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Ezetimibe markedly attenuates hepatic cholesterol accumulation and improves liver function in the lysosomal acid lipase-deficient mouse, a model for cholesteryl ester storage disease

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ABSTRACT

Lysosomal acid lipase (LAL) plays a critical role in the intracellular handling of lipids by hydrolyzing cholesteryl esters (CE) and triacylglycerols (TAG) contained in newly internalized lipoproteins. In humans, mutations in the LAL gene result in cholesteryl ester storage disease (CESD), or in Wolman disease (WD) when the mutations cause complete loss of LAL activity. A rat model for WD and a mouse model for CESD have been described. In these studies we used LAL-deficient mice to investigate how modulating the amount of intestinally-derived cholesterol reaching the liver might impact its mass, cholesterol content, and function in this model. The main experiment tested if ezetimibe, a potent cholesterol absorption inhibitor, had any effect on CE accumulation in mice lacking LAL. In male *Lal*$^{-/-}$ mice given ezetimibe in their diet (20 mg/day/kg bw) for 4 weeks starting at 21 days of age, both liver mass and hepatic cholesterol concentration (mg/g) were reduced to the extent that whole-liver cholesterol content (mg/organ) in the treated mice (74.3 ± 3.4) was only 56% of that in those not given ezetimibe (133.5 ± 6.7). There was also a marked improvement in plasma alanine aminotransferase (ALT) activity. Thus, minimizing cholesterol absorption has a favorable impact on the liver in CESD.

1. Introduction

Cholesteryl esters (CE), triacylglycerols (TAG), unesterified cholesterol (UC), other lipids, and proteins contained in various types of lipoproteins enter lysosomes during their clearance from the plasma largely via receptor-mediated endocytosis [1,2]. The CE and TAG are hydrolyzed by lysosomal acid lipase (LAL) (EC 3.1.1.13) thereby generating UC, free fatty acids, and amino acids for cellular utilization [3]. In humans, loss of function mutations in the LIPA gene result in Wolman disease (WD) or cholesteryl ester storage disease (CESD). WD is a severe, early-onset illness caused by complete absence of LAL activity and typically leads to death in infancy, whereas CESD is a milder, later-onset disease resulting from partial LAL deficiency [4,5]. In both disorders, hepatomegaly and a marked increase in tissue CE levels are evident [6–8]. A rat model for WD has been described [6], and for CESD, a mouse model has been extensively characterized [7–9].

In LAL deficiency, the degree of CE accumulation is especially pronounced in the liver because this organ is not only the major site of clearance of low density lipoproteins (LDL) and very low density lipoprotein remnants (VLDLr), all of which contain a lot of CE [10,11], but also it receives essentially all of the cholesterol that is absorbed from the small intestine, 70–80% of which is esterified [12,13]. Ezetimibe, a potent and selective inhibitor of cholesterol absorption, profoundly alters intrahepatic cholesterol handling in multiple ways that result in lowering of plasma LDL-cholesterol levels [14–16]. Surprisingly, ezetimibe treatment has also been shown to be effective in reducing development of non-alcoholic fatty liver disease (NAFLD) and related conditions in humans [17,18] and in multiple animal models for this disorder [19–26]. While the fundamental causes of NAFLD are different from the defects that lead to hepatic CE and TAG accumulation in CESD and WD, we were nevertheless interested in determining how a mouse model for CESD might respond to ezetimibe, particularly as we had previously found a favorable effect of this agent in a mouse model for NPC1 disease, another lysosomal cholesterol storage disorder [27].

Abbreviations: ALT, alanine aminotransferase; CE, cholesteryl ester; LAL, lysosomal acid lipase; LIPA, gene that encodes LAL; NPC1L1, Niemann-Pick C1-Like1; SI, small intestine; TAG, triacylglycerol; UC, unesterified cholesterol.

