a well-established risk factor of atherosclerosis and major cardiovascular events [1]. Plasmatic levels of cholesterol are tightly regulated by three major metabolic processes; dietary absorption, de novo synthesis and biliary clearance and excretion [2]. About 50 % of dietary cholesterol is absorbed, where two-thirds is from the bile while the other one-third is derived from the diet [3], influencing endogenous cholesterol biosynthesis as well [4]. In contrast to the long-standing hypothesis indicating that cholesterol uptake was related to a passive diffusion process, in the early 1990s evidence showed that dietary cholesterol was absorbed through a protein-mediated mechanism [5], and discovery of inhibitors selectively blocking cholesterol absorption supported that this process is via a specific transporter [6].

Ezetimibe is a drug that blocks dietary and biliary cholesterol absorption by the small intestine [7]. It was approved by the US Food and Drug Administration (FDA) in 2002 for the treatment of primary hypercholesterolemia [8]. Contrary to the conventional rationale in drug design, it started being used as a lipid-lowering drug without a clear understanding of the molecular target and long before it was identified. Ezetimibe undergoes rapid and extensive metabolization to its phenolic glucuronide in the intestinal wall and is excreted into the bile, delivering the drug back to the intestinal lumen where it can inhibit cholesterol absorption. It has a plasma half-life of approximately 22 h, which allows for once-daily dosing [9]. Both, ezetimibe and its glucuronide are excreted mostly by feces (90 %) and urine (10 %). Ezetimibe does not influence the cytochrome P450 enzymes. Hence, no significant interactions occur with most medications [10, 11]. Ezetimibe also reduces plasma phytosterol levels, sitosterol and campesterol in patients with sitosterolemia, a condition caused by mutations in ABCG5/G8 co-transporters [12, 13]. Identification of ezetimibe, a potent cholesterol absorption inhibitor, finally confirmed that cholesterol uptake is mediated by a specific transport protein [14]. Intense efforts were made to elucidate this putative protein, hypothesizing that it should be in direct contact with the intestinal luminal content, expressed on the brush border membrane surface of enterocytes and to contain sequence motifs known to interact with sterols. Through a genomic/bioinformatics approach [15], the only candidate gene resulted in Niemann-Pick C1 Like 1 (NPC1L1), known to share diverse features related to a sterol transporter [16, 17].

NPC1L1 plays a fundamental role in cholesterol homeostasis, reducing up to 70 % of cholesterol intake by the intestinal epithelium in NPC1L1 knockout mice [15]. In Npc1l1(−/−)/ApoE (−/−) mice, lack of NPC1L1 inhibits cholesterol absorption, reduces plasma cholesterol and inhibits the development and progression of atherosclerosis [18], thereby establishing that NPC1L1 is critical for cholesterol uptake. A following investigation determined that NPC1L1 is the unequivocal direct molecular target of ezetimibe [19]. In humans, ezetimibe effectively lowers plasmatic cholesterol by 15–20 % and is well tolerated [20–22]. It also promotes a compensatory increase of cholesterol synthesis [23]. However, a significant interindividual variability has been documented in terms of intestinal cholesterol absorption and low-density lipoprotein (LDL)-C reduction at baseline and after ezetimibe treatment. LDL-C levels in response to ezetimibe therapy are reported to differ according to NPC1L1 variants. Several genetic variants of this protein were found in low cholesterol absorbers [24] and are consistent with a reduction in plasma levels of low-density lipoproteins [25]. In previous studies, a relationship between ezetimibe treatment and variations at the *NPC1L1* locus was demonstrated [26, 27]. However, to date there are still limited data in relation to NPC1L1 polymorphisms, and no evidence exists in Chilean populations. Therefore, the aim of this study was to investigate the possible effect of two common variants on the response to 10 mg/day/1 month of ezetimibe monotherapy in Chilean hypercholesterolemic subjects.

2 Materials and Methods

2.1 Subjects

Sixty unrelated male and female Chilean individuals, over 18 years old, with a diagnosis of hypercholesterolemia, according to NCEP norms [28] were enrolled. After a washout period of four weeks, these subjects underwent treatment with 10 mg/day of ezetimibe (Zient*, Schering-Plough, Puerto Rico) for 1 month, sufficient time to achieve a significant improvement in the lipid profile as demonstrated elsewhere [21, 29, 30]. Subjects with diabetes mellitus, hypothyroidism, pregnancy, kidney and hepatic dysfunction, and those taking oral contraceptives and concomitant hypolipemiant medication, such as statins, niacin or fibrates, were discarded. Characteristics of the study group such as age, hypertension and body mass index were annotated. The study design was approved by the local ethics committee. All individuals gave their written informed consent to participate in the study.

