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Ezetimibe as a potential treatment for non-alcoholic fatty liver disease: is the intestine a modulator of hepatic insulin sensitivity and hepatic fat accumulation?

Mohamed H. Ahmed[1] **and Christopher D. Byrne**[2,3], cdtb@southampton.ac.uk

Non-alcoholic fatty liver disease (NAFLD) is the hepatic component of the metabolic syndrome and is known to be associated with marked insulin resistance and increased risk of cardiovascular disease. Ezetimibe, an inhibitor of intestinal cholesterol absorption, inhibits Niemann-Pick C1-like 1 (NPC1L1). Interestingly, NPC1L1 is abundantly expressed in human liver, as well as in the intestine. Recent reports suggest a potential benefit of ezetimibe in improving hepatic insulin sensitivity and decreasing hepatic inflammation and lipid accumulation. Insulin resistance and excess hepatic fat accumulation are regarded as key factors in the pathogenesis of NAFLD. We suggest, therefore, that urgent studies are needed to assess the potential therapeutic benefit of ezetimibe in treating NAFLD.

Introduction

Ezetimibe inhibits intestinal uptake of cholesterol and is used in clinical practice to lower low-density lipoprotein cholesterol. The major metabolic pathway for ezetimibe consists of glucuronidation of the 4-hydroxyphenyl group by uridine 5′-diphosphate-glucuronosyltransferase isoenzymes to form ezetimibe-glucuronide in the intestine and liver. Approximately 78% of the ezetimibe dose is excreted in the faeces as ezetimibe, and the remainder is excreted in the urine, mainly as ezetimibe-glucuronide [1]. Niemann-Pick C1-like 1 (NPC1L1) is highly expressed in jejunum of different species and in human liver. In the intestine, NPC1L1 is the main transporter of intestinal cholesterol. Mice deficient in NPC1L1 show a marked reduction in cholesterol absorption (>70%) without a decrease in plasma with ezetimibe treatment, leading to the conclusion that ezetimibe reduces intestinal absorption via inhibition of the action of NPC1L1 [2] (Fig. 1).

Non-alcoholic fatty liver disease (NAFLD) represents a spectrum of liver diseases associated with fatty infiltration in hepatocytes (steatosis). Once hepatic steatosis is established, other factors – including oxidative stress, mitochondrial dysfunction and inflammation – can promote further cellular damage and might lead to non-alcoholic steatohepatitis (NASH), increasing fibrosis and cirrhosis. Major risk factors for NAFLD are obesity and insulin resistance, and, to date, the only effective treatment for NAFLD is weight loss with attention to lifestyle, which is difficult to achieve for most NAFLD patients [3]. In this article, our focus is the theoretical benefit of ezetimibe use in the treatment of NAFLD.

Ezetimibe and NAFLD

Recent evidence suggests that ezetimibe has a beneficial effect in the treatment of NAFLD. Zheng *et al.* [4] have shown that ezetimibe treatment for four weeks reduced alanine aminotransferase (ALT), hepatic triglyceride (TG), hepatomegaly, cholesterol ester and free cholesterol in diet-induced obese mice fed a high-fat and high-cholesterol diet for seven months. Importantly, administration of ezetimibe in obese Zucker rats (a model of NAFLD and metabolic syndrome) produced a marked improvement in plasma cholesterol and TG