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2. Materials and methods

2.1. Animals and diets

Lal$^{+/+}$ and *Lal*$^{-/-}$ mice were generated from heterozygous breeding stock, all on an FVB/N strain background. The litters were weaned at 21 days and genotyped using an ear notch. Primer sequences for the PCR method used were kindly supplied by Dr. Hong Du. Depending on the type of experiment, some of the mice were maintained on a pelleted chow diet from weaning until 10 weeks of age. However, the majority of mice were started on their respective experimental diet from the day of weaning until studied four weeks later at 49 days of age. The gender of the mice varied with each experiment but in most instances males were used. A basal low-cholesterol, low-fat rodent chow diet (No. 7001, Harlan Teklad, Madison, WI) was used in all experiments. This formulation had an inherent cholesterol content of 0.02% (w/w) [28]. For one study, the meal form of this diet was made to contain either additional cholesterol (0.5% w/w) or surfomer (AOMA, a co-polymer of maleic acid and an 18-carbon α-olefin) [29] at a level of 2% (w/w) [15]. Ezetimibe was added to the diet at a level of 0.0125% (w/w) which provided an approximate dose of 20 mg/day/kg bw [27]. All mice were group-housed in a light-cycled room and were studied in the fed state toward the end of the dark phase of the lighting cycle. All experiments were approved by the Institutional Animal Care and Use Committee at the University of Texas Southwestern Medical Center.

2.2. Quantitation of total, unesterified and esterified cholesterol in tissues and plasma, and of plasma ALT activity

After exsanguination of the mice, the liver, and in some experiments the entire small intestine as well, were removed, rinsed, blotted and weighed. Depending on the planned measurements, aliquots of liver, and the whole small intestine were placed in chloroform:methanol (2:1 v/v) for measurement of the esterified (CE) and unesterified cholesterol (UC) fractions, and the total triacylglycerol (TAG) concentration [27,30]. When only a direct measure of the total cholesterol (TC) concentration (CE + UC) was required, an aliquot of liver tissue or plasma was digested directly in alcoholic KOH. All cholesterol quantitation was done using gas chromatography [31]. Plasma total cholesterol concentrations were expressed as mg/dl. For the liver and small intestine, the total cholesterol concentration was expressed as mg/g tissue. To obtain whole organ cholesterol content (mg/organ), the total cholesterol concentration was multiplied by the respective whole organ weight. Plasma ALT activities (units/L) were measured by a commercial laboratory.

2.3. Data analysis

With one exception, all values are the mean ± SEM for the specified number of individual animals. For the plasma ALT data in the *Lal*$^{-/-}$ mice at different ages, the values are for individual mice. GraphPad Prism 6 software (GraphPad Software Inc., San Diego, CA) was used for all statistical analyses. Depending on the design of the experiment, differences between mean values were tested for statistical significance ($p < 0.05$) by an unpaired Student's *t*-test, or a 2-way analysis of variance (ANOVA) with genotype and type of dietary addition as variables.

3. Results

The main objective of the first experiment was to define, in quantitative terms, the impact of LAL deficiency on liver mass and cholesterol content in young adult mice of the FVB/N strain