2.2 Biochemical and Molecular Analysis

Two venous blood samples were obtained after a 12-h fast and a third sample obtained for biochemical determinations after ezetimibe treatment was completed. The first sample was collected using EDTA tubes (1 mg/mL) to obtain

genomic DNA from leukocytes by a protocol described elsewhere [31]. Second and last samples were obtained without the addition of anticoagulant for determination of serum glucose and lipid profile using routine enzymatic colorimetric assays [32, 33] before and after ezetimibe medication. LDL-C was determined using Friedewald's formula when triglycerides did not exceed 400 mg/dL (4.8 mmol/L). All determinations were performed in the semi-automatic biochemistry analyzer Humalyzer 3000 (Human, Germany). Accuracy of biochemical procedures was controlled using normal and pathological commercial serums (Human, Germany).

Polymorphisms rs2072183 and rs17655652 located in exon 2 and in the promoter region of *NPC1L1*, respectively, were determined by polymerase chain reaction (PCR) followed by restriction fragment length polymorphism (RFLP). Primers for the rs2072183 single nucleotide polymorphism (SNP) were as follow: 5′-TGCAATGAGTC CCAAGGTGACG-3′ (forward) and 3′-ACCATCTTGCTT TTGTCCCTGGCG-5′ (reverse). For the rs17655652 variant, the primers were 5′-AGGAACAGCCAAGGGCTGA A-3′ (forward) and 3′-AGCAGTGTTAGGGGCTAATAG CGT-5′ (reverse). Each reaction was completed using 10 mM of each dNTP, 1 U *Taq* polymerase and PCR buffer (50 mmol/L KCL, 2 mmol/L $MgCl_2$, 20 mmol/L $(NH_4)_2SO_4$, 75 mmol/L Tris–HCl, pH 9.0, in a 50 µL final reaction volume.

Once the PCR reaction was concluded, products were incubated with *Taq*I (rs2072183) or *Blp*I (rs17655652) for enzymatic digestion, during 60 min, at 37° C in a final volume of 30 µL. The resulting fragments were separated on 2 % agarose gel for 45 min at 100 V, stained with ethidium bromide and visualized on a digital photo-documentation system E-Box 1000 (VilberLourmat, France). Twenty percent of the samples were selected randomly and reanalyzed as quality control criteria. Gels were re-read blindly by two more persons without any change in the genotypes.

2.3 Statistical Analysis

Data analysis was completed using the Sigma Stat software v. 3.5 (SPSS Inc., Chicago IL, USA). Allelic frequencies and genotype distribution were estimated by gene counting and tested for Hardy–Weinberg equilibrium. Lipid values before and after ezetimibe treatment were evaluated by paired *t* test. Gaussian distribution of variables was assessed by Shapiro–Wilk normality test ($\alpha = 0.05$). Coefficient of variation (CV) was also obtained. Since the after-treatment values of triglycerides were the only borderline data ($P = 0.04$), along with similar CVs between therapies, these values were considered and analyzed in the same manner as before-treatment, using the standard deviation as a scatter measure. Due to the low frequency of the recessive genotypes, further association analyses were concluded by clustering genotypes into a dominant inheritance model. Differences in non-continuous variables, genotype and allelic distributions were compared by Chi-square test. Statistical significance was considered at $P < 0.05$.

3 Results

3.1 Clinical Data

Clinical characteristics, along with changes in lipid profile before and after completion of lipid-lowering treatment in the Chilean cohort are summarized in Table 1. As expected, ezetimibe was effective in reducing plasma lipids after 4 weeks, observing an 18 % reduction in TC and a 23 % of LDL-C reduction ($P < 0.0001$). No significant differences were found regarding HDL-C nor triglycerides response after the treatment was completed (P = NS). Despite a mean 23 % reduction in LDL-C levels following lipid-lowering therapy, we observed large interindividual

Table 1 Clinical and laboratory determinations

Variables	HC ($n = 60$)	*P* value
Age, years	51.3 ± 9.4	–
Gender (Female), %	72	–
Ethnicity (Amerindian), %	90	–
Menopause, %	28	–
Cigarette smoking, %	20	–
Systolic pressure, mmHg	132.4 ± 22.6	–
Diastolic pressure, mmHg	79.1 ± 12.0	–
Body mass index, kg/m^2	28.7 ± 4.3	–
WHR	0.918 ± 0.08	–
Total cholesterol, mg/dL		
Basal	271.9 ± 29.1	<0.0001
Treatment	222.4 ± 33.6	
LDL cholesterol, mg/dL		
Basal	180.7 ± 28.5	<0.0001
Treatment	139.7 ± 28.3	
HDL cholesterol, mg/dL		
Basal	44.0 ± 14.2	0.275
Treatment	42.8 ± 12.9	
Triglycerides, mg/dL		
Basal	165.7 ± 56.9	0.053
Treatment	155.9 ± 57.3	

Continuous variables presented as mean ± SD. Ezetimibe treatment results were evaluated by paired *t* test

HC hypercholesterolemic, *BMI* body mass index, *WHR* waist-hip ratio, *LDL* low-density lipoprotein, *HDL* high-density lipoprotein, *WHR* waist-hip ratio