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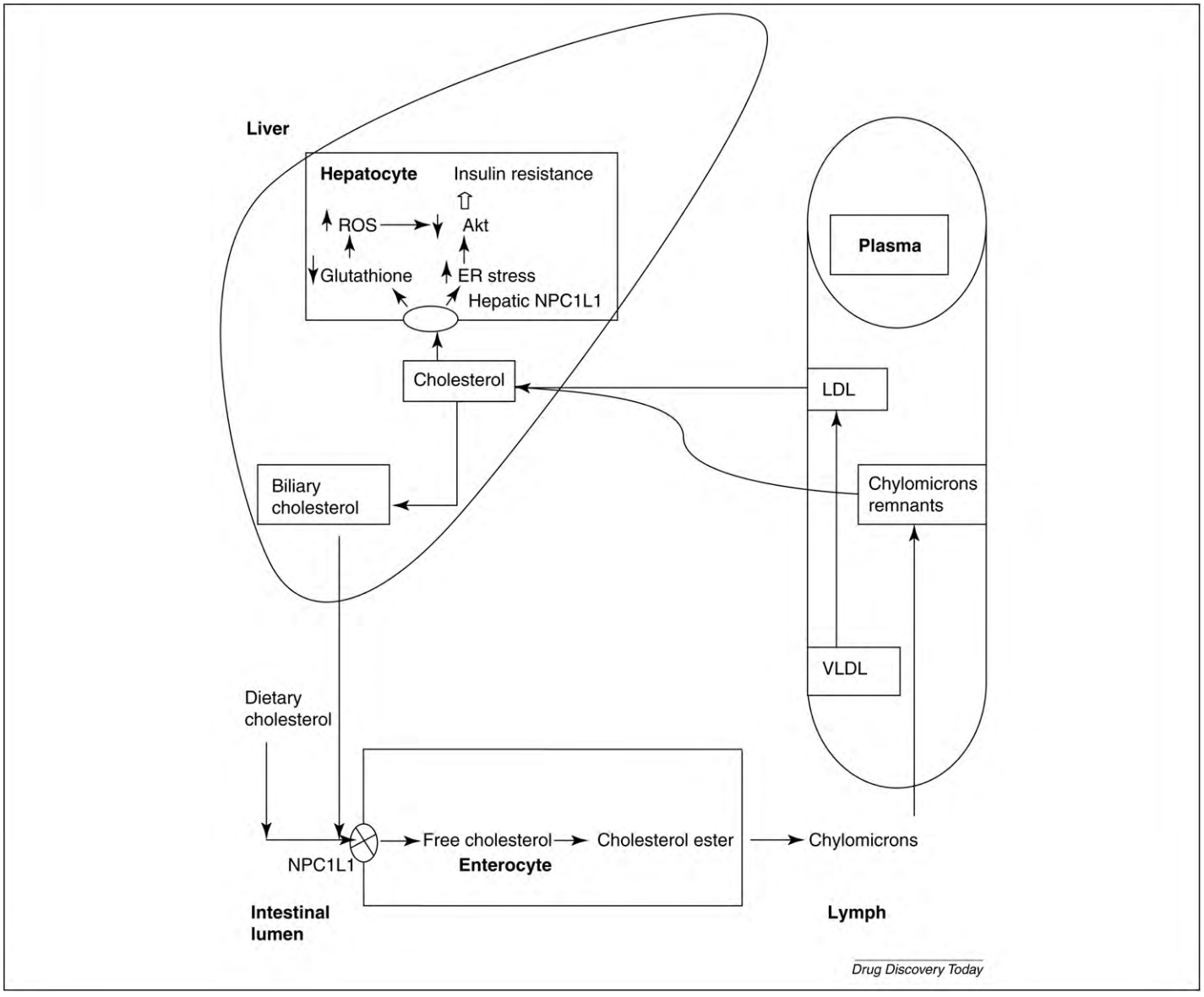

FIGURE 1

Illustration showing actions of ezetimibe treatment in the intestine and liver, suggesting that ezetimibe might be a potential treatment for insulin resistance. Abbreviations: ES, endoplasmic reticulum stress, ROS, reactive oxygen species.