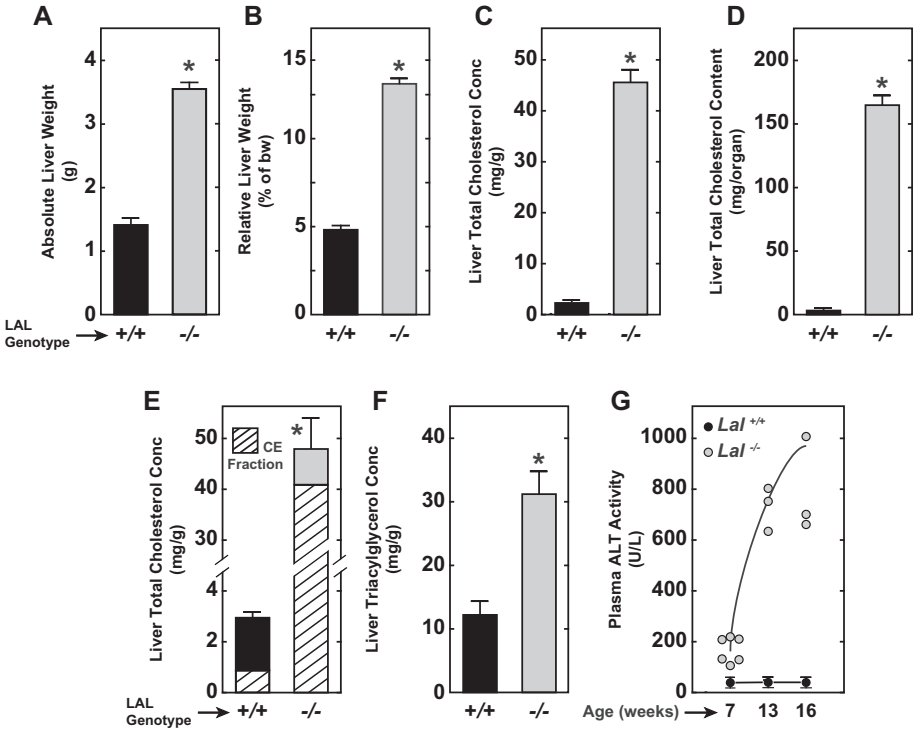

Fig. 1. *Lal*$^{-/-}$ mice manifest marked hepatomegaly and elevations in hepatic cholesterol and triacylglycerol content, and in plasma ALT activity. Absolute (A) and relative (B) liver weights, liver total cholesterol concentration (C) and total cholesterol content (D) in 10-week-old male *Lal*$^{-/-}$ and *Lal*$^{+/+}$ mice fed a basal rodent chow diet. Most of the additional cholesterol in the livers of *Lal*$^{-/-}$ mice was esterified (E), and this, together with an increased triacylglycerol content (F), markedly raised hepatic lipid content (data not shown). Plasma ALT activity in the *Lal*$^{-/-}$ mice increased dramatically with age (G). The data in E and F are from 10-week-old female, and 7-week old male mice, respectively. In G, all values are for male mice at 7, 13 and 16 weeks. In A–F all values are the mean ± SEM of data from 4 to 6 animals in each group. For the ALT activity data, values for the *Lal*$^{+/+}$ mice are the mean ± SEM of data from 4 to 6 mice at each age, whereas for the *Lal*$^{-/-}$ mice the data represent values in individual animals $^{*}p < 0.05$.

maintained on a basal rodent chow diet with no additions. As shown in Fig. 1A and B, respectively, the absolute and relative liver weights in *Lal*$^{-/-}$ males at 10 weeks of age were 2.5- and 2.7-fold greater than in their matching *Lal*$^{+/+}$ controls. The total (esterified + unesterified) cholesterol concentration in the LAL-deficient mice was 20.4-fold greater than in their *Lal*$^{+/+}$ controls (Fig. 1C). Even more striking was the genotypic difference in whole-liver cholesterol content (mg/organ); 164.8 ± 7.4 in the *Lal*$^{-/-}$ mice vs 3.2 ± 0.2 in their *Lal*$^{+/+}$ controls (Fig. 1D). Although the data are not shown, at the time of weaning (21 days of age), whole-liver cholesterol content in the *Lal*$^{-/-}$ mice averages about 22 mg/organ vs about 2 mg/organ in their *Lal*$^{+/+}$ littermates. Thus, between weaning and 70 days of age, whole-liver cholesterol content in the LAL-deficient animals expands by more than ~140 mg, while it remains almost unchanged in the *Lal*$^{+/+}$ mice.

In female mice at 10 weeks of age, the genotypic difference in hepatic total cholesterol concentration (Fig. 1E) was similar to that found for males (Fig. 1C). What Fig. 1E also illustrates is the fraction of the total cholesterol that is present in esterified form. This was 85.3% and 29.6% in the *Lal*$^{-/-}$ and *Lal*$^{+/+}$ mice, respectively. Triacylglycerol concentrations were not determined in those livers but were measured in the livers of 7-week-old males. As shown in Fig. 1F, the liver TAG concentration was elevated 2.6-fold in the LAL-deficient mice. The data in Fig. 1G show that by 7 weeks of age the *Lal*$^{-/-}$ mice manifested higher plasma ALT levels which continued to rise with age. In their *Lal*$^{+/+}$ controls, ALT levels remained at <40 units/L.

The changes in the small intestine of the LAL-deficient mice paralleled those in the liver but were less pronounced, as illustrated by the data for 10 week-old females (Table 1). Most striking was the 12.8-fold greater CE content in the small intestine of the *Lal*$^{-/-}$ mice. Overall, the data in Fig. 1 and Table 1 are comparable to those initially reported by Dr. Grabowski's laboratory for *Lal*$^{-/-}$ mice on a mixed strain (129/Sv:CF-1) background [7,8].