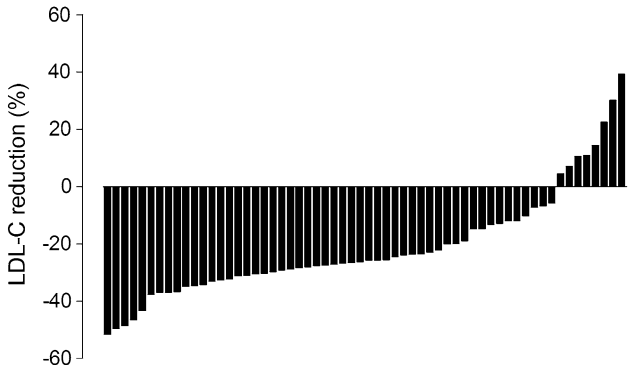

Fig. 1 Individual response to ezetimibe treatment using a 10 mg/day dose during 4 weeks in the studied population. Evaluation was performed using LDL-C as the variable of efficacy

Table 2 Allelic and genotype frequencies for *NPC1L1* polymorphisms in hypercholesterolemic subjects

SNP	Genotype			HWE (*P*)	Alleles	
	AA	*AG*	*GG*		*A*	*G*
−133A>G	57 %	40 %	3 %	0.360	77 %	23 %
(rs17655652)	(34)	(24)	(2)			
	CC	*CG*	*GG*		*C*	*G*
1679C>G	57 %	35 %	8 %	0.502	74 %	26 %
(rs2072183)	(34)	(21)	(5)			

Number of individual in parentheses

HWE Hardy–Weinberg equilibrium

variability when individual response to medication was examined (Fig. 1).

3.2 *NPC1L1* Single Nucleotide Polymorphisms

Unbiased selection of patients was confirmed accordance to Hardy-Weinberg equilibrium when the genotype frequencies of the *NPC1L1* variants were evaluated. Genotype distribution and minor allele frequency (MAF) for the rs17655652 and rs2072183 polymorphisms are shown in Table 2. In addition, MAFs registered in this study and from different cohorts previously reported were listed in Table 3.

3.3 Single Nucleotide Polymorphisms in NPC1L1 and Lipid-Lowering Response to Ezetimibe

Lipid levels according to genotypes are shown in Table 4. As observed, the rs2072183 variant did not influence the lipid profile. Also, SNP rs17655652 was not related to lipid levels in hypercholesterolemic subjects. Furthermore, when using the dominant inheritance model, no significant association was observed between ezetimibe treatment and the two polymorphisms investigated ($P > 0.05$).

4 Discussion

In this study, we have evaluated two single nucleotide polymorphisms in the *NPC1L1* locus, and their relation with plasma lipids and lipid-lowering response to ezetimibe in a cohort of Chilean hypercholesterolemic subjects.

Absorption of cholesterol and plasma levels of the low-density lipoprotein fraction are complicated traits strongly influenced by environmental and but genetic factors. It has been estimated that up to 50 % of the inter-individual plasma LDL-C variation is due to genetic factors [34]. Furthermore, there are multiple genes involved in LDL regulation contributing to the dislypidemic phenotype [24]. After its discovery, NPC1L1 has acquired great relevance as the pharmacological target of ezetimibe, a potent and selective cholesterol absorption inhibitor [35]. However, blocking intestinal cholesterol uptake results in an increased rate of endogenous cholesterol synthesis [36]. This outcome is taken as the norm for the use of combined therapy between ezetimibe and 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGCR) inhibitors known as statins, inducing an additional and substantial decrease of plasma LDL-C [37–41]. Our data support the extensive evidence documenting the potent hypolipemiant action of ezetimibe [39, 40, 42]. As shown in Table 1, four weeks of therapy was sufficient time to achieve a considerable decrease in total and LDL-cholesterol. Although it has been confirmed that four weeks of treatment are adequate to observe significant lipid diminutions [30], two independent reports demonstrated an even earlier and equally effective reduction, starting from the 2nd week of ezetimibe therapy [21, 29]. In spite of studies showing that ezetimibe also improves non-LDL cholesterol fractions, raising high-density lipoprotein cholesterol and reducing triglycerides levels [20–22], we did not observe these effects in the cohort evaluated. Additionally, response to this drug showed high inter-individual variability, resulting in not all patients achieving an adequate clinical response [43]. Since NPC1L1 is considered to be an important factor affecting plasma cholesterol levels and clinical response to ezetimibe, exploring genetic variations in this gene are mandatory to value more precisely this response disparity. Such studies ultimately will lead to improved understanding of the response-related dynamics of the drug. Determination of the frequency of SNPs in a particular population is also practical for translating the variants into clinical use. Factors such as ethnicity and gender, affecting the allelic association with disease, are unlikely to be determined without population based allele frequencies [44].