levels and hepatic steatosis and improved insulin sensitivity [5]. These data are in accordance with a recent study by Nomura *et al.* [6], which showed that ezetimibe improved hepatic insulin sensitivity in obese Zucker fatty rat. Hughes *et al.* [7] have also demonstrated in human that administration of ezetimibe in six patients with ultrasound evidence of NAFLD resulted in the normalization of ALT. Administration of ezetimibe for six months in non-obese patients with NAFLD has resulted in normalization of the ALT [8]. Importantly, administration of ezetimibe with moderate weight loss in 15 patients with fatty liver for 16 weeks was associated with improvement of hepatic steatosis, inflammation and LDL-apoB100 metabolism [9], and administration of ezetimibe for six months in patients with NASH and dyslipidaemia was associated with marked histological improvement of NASH score [10]. Furthermore, administration of ezetimibe with other lipid-lowering, glucose-lowering or insulin-sensitizing agents has resulted in a marked improvement in NAFLD. For example, administration of ezetimibe with the alpha-glucosidase inhibitor acarbose for 24 weeks improved histological findings in a mouse model of NAFLD [11]. In addition, administration of a combination of valsartan, metformin and rosiglitazone for a total of 15 weeks in a rat model of NAFLD produced a marked decrease in liver steatosis (−54%), hepatic TG (−64%), hepatic cholesterol (−31%) and hepatic MDA (−70%; MDA is widely used as an indicator of free-radical-mediated lipid peroxidation injury) [12]. Interestingly, the administration of ezetimibe and simvastatin for six months in 19 people with type 2 diabetes and NAFLD was associated with a marked improvement in liver enzymes (ALT and aspartate aminotransferase) [13]. Because statins have no therapeutic or toxic effect in NAFLD, it is likely that ezetimibe treatment *per se* has the potential to reduce hepatic lipid accumulation. Moreover, the data discussed above suggest that ezetimibe improves hepatic insulin sensitivity; hepatic insulin resistance and hepatic fat accumulation are two key factors occurring early in the pathogenesis of NAFLD.

Ezetimibe and insulin resistance

Ezetimibe treatment for 24 weeks in a rat model of NAFLD was associated with a marked decrease

in insulin resistance. This improvement in insulin sensitivity was associated with a marked decrease in liver weight and in fasting glucose; however, only ezetimibe in combination with acarbose induced marked upregulation of liver SREBP-2, liver peroxisome proliferator-activated receptor (PPAR)α, liver MTP and liver LDRmRNA expression levels. In mice, ezetimibe in combination with acarbose had no effect on SREBP-1c (which is known to be a key transcriptional regulator of hepatic fatty acid synthesis) [11] and ezetimibe administration for four weeks reduces hepatic TG considerably in a rat model of NAFLD [4]. In a recent study, Nomura *et al.* [6] showed that ezetimibe treatment in the obese Zucker rat markedly lowered both SREBP-1c and ChREBP, two key transcription factors regulated by insulin and carbohydrate that are potentially very important regulators of lipogenic gene expression in the liver [3]. In Zucker obese rats, administration of ezetimibe improved insulin response and lower plasma glucose response after intraperitoneal glucose injection. This effect was thought to be due to ezetimibe treatment augmenting insulin-induced phosphorylation (insulin signalling) of insulin receptor-β, insulin receptor substrate-1 and Akt-1 [5]. Nomura *et al.* [6] showed that ezetimibe-specific inhibition of NPC1L1 restored hepatic insulin sensitivity *in vivo* and in steatotic hepatocytes *in vitro*. Ezetimibe also decreased gluconeogenic gene expression in the liver. The authors concluded that this effect of ezetimibe was likely to be mediated, at least in part, by a reduction in hepatic reactive oxygen species generation, JNK activation and ER stress. Nomura *et al.* [6] showed that transfection of NPC1L1 Short hairpin RNA (shRNA) into hepatocytes also reduced reactive oxygen species generation and ER stress. Human studies suggest there may be a benefit of ezetimibe per se to improve insulin sensitivity. For example, ezetimibe treatment in combination with low dose pravastatin (10 mg for six months in 50 individuals) resulted in a marked decrease in insulin resistance compared with 40 mg pravastatin treatment alone [14]. To date, however, it has not been proven that ezetimibe treatment improves insulin sensitivity and specifically whole-body insulin sensitivity. In another study, ezetimibe treatment alone for three months in 12 obese dyslipidaemic patients decreased total cholesterol and low-density lipoprotein cholesterol without affecting insulin sensitivity [15]. In this study, plasma levels of Free fatty acid (FFA) and insulin were not described. Whether there is a differential effect of ezetimibe according to baseline levels of hepatic insulin sensitivity or according to whether treatment with a specific TG-lowering agent is required needs to be determined. We suggest, therefore, that urgent clinical studies are needed to assess the impact of ezetimibe alone and in combination with other lipid-lowering medication on insulin sensitivity and hepatic fat. Insulin resistance increases TG lipolysis and release of FFA, which potentially influences insulin sensitivity. FFA also activates the pro-inflammatory NF-κB pathway in the liver [1] and, interestingly, administration of ezetimibe is associated with a marked reduction in inflammatory and liver fibrosis markers such as CRP, TGFβ, TNF-α and liver collagen 1 mRNA [4–10].