The next set of experiments compared the effects of manipulating the amount of cholesterol reaching the liver from the small intestine. This was done by feeding female LAL-deficient mice, along with their *Lal*$^{+/+}$ controls, either a cholesterol-enriched diet, or one containing a high level of surfomer, a polymeric inhibitor of cholesterol absorption that has been tested in humans and animal models [15,29,32]. The mice received these diets from 3 to 7 weeks of age. In the cholesterol-fed groups, relative liver weight increased significantly in the *Lal*$^{-/-}$ mice but was unchanged in their *Lal*$^{+/+}$ counterparts. In the surfomer-treated *Lal*$^{-/-}$ mice, there was a marginal, but significant reduction compared to the value for the LAL-deficient mice given the basal diet alone (Fig. 2A). The high-cholesterol diet clearly raised the liver total cholesterol content in mice of both genotypes, whereas for the surfomer-treated groups there was no significant effect in the mice of either genotype (Fig. 2B). A trend toward a lower TC content was nevertheless evident in the *Lal*$^{-/-}$ mice given surfomer. The diet-related changes in plasma ALT activity amongst the three groups of LAL-deficient mice (Fig. 2C) paralleled those seen for liver TC content.

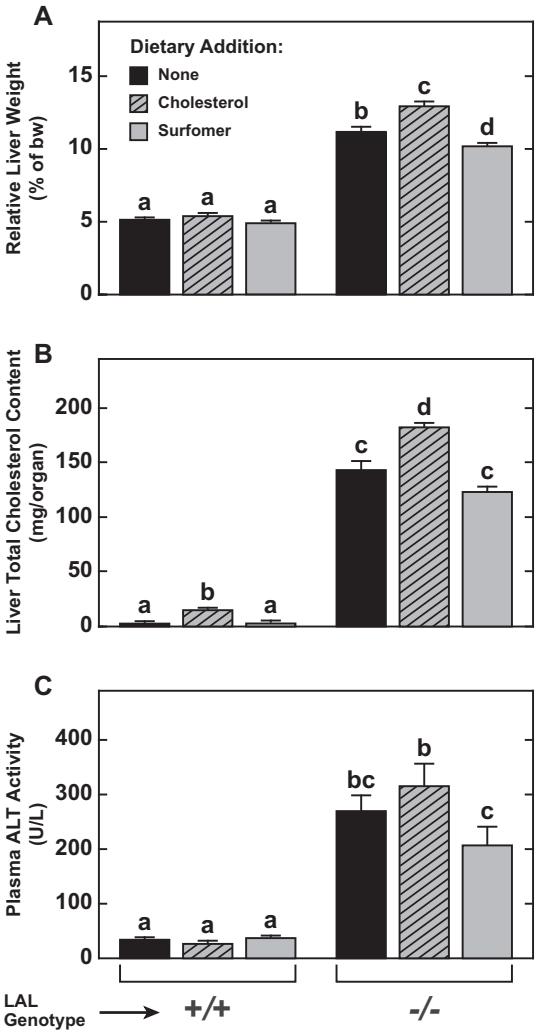

Fig. 2. Modulation of liver weight, cholesterol content and function in *Lal*$^{-/-}$ mice by manipulation of the amount of cholesterol absorbed from the small intestine. Female *Lal*$^{-/-}$ and matching *Lal*$^{+/+}$ mice were fed a rodent chow diet alone or containing either added cholesterol (0.5% w/w) or surfomer (2% w/w) for 28 days starting at 21 days of age. All data are for female mice. Values are the mean ± SEM of data from 5 or 6 mice for every group except the *Lal*$^{-/-}$ mice fed the high cholesterol diet where there were 4 mice. Different letters (a–d) denote statistically different values ($p < 0.05$) as determined by 2-way ANOVA with genotype and type of dietary addition as variables.