The minor allele frequencies (MAFs) determined in this study were similar to those previously described in a Spanish population [45]. A second larger study, including Spanish subjects, also showed MAF similarities [46],

Table 3 MAF for *NPC1L1* polymorphisms from ethnically diverse individuals

SNP	Chilean	[45]	[46]			[55]	[58]	
		Hispanic	Caucasian	Hispanic	African American	Taiwanese	Mulao	Han
−133A>G (rs17655652)	23.0	25.0	29.9	18.6	9.2	–	–	–
1679C>G (rs2072183)	26.0	24.0	21.9	28.3	17.9	36.0	29.7	37.2

MAF minor allele frequency

Table 4 Influence of *NPC1L1* polymorphisms on serum lipid profile in HC patients treated with ezetimibe

Variables, mg/dL	−133A>G (rs17655652)			1679C>G (rs2072183)		
	AA (34)	AG+GG (26)	*P* value	CC (34)	CG+GG (26)	*P* value
Total cholesterol						
Basal	267.1 ± 23.2	280.0 ± 34.7	*0.095*	271.6 ± 31.0	272.1 ± 27.5	*0.945*
Treatment	221.4 ± 31.5	224.5 ± 37.8	*0.736*	228.3 ± 35.1	218.5 ± 32.6	*0.301*
% Change	−16.8 ± 11.9	−19.2 ± 13.5	*0.463*	−15.4 ± 13.1	−19.4 ± 11.2	*0.240*
LDL cholesterol						
Basal	180.2 ± 25.1	182.6 ± 33.8	*0.768*	175.4 ± 29.6	186.1 ± 26.8	*0.193*
Treatment	138.8 ± 28.7	140.0 ± 28.8	*0.877*	143.0 ± 31.6	136.7 ± 26.7	*0.454*
% Change	−24.4 ± 23.7	−21.5 ± 19.9	*0.637*	−20.5 ± 25.4	−25.5 ± 18.9	*0.452*
HDL cholesterol						
Basal	47.2 ± 16.9	41.1 ± 8.2	*0.103*	43.0 ± 15.7	47.1 ± 13.0	*0.322*
Treatment	45.2 ± 14.6	41.0 ± 9.6	*0.218*	42.7 ± 14.2	45.7 ± 11.4	*0.421*
% Change	−1.0 ± 23.3	−0.5 ± 19.3	*0.785*	−1.9 ± 23.1	−0.5 ± 21.1	*0.687*
Triglycerides						
Basal	161.1 ± 57.1	168.7 ± 57.0	*0.695*	175.4 ± 60.5	141.1 ± 42.5	*0.061*
Treatment	150.4 ± 56.1	161.9 ± 60.8	*0.515*	166.0 ± 55.9	143.0 ± 56.8	*0.199*
% Change	−8.4 ± 32.9	−7.7 ± 38.0	*0.951*	−5.3 ± 33.1	−8.0 ± 35.5	*0.813*

Number of subjects in parentheses. Values are expressed as mean ± SD and compared by *t* test

LDL low-density lipoprotein, *HDL* high-density lipoprotein

indicative of the common ethnic background as the southern Chilean population had a strong influx of Spanish genes during the colonization of the country [47]. In contrast, MAFs from Caucasians, African American and Asian populations were dissimilar from ours, showing interethnic differences in allele frequencies and highlighting the importance of MAF determination across diverse populations.

The polymorphisms studied in this investigation were not related to baseline low-density lipoprotein levels. This result is likely to be due to the hypercholesterolemic nature of the cohort studied, reducing the power to detect baseline associations. Concerning the lipid-lowering response, our results point out that the two polymorphisms in *NPC1L1*are unrelated to lipid-lowering response using 10 mg/day for 1 month of ezetimibe monotherapy in Chilean hypercholesterolemic individuals.

In relation to the SNP located in the promoter region of *NPC1L1*, results are controversial. This variant was shown to have a significant effect on the gene promoter activity, linking this variation to autosomal dominant hypercholesterolemia (ADH) [45]. In the PROSPER trial [48] the −133A>G polymorphism—or rs17655652—and not other variants, was associated with 6-month LDL-C lowering. However, subjects evaluated in the PROSPER cohort were mostly elderly with a mean age of 75 years, while individuals enrolled in our study had a mean age more than 20 years younger, a covariate that can define lipid response [49]. Also, the MAFs found in the PROSPER trial were more related to a Caucasian phenotype, contrasting with the Amerindian background reported for Chilean subjects [50]. Ethnicity has been reported to be a decisive factor contributing to control of dyslipidemia [51]. Finally, another difference between the studies was that, response in PROSPER was evaluated using pravastatin instead of ezetimibe. Hence, reduction of LDL-C levels was not achieved only by a cholesterol absorption inhibition mechanism. On the contrary, a recently published study supported our results demonstrating that the −133A>G SNP did not influence lipid parameters after 3, 6 or

12 months of ezetimibe monotherapy medication in hyperlipidemic subjects [52]. This publication did find elevation in Apo-A1 levels associated with a particular allele of NPC1L1.