In people with type 2 diabetes, as well as in animal models with diabetes, a marked increase in expression of NCP1L1 has been demonstrated [16]. This observation might explain, in part, the dyslipidaemia was associated with the metabolic syndrome. Ligands of PPARγ (subtypes α, δ) promote adipogenesis, stimulate glucose disposal in skeletal muscle and suppress glucose production from the liver. Current evidence suggests that the treatments showing the most promise in treating NAFLD are the PPARγ agonists [3]. Pioglitazone (PPARγ agonist) treatment produces histological improvement in NAFLD, particularly decreasing hepatic fat [17]. Interestingly, administration of pioglitazone to diabetic Zucker obese rats reduced intestinal and hepatic expression of NCP1L1, and there was a strong positive correlation between chylomicron cholesterol and intestinal NCP1L1 and between hepatic NCP1L1 and VLDL [18].

From the above discussion, it is possible to postulate that intestinal function (or at least NCP1L1 function) might influence hepatic insulin sensitivity. The concept of the intestine influencing insulin sensitivity is also suggested by the effects of gastric bypass surgery, which produces normalization of insulin sensitivity very quickly post-surgery, before weight loss has occurred. It is not yet clear how NCP1L1 (a cholesterol transporter in liver and intestine) functions to regulate SREBP-1c, ChREBP and PPAR receptors and ultimately impact on hepatic insulin sensitivity. We suggest further investigation is also required to determine the impact of AMP-activated protein kinase and leptin on NCP1L1. Recently, it has been shown in a study of 72 healthy men with a normal BMI that neither ezetimibe nor simvastatin, or a combination of both for 14 days, changed serum leptin, adiponectin or resistin [19]. Nevertheless, it remains to be proven whether there is an effect of NCP1L1 function on leptin, adiponectin and resistin in various insulin-resistant states.

Ezetimibe and lipid metabolism

Fat accumulation in the liver is influenced by the delivery of dietary fat to the liver (contribution to liver fat ~5%); delivery of extra-hepatic non-esterified fatty acids to the liver (contribution to liver fat ~60%) and the remainder of liver fat accumulation are affected by hepatic *de novo* lipogenesis [20]. Because ezetimibe decreases dietary cholesterol absorption and has the potential to improve insulin sensitivity (thus affecting adipocyte lipolysis and the delivery of fatty acids to the liver), it is conceivable that ezetimibe might influence considerably the delivery of fat to the liver. Given the potential importance of extra hepatic fat delivery to the liver to affect hepatic fat accumulation, however, further research is needed to determine more precisely the effects of ezetimibe on liver fat.