The data in Fig. 2 prompted the question of what impact ezetimibe, a highly potent and selective inhibitor of cholesterol absorption, might have on hepatic cholesterol content in LAL-deficient mice, particularly if treatment were started when they were weaned at 21 days of age. After 4 weeks of ezetimibe treatment, the *Lal*$^{-/-}$ mice showed significantly lower absolute (Fig. 3A) and relative liver weights (Fig. 3B). No such change was seen in the *Lal*$^{+/+}$ controls. Plasma total cholesterol concentrations varied little with ezetimibe treatment in mice of either genotype (Fig. 3C). In the case of hepatic TC concentrations, however, there was a decisive reduction in the LAL-deficient group given ezetimibe (Fig. 3D). In these same livers the TAG concentration fell by 20% with ezetimibe treatment (data not shown). When the reduction in liver mass in these mice was taken into account, and the liver cholesterol data were expressed on a whole-organ basis, there was essentially a halving of the amount of cholesterol in the livers of the *Lal*$^{-/-}$ mice receiving ezetimibe (Fig. 3E). This reduction was

Table 1
Weight and cholesterol content of small intestine in *Lal*$^{+/+}$ and *Lal*$^{-/-}$ mice.

Parameters	*Lal*$^{+/+}$	*Lal*$^{-/-}$
Whole animal weight (g)	23.3 ± 0.6	25.0 ± 0.9
Absolute SI weight (g)	0.901 ± 0.040	1.201 ± 0.06*
Relative SI weight (% of bw)	3.87 ± 0.20	4.80 ± 0.11*
Total cholesterol concentration (mg/g)	2.87 ± 0.11	6.73 ± 0.32*
Total cholesterol content (mg/organ)	2.59 ± 0.33	8.03 ± 0.25*
Proportion of cholesterol esterified (%)	4.0 ± 0.3	51.2 ± 2.1*

Data are for female mice at 10 weeks of age. Values are mean ± 1 SEM of data from 5 mice of each genotype.
* $p < 0.05$.

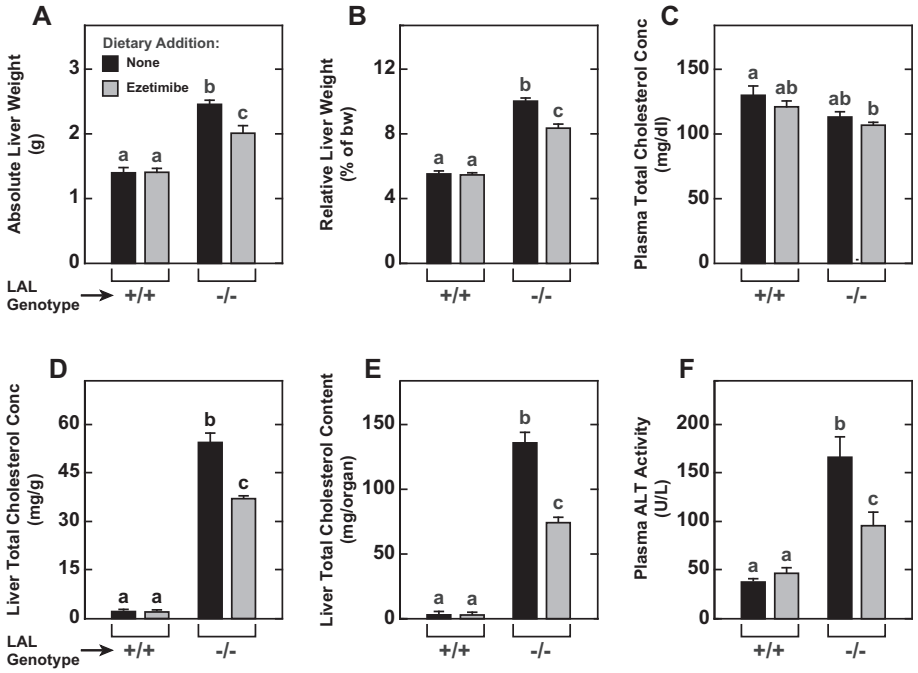

Fig. 3. Ezetimibe, a sterol absorption inhibitor, significantly reduced liver mass and cholesterol content and improved plasma alanine aminotransferase levels in *Lal*$^{-/-}$ mice. Male *Lal*$^{-/-}$ and matching *Lal*$^{+/+}$ control mice were fed a rodent chow diet either alone or containing ezetimibe (20 mg/day/kg bw) for 28 days starting at 21 days of age. The data for whole liver cholesterol content (E) were obtained by multiplying the liver total cholesterol concentration (D) in each mouse by the whole liver weight for that same animal (A). Values are the mean ± SEM of data from 6 mice in each group. Different letters (a–c) denote statistically significant values ($p < 0.05$) as determined by 2-way ANOVA with genotype and type of dietary addition as variables.

almost all in the esterified fraction (data not shown). Finally, this highly favorable effect of ezetimibe on hepatic cholesterol content was paralleled by an equally impressive reduction in plasma ALT activity (Fig. 3F).