In 2010, a large meta-analysis strongly correlated the rs2072183 variant with total and LDL-C levels [53]. The PROSPER study also reported an association between the rs2072183 SNP with slightly higher baseline LDL-C levels, indicating that *NPC1L1* variants do associate with LDL-C. Maeda et al. [54] demonstrated this variant to be correlated with significantly higher levels of campesterol, a marker of cholesterol absorption, thus enhancing this process in Japanese subjects. In a prior report, this variant was absent in responders to ezetimibe and normal subjects, and was associated with non-responsiveness to this drug [27], probably due to the higher cholesterol absorption by these individuals. There was no relation reported between this allele and basal cholesterol levels in control subjects from different ethnic backgrounds [46]. Similarly, it was not associated with cholesterol levels in subjects from Taiwan [55], similar to results obtained elsewhere [56]. Nevertheless, this variant was associated with higher total cholesterol levels in 127 Japanese subjects with Crohn's disease [57] and was documented to cause different plasma lipid profiles between the Mulao and Han populations in China [58]. Recently, the rs2072183 genotype distribution was found to be significantly different in dyslipidemic vs. healthy Japanese individuals [59]. Furthermore, a significant association was shown between this SNP and LDL-C response using an extreme responder analysis, defined as the upper and lower 10th percentiles of LDL-C responders [46]. However, the influence of this polymorphism was evaluated in a large Caucasian cohort using a combined ezetimibe-statin therapy, which provides greater reductions in LDL-C as combined therapy achieves inhibition in both cholesterol synthesis and absorption [60], thus allowing a marked extreme response to lipid-lowering medication. Moreover, association between the SNP and LDL-C was further supported through haplotype analyses, which was not explored in this study.

According to the common disease-common variant hypothesis, we explored two frequent polymorphisms in a Chilean cohort. Although we did not find an association between the polymorphisms and lipid variability, we cannot exclude that those SNPs with MAF <5 % in *NPC1L1* may be affecting the lipid profile, since it was demonstrated that rare variants account for a significant effect on lipid levels [25]. Additionally, a unique Amerindian component has been demonstrated in our population, which besides affecting the allelic distribution in our cohort, as discussed above, may influence control of plasma lipid levels. Moreover, cholesterol response to lipid-lowering therapies is variable. Many reasons for this variability can be conjectured. Absorption, activation, modification, binding, interactions, transport, degradation and elimination are just a few processes that can influence a particular drug, modifying its action. The two polymorphisms studied only account for a small percentage of the more than 25 previously described variants in NPC1L1 gene [61]. Also, is important to remember that cholesterol absorption is not exclusively regulated by NPC1L1, although explaining about 70 % of the reduction in absorption of dietary cholesterol, other proteins, such as SR-B1 and CD36, are also involved in this process [62, 63]. Lastly, efficiency of cholesterol absorption is also critical, which ranges from 29 to 80 % among healthy individuals [64] and also contributes to differences in plasma levels of LDL-C.

A major limitation in this study is the small sample size, which could contribute some bias of the final results. However, when sample sizes are restricted, statistical power can be overcome by evaluating single nucleotide polymorphisms with MAFs superior than 5 % [65]. Additional studies including a bigger cohort and analyzing simultaneously multiple genes critical to cholesterol homeostasis are necessary. To our knowledge, this is the first study reporting the influence of the *NPC1L1* rs2072183 and rs17655652 single nucleotide polymorphisms on ezetimibe therapy in Chilean subjects. In summary, the results obtained indicate that the two variants studied were not related to cholesterol variability. Additionally, none of the polymorphisms influenced lipid-lowering therapy with ezetimibe.

Acknowledgments This study was supported by Grants from FONDECYT-Chile (No. 1130675). T. Z. was recipient of a fellowship from CONICYT-Chile (No. 21090417).

Disclosure statement None of the authors has a relationship with a for-profit company or institution. No competing financial interests exist.

Conflict of interest The authors have no conflict of interest relevant to the content of this article or a relationship with a for-profit company or institution.

Author contributions Conceived and designed the experiment: LAS TZ FL; Performed the experiments: TZ NS JC; Analyzed the data: TZ NS LAS; Contributed reagents/materials/analysis tools: JC FL LAS; Wrote the paper: TZ LAS.

References

1. Go AS, Mozaffarian D, Roger VL, Benjamin EJ, Berry JD, Borden WB, et al. Heart disease and stroke statistics—2013 update: a report from the American Heart Association. Circulation. 2013;127(1):e6–245.
2. Dietschy JM, Turley SD, Spady DK. Role of liver in the maintenance of cholesterol and low density lipoprotein homeostasis in