Recently, it has been shown in humans and animals that high dietary cholesterol was associated with NAFLD [21,22]. Importantly, dietary cholesterol was thought to be an important risk factor for the progression to hepatic inflammation in diet-induced NASH in mice [23]. Recent studies suggest a potential benefit of ezetimibe in treating NAFLD through decreasing dyslipidaemia. Deushi *et al.* [5] showed that ezetimibe treatment in an animal model of NAFLD decreased plasma cholesterol by 27%, TG by 32% and free fatty acid by 31%. The decrease in TG occurred predominately in chylomicron and Very low density lipoprotein (VLDL) [5]. Ezetimibe treatment for four weeks improved high-fat and -cholesterol diet-induced fatty liver in mice. This effect was associated with a marked reduction in the liver-to-body-weight ratio, hepatic TG, hepatic cholesteryl esters and hepatic cholesterol [4]. The administration of ezetimibe alone or in combination with acarbose for 24 weeks in an animal model of NAFLD considerably decreased total cholesterol, serum TG, chylomicron and VLDL [8]. Furthermore, administration of ezetimibe in a rat model of NAFLD for 15 weeks considerably decreased hepatic and plasma TG by 53%, and hepatic cholesterol was decreased by 25% and plasma cholesterol by 30% [9].

The liver plays a crucial part in the maintenance of lipid homeostasis, not only because it is the organ that receives most of the cholesterol absorbed by the small intestine but also because it is the site for the excretion of cholesterol in bile. The liver actively synthesizes cholesterol, and this is affected by the amount of cholesterol being delivered to it from the small intestine. It is well documented that dietary or pharmacological manipulation of the enterohepatic flux of either cholesterol or bile acids can cause marked changes in the rate at which the liver synthesizes cholesterol, converts cholesterol to bile acids, incorporates cholesterol into very low-density

lipoproteins, esterifies and stores cholesterol or secretes unesterified cholesterol directly into bile.

Recent studies also showed the potential benefit of ezetimibe as a treatment for gallstones. Ezetimibe acts through decreasing intestinal cholesterol absorption and biliary cholesterol secretion. Ezetimibe preserves gallbladder motility function by desaturating bile in mice, promoting the dissolution of gallstones by forming an abundance of unsaturated micelles, reducing biliary cholesterol saturation and, thus, retarding cholesterol crystallization in bile [24–28].

Various nuclear transcription factors can play a key role in modulating expression of NPC1L1. Hepatic nuclear factors (HNFs) are expressed in various organs including the liver, intestine and pancreas. Deficiency of HNF1α in mice results in a defect of bile acid transport, increased bile acid and liver cholesterol synthesis, and impaired HDL metabolism. Liver-specific disruption of HNF4α results in hepatomegaly, lipid deposition in the liver, reduced serum cholesterol and TG levels, and elevated serum bile acid concentrations. Thus, both HNF1α and HNF4α have important roles in lipid homeostasis. Pramfalk *et al.* [29] showed that SREBP2 and HNF1α are important transcription factors for hepatic NPC1L1 promoter activity and can bind to and regulate its expression in humans. Interestingly, mutation in HNF1α, HNF4α and glucokinase genes is associated with maturity onset diabetes of the young [30]. In a population study of approximately 17,000 individuals, polymorphism of both HNF1α and HNF4α, but not glucokinase, predicted future type 2 diabetes [30]. Whether ezetimibe is of any benefit in people with HNFα mutations who are at risk of diabetes now needs to be tested.

The nuclear transcription factors PPARs and Liver X receptors (LXR) might also play a part in the regulation of NPC1L1 expression. PPARs are thought to play a crucial part in the regulation of cholesterol homeostasis, insulin sensitivity and fatty acid oxidation. PPARα and PPARδ are largely expressed in the liver and skeletal muscles, and both can regulate NPC1L1 [12]. PPARα is predominately expressed in the liver and is involved in promoting gluconeogenesis and regulation of expression of genes involved in mitochondrial fatty acid oxidation. The fibrate class of drugs acts as PPARα agonists, decreases plasma TG and increases HDL-c levels. Interestingly, activation of PPARα by fenofibrate also decreases intestinal cholesterol absorption via an inhibitory effect on NCP1L1 expression in the small intestine [31].