4. Discussion

In evaluating these initial findings, several points regarding both the model and the experimental design are noteworthy. First, with regard to the lifespan of *Lal*$^{-/-}$ mice, it is surprisingly long considering the increasing severity of disease as the mice age. In their formative studies with this model, Du et al. reported that LAL-deficient mice (on a mixed 129/Sv:CF-1 background) lived for 7 to 8 months [8]. In our colony of LAL mice (FVB/N background), the median age of the *Lal*$^{-/-}$ mice at death is closer to 10 months (unpublished observation). This contrasts considerably with NPC1-deficient mice which generally have a lifespan of no more than 3 months [33]. This is largely the result of the neurodegeneration that occurs with loss of NPC1 function. Another feature of LAL-deficient mice warranting comment is that, while there are many similarities in how cholesterol metabolism changes in CESD patients and in the mouse model, the latter does not develop the type of dyslipidemia described in humans with this disorder [4,34], although they do manifest a shift in their lipoprotein composition [7,8].

Two aspects of the design of the present experiments require emphasis. First, there is the general point that in all experiments, except the one involving the feeding of a high-cholesterol diet, the mice were fed ad libitum a cereal-based rodent chow diet with a very low inherent cholesterol and fat content. Added to this, all mice were in the fed state at the time they were studied. Importantly, none of the agents added to the diets adversely affected body weight gain. The other aspect of these studies warranting comment relates to our decision to test surfomer, a now disused cholesterol absorption inhibitor, even though our focus was on ezetimibe treatment. Surfomer, which disrupts cholesterol absorption at the mucosal surface, can inhibit cholesterol absorption in chow-fed mice by at least 50% but it requires a dose that is about 160-fold greater than that of ezetimibe which acts by specifically inhibiting a sterol transporter in the brush-border membrane [15].

In designing these experiments we could not forecast with any certainty how hepatic cholesterol content in the *Lal*$^{-/-}$ mice might change with ezetimibe given the possibility that the anticipated reduction in chylomicron remnant-CE delivery to the liver might be offset by changes in intrahepatic cholesterol metabolism, most particularly a compensatory increase in cholesterol synthesis within the hepatocytes. If this happened, as is well documented to be the case in other ezetimibe-treated animal models without lysosomal storage disorders [14,30], it was conceivable that this might in turn alter hepatic VLDL-cholesterol secretion rates and the rate of formation of LDL particles. Furthermore, the finding of a reduced hepatic cholesterol level in some animal models of fatty liver disease given ezetimibe [21,23,25] could not be taken as an indicator of what would potentially happen in the LAL-deficient mice because in those models the accumulation of CE in the liver is within lipid droplets in the cytoplasmic compartment whereas in CESD the CE is trapped in lysosomes.

These initial findings raise the question as to what types of additional experiments need to be done to further evaluate the cholesterol-lowering action of ezetimibe in this model of CESD. Apart from testing a dose lower than 20 mg/day/kg bw, it will also be important to establish whether starting treatment early in the suckling period might yield an even more favorable outcome for the liver and possibly increase lifespan. The converse of this will be to investigate if there is any benefit when the start of treatment is delayed until adulthood. There is also the related question of what happens to liver cholesterol content in *Lal*$^{-/-}$ mice that had received treatment from weaning to 7 weeks of age, but are then taken off ezetimibe for several weeks before study. While the liver

will be the target organ of such experiments, cholesterol measurements should be made in several other key organs, especially the small intestine and spleen. Studies on the actions of ezetimibe in this cholesterol storage disorder should also focus on what is happening at a biochemical and molecular level. Given the well documented effect of ezetimibe in markedly stimulating hepatic, intestinal, and whole-body sterol synthesis [14,30,35], a particularly interesting study would be to measure, in vivo, the rates of cholesterol and fatty acid synthesis, and the mRNA expression levels for related genes, in the liver and small intestine of ezetimibe-treated *Lal*$^{-/-}$ mice. Such studies might provide clearer insights into the reported benefit of ezetimibe in a CESD patient on statin therapy [36].

Acknowledgments

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