different animal species, including humans. J Lipid Res. 1993;34(10):1637–59.
3. Lammert F, Wang DQ. New insights into the genetic regulation of intestinal cholesterol absorption. Gastroenterology. 2005;129(2):718–34.
4. Jones PJ, Pappu AS, Hatcher L, Li ZC, Illingworth DR, Connor WE. Dietary cholesterol feeding suppresses human cholesterol synthesis measured by deuterium incorporation and urinary mevalonic acid levels. Arterioscler Thromb Vasc Biol. 1996;16(10):1222–8.
5. Thurnhofer H, Hauser H. Uptake of cholesterol by small intestinal brush border membrane is protein-mediated. Biochemistry. 1990;29(8):2142–8.
6. Hernandez M, Montenegro J, Steiner M, Kim D, Sparrow C, Detmers PA, et al. Intestinal absorption of cholesterol is mediated by a saturable, inhibitable transporter. Biochim Biophys Acta. 2000;1486(2–3):232–42.
7. Clader JW. The discovery of ezetimibe: a view from outside the receptor. J Med Chem. 2004;47(1):1–9.
8. Earl J, Kirkpatrick P. Fresh from the pipeline. Ezetimibe. Nat Rev Drug Discov. 2003;2(2):97–8.
9. Bruckert E, Giral P, Tellier P. Perspectives in cholesterol-lowering therapy: the role of ezetimibe, a new selective inhibitor of intestinal cholesterol absorption. Circulation. 2003;107(25): 3124–8.
10. Patrick JE, Kosoglou T, Stauber KL, Alton KB, Maxwell SE, Zhu Y, et al. Disposition of the selective cholesterol absorption inhibitor ezetimibe in healthy male subjects. Drug Metab Dispos. 2002;30(4):430–7.
11. Kosoglou T, Statkevich P, Johnson-Levonas AO, Paolini JF, Bergman AJ, Alton KB. Ezetimibe: a review of its metabolism, pharmacokinetics and drug interactions. Clin Pharmacokinet. 2005;44(5):467–94.
12. Davis HR Jr, Zhu LJ, Hoos LM, Tetzloff G, Maguire M, Liu J, et al. Niemann-Pick C1 Like 1 (NPC1L1) is the intestinal phytosterol and cholesterol transporter and a key modulator of whole-body cholesterol homeostasis. J Biol Chem. 2004;279(32): 33586–92.
13. Klett EL, Patel SB. Biomedicine. Will the real cholesterol transporter please stand up. Science. 2004;303(5661):1149–50.
14. van Heek M, Farley C, Compton DS, Hoos L, Davis HR. Ezetimibe selectively inhibits intestinal cholesterol absorption in rodents in the presence and absence of exocrine pancreatic function. Br J Pharmacol. 2001;134(2):409–17.
15. Altmann SW, Davis HR Jr, Zhu LJ, Yao X, Hoos LM, Tetzloff G, et al. Niemann-Pick C1 Like 1 protein is critical for intestinal cholesterol absorption. Science. 2004;303(5661):1201–4.
16. Davies JP, Levy B, Ioannou YA. Evidence for a Niemann-pick C (NPC) gene family: identification and characterization of NPC1L1. Genomics. 2000;65(2):137–45.
17. Carstea ED, Morris JA, Coleman KG, Loftus SK, Zhang D, Cummings C, et al. Niemann-Pick C1 disease gene: homology to mediators of cholesterol homeostasis. Science. 1997;277(5323): 228–31.
18. Davis HR Jr, Hoos LM, Tetzloff G, Maguire M, Zhu LJ, Graziano MP, et al. Deficiency of Niemann-Pick C1 Like 1 prevents atherosclerosis in ApoE−/− mice. Arterioscler Thromb Vasc Biol. 2007;27(4):841–9.
19. Garcia-Calvo M, Lisnock J, Bull HG, Hawes BE, Burnett DA, Braun MP, et al. The target of ezetimibe is Niemann-Pick C1-Like 1 (NPC1L1). Proc Natl Acad Sci USA. 2005;102(23): 8132–7.
20. Bays HE, Moore PB, Drehobl MA, Rosenblatt S, Toth PD, Dujovne CA, et al. Effectiveness and tolerability of ezetimibe in patients with primary hypercholesterolemia: pooled analysis of two phase II studies. Clin Ther. 2001;23(8):1209–30.
21. Dujovne CA, Ettinger MP, McNeer JF, Lipka LJ, LeBeaut AP, Suresh R, et al. Efficacy and safety of a potent new selective cholesterol absorption inhibitor, ezetimibe, in patients with primary hypercholesterolemia. Am J Cardiol. 2002;90(10):1092–7.