The liver X receptors are also key regulators of cholesterol homeostasis belonging to the nuclear receptor superfamily. These nuclear receptors mediate ligand-induced transcriptional regulation of genes involved in HDL metabolism, in reverse cholesterol transport from peripheral tissues, in catabolism of cholesterol to bile acids and in cholesterol excretion into bile or intestinal lumen. Administration of the LXR agonist, both *in vitro* and *in vivo*, was associated with marked decrease in NPC1L1 [32]. Interestingly, administration of an LXR agonist in NPC1L1 knockout mice failed to raise HDL-c but caused increased faecal cholesterol loss, suggesting that NPC1L1 is necessary to mediate an LXR-agonist-induced increase in plasma HDL-c in mice [33].

Importantly, overexpression of PPARδ is associated with reduced expression of NPC1L1. Overexpression of PPARδ in skeletal muscle led to a remarkable increase in energy endurance capacity, improved insulin sensitivity and activated reverse cholesterol transport to increase HDL-c [30]. Recently, it has been suggested that PPARδ activation leads to direct transintestinal cholesterol efflux (increased faecal neutral sterol secretion, the last step in reverse cholesterol transport) [34,35]. Thus, we can speculate that *in vivo* activation of LXR, PPARδ, PPARα and PPARγ modulate the expression of NPC1L1; however, further investigations are required to explore whether ezetimibe treatment modulates nuclear receptor activity via inhibitory effects on NPC1L1 (Fig. 2). It is also important to determine the exact function of hepatic NPC1L1. That NPC1L1 is highly expressed in human liver with trivial amounts of NPC1L1 expressed in rodent liver suggests a unique function for hepatic NPC1L1 in humans that might have arisen as an adaptation to deal with increased amounts of dietary fat in humans. Interestingly, in the liver, NPC1L1 localizes to the bile canalicular membrane, and this suggests an important role for NPC1L1 in the regulation of biliary cholesterol. This is only speculation, however, and further research is urgently needed. Another important function of hepatic NPC1L1 suggested by Nomura *et al.* [6] is that NPC1L1 activity might promote hepatic insulin resistance by enhancing hepatic free cholesterol accumulation. From the above discussion, it is reasonable to suggest that further research is needed to determine the role of hepatic NPC1L1 on glucogenic genes, lipogenic genes and inflammatory pathways. Diet can also modulate the expression of NPC1L1; for example, a diet rich in fish oil (but not olive oil) decreases expression of NPC1L1 [36]. Fish oil and fenofibrate are known to decrease plasma TG levels, and it is still to be established whether TG is also transported by NPC1L1. Interestingly, ezetimibe decreases the CD36 fatty acid transporter dominant CD36, fatty acid transport protein 4 (FATP4) and liver fatty acid binding protein (L-FABP) [33]. CD36 is known to be important for fat absorption, especially of very long-chain fatty acids.

FATP4 is an important protein in the absorption of very long-chain fatty acids and might have a role in dietary fat absorption. L-FABP is another important protein that might have a role in fatty acid transport and chylomicron production. Importantly, the decrease in the expression of FATP4, CD36 and L-FABP induced by ezetimibe is associated with protection against diet-induced hyperglycaemia and insulin resistance [37]. These data support the notion that the intestine modifies insulin sensitivity and that there is a need to consider intestinal fat absorption when assessing insulin sensitivity.

The use of a standardized OLTT to assist in studying the relationship between hepatic insulin sensitivity and intestinal fat absorption

Currently, there is no standardised oral lipid tolerance test (OLTT) for assessing individual variation in intestinal absorption of dietary fats and cholesterol. Bahceci *et al.* [38] have shown that in 20 women with PCOS who had an OLTT, there was an increase in postprandial TG, VLDL and cholesterol and total cholesterol. Importantly, postprandial hyperlipidaemia and hyperglycaemia are associated with insulin resistance, metabolic