22. Knopp RH, Gitter H, Truitt T, Bays H, Manion CV, Lipka LJ, et al. Effects of ezetimibe, a new cholesterol absorption inhibitor, on plasma lipids in patients with primary hypercholesterolemia. Eur Heart J. 2003;24(8):729–41.
23. Sudhop T, Lutjohann D, Kodal A, Igel M, Tribble DL, Shah S, et al. Inhibition of intestinal cholesterol absorption by ezetimibe in humans. Circulation. 2002;106(15):1943–8.
24. Burnett JR, Hooper AJ. Common and rare gene variants affecting plasma LDL cholesterol. Clin Biochem Rev. 2008;29(1):11–26.
25. Cohen JC, Pertsemlidis A, Fahmi S, Esmail S, Vega GL, Grundy SM, et al. Multiple rare variants in NPC1L1 associated with reduced sterol absorption and plasma low-density lipoprotein levels. Proc Natl Acad Sci USA. 2006;103(6):1810–5.
26. Hegele RA, Guy J, Ban MR, Wang J. NPC1L1 haplotype is associated with inter-individual variation in plasma low-density lipoprotein response to ezetimibe. Lipids Health Dis. 2005;4:16.
27. Wang J, Williams CM, Hegele RA. Compound heterozygosity for two non-synonymous polymorphisms in NPC1L1 in a non-responder to ezetimibe. Clin Genet. 2005;67(2):175–7.
28. Executive summary of the third report of The National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, And Treatment of High Blood Cholesterol In Adults (Adult Treatment Panel III). JAMA. 2001;285(19):2486–97.
29. Jeu L, Cheng JW. Pharmacology and therapeutics of ezetimibe (SCH 58235), a cholesterol-absorption inhibitor. Clin Ther. 2003;25(9):2352–87.
30. Kishimoto M, Sugiyama T, Osame K, Takarabe D, Okamoto M, Noda M. Efficacy of ezetimibe as monotherapy or combination therapy in hypercholesterolemic patients with and without diabetes. J Med Invest. 2011;58(1–2):86–94.
31. Salazar LA, Hirata MH, Cavalli SA, Machado MO, Hirata RD. Optimized procedure for DNA isolation from fresh and cryopreserved clotted human blood useful in clinical molecular testing. Clin Chem. 1998;44(8 Pt 1):1748–50.
32. Burstein M, Scholnick HR, Morfin R. Rapid method for the isolation of lipoproteins from human serum by precipitation with polyanions. J Lipid Res. 1970;11(6):583–95.
33. Fossati P, Prencipe L. Serum triglycerides determined colorimetrically with an enzyme that produces hydrogen peroxide. Clin Chem. 1982;28(10):2077–80.
34. Heller DA, de Faire U, Pedersen NL, Dahlen G, McClearn GE. Genetic and environmental influences on serum lipid levels in twins. N Engl J Med. 1993;328(16):1150–6.
35. Miura S, Saku K. Ezetimibe, a selective inhibitor of the transport of cholesterol. Intern Med. 2008;47(13):1165–70.
36. Santosa S, Varady KA, AbuMweis S, Jones PJ. Physiological and therapeutic factors affecting cholesterol metabolism: does a reciprocal relationship between cholesterol absorption and synthesis really exist? Life Sci. 2007;80(6):505–14.
37. Ballantyne CM, Weiss R, Moccetti T, Vogt A, Eber B, Sosef F, et al. Efficacy and safety of rosuvastatin 40 mg alone or in combination with ezetimibe in patients at high risk of cardiovascular disease (results from the EXPLORER study). Am J Cardiol. 2007;99(5):673–80.
38. Rodney RA, Sugimoto D, Wagman B, Zieve F, Kerzner B, Strony J, et al. Efficacy and safety of coadministration of ezetimibe and simvastatin in African–American patients with primary hypercholesterolemia. J Natl Med Assoc. 2006;98(5): 772–8.
39. Pearson TA, Denke MA, McBride PE, Battisti WP, Brady WE, Palmisano J. A community-based, randomized trial of ezetimibe added to statin therapy to attain NCEP ATP III goals for LDL

cholesterol in hypercholesterolemic patients: the ezetimibe add-on to statin for effectiveness (EASE) trial. Mayo Clin Proc. 2005;80(5):587–95.
40. Davidson MH, Ballantyne CM, Kerzner B, Melani L, Sager PT, Lipka L, et al. Efficacy and safety of ezetimibe coadministered with statins: randomised, placebo-controlled, blinded experience in 2382 patients with primary hypercholesterolemia. Int J Clin Pract. 2004;58(8):746–55.
41. Davidson MH, McGarry T, Bettis R, Melani L, Lipka LJ, LeBeaut AP, et al. Ezetimibe coadministered with simvastatin in patients with primary hypercholesterolemia. J Am Coll Cardiol. 2002;40(12):2125–34.
42. Stein E, Stender S, Mata P, Sager P, Ponsonnet D, Melani L, et al. Achieving lipoprotein goals in patients at high risk with severe hypercholesterolemia: efficacy and safety of ezetimibe coadministered with atorvastatin. Am Heart J. 2004;148(3):447–55.
43. Pisciotta L, Fasano T, Bellocchio A, Bocchi L, Sallo R, Fresa R, et al. Effect of ezetimibe coadministered with statins in genotype-confirmed heterozygous FH patients. Atherosclerosis. 2007;194(2):e116–22.
44. Cross DS, Ivacic LC, Stefanski EL, McCarty CA. Population based allele frequencies of disease associated polymorphisms in the Personalized Medicine Research Project. BMC Genet. 2010;11:51.
45. Martin B, Solanas-Barca M, Garcia-Otin AL, Pampin S, Cofan M, Ros E, et al. An NPC1L1 gene promoter variant is associated with autosomal dominant hypercholesterolemia. Nutr Metab Cardiovasc Dis. 2010;20(4):236–42.
46. Simon JS, Karnoub MC, Devlin DJ, Arreaza MG, Qiu P, Monks SA, et al. Sequence variation in NPC1L1 and association with improved LDL-cholesterol lowering in response to ezetimibe treatment. Genomics. 2005;86(6):648–56.
47. Greenberg JH. The settlement of the Americas; a comparison of the linguistic, dental, and genetic evidence. Curr Antrophol. 1986;27(5):18.
48. Polisecki E, Peter I, Simon JS, Hegele RA, Robertson M, Ford I, et al. Genetic variation at the NPC1L1 gene locus, plasma lipoproteins, and heart disease risk in the elderly. J Lipid Res. 2010;51(5):1201–207.
49. Talmud PJ, Berglund L, Hawe EM, Waterworth DM, Isasi CR, Deckelbaum RE, et al. Age-related effects of genetic variation on lipid levels: The Columbia University BioMarkers Study. Pediatrics. 2001;108(3):E50.
50. Lagos J, Zambrano T, Rosales A, Salazar LA. Influence of SREBP-2 and SCAP gene polymorphisms on lipid-lowering response to atorvastatin in a cohort of Chilean subjects with Amerindian background. Mol Diagn Ther. 2014;18(4):435–43.
51. Hendrix KH, Riehle JE, Egan BM. Ethnic, gender, and age-related differences in treatment and control of dyslipidemia in hypertensive patients. Ethn Dis. 2005;15(1):11–6.
52. Zsiros N, Bodor M, Varga V, Berta E, Balogh I, Seres I, et al. The c.−133A>G polymorphism in NPC1L1 gene influences the efficacy of ezetimibe monotherapy on apolipoprotein A1 in hyperlipidemic patients. Pharmazie. 2014;69(6):424–9.
53. Teslovich TM, Musunuru K, Smith AV, Edmondson AC, Stylianou IM, Koseki M, et al. Biological, clinical and population relevance of 95 loci for blood lipids. Nature. 2010;466(7307):707–13.
54. Maeda T, Honda A, Ishikawa T, Kinoshita M, Mashimo Y, Takeoka Y, et al. A SNP of NPC1L1 affects cholesterol absorption in Japanese. J Atheroscler Thromb. 2010;17(4):356–60.
55. Chen CW, Hwang JJ, Tsai CT, Su YN, Hsueh CH, Shen MJ, et al. The g.−762T>C polymorphism of the NPC1L1 gene is common in Chinese and contributes to a higher promoter activity and higher serum cholesterol levels. J Hum Genet. 2009;54(4):242–7.
56. Zhao HL, Houweling AH, Vanstone CA, Jew S, Trautwein EA, Duchateau GS, et al. Genetic variation in ABC G5/G8 and NPC1L1 impact cholesterol response to plant sterols in hypercholesterolemic men. Lipids. 2008;43(12):1155–64.
57. Osaki R, Imaeda H, Takahashi K, Fujimoto T, Takeuchi T, Fujiyama Y, et al. Polymorphisms of the Niemann-Pick C1-like 1 gene in a Japanese population. Biomed Rep. 2013;1(1):156–60.
58. Miao L, Yin RX, Hu XJ, Wu DF, Cao XL, Li Q, et al. Association of rs2072183 SNP and serum lipid levels in the Mulao and Han populations. Lipids Health Dis. 2012;11:61.
59. Kashiwabara Y, Kobayashi Y, Koba S, Kohyama N, Ohbayashi M, Murayama JI, et al. Gene polymorphism and frequencies of the NPC1L1 Gene (rs2072183, rs217434 and rs217428) in Japanese patients with dyslipidemia. J Clin Pharm Ther. 2014;39(5):551–4.
60. Grigore L, Norata GD, Catapano AL. Combination therapy in cholesterol reduction: focus on ezetimibe and statins. Vasc Health Risk Manag. 2008;4(2):267–78.
61. Huff MW, Pollex RL, Hegele RA. NPC1L1: evolution from pharmacological target to physiological sterol transporter. Arterioscler Thromb Vasc Biol. 2006;26(11):2433–8.
62. Wang DQ. Regulation of intestinal cholesterol absorption. Annu Rev Physiol. 2007;69:221–48.
63. Ge L, Wang J, Qi W, Miao HH, Cao J, Qu YX, et al. The cholesterol absorption inhibitor ezetimibe acts by blocking the sterol-induced internalization of NPC1L1. Cell Metab. 2008;7(6):508–19.
64. Bosner MS, Wolff AA, Ostlund RE Jr. Lack of effect of cholesterol esterase inhibitor CVT-1 on cholesterol absorption and LDL cholesterol in humans. Cardiovasc Drugs Ther. 1999;13(5):449–54.
65. Gorlov IP, Gorlova OY, Sunyaev SR, Spitz MR, Amos CI. Shifting paradigm of association studies: value of rare single-nucleotide polymorphisms. Am J Hum Genet. 2008;82(1):100–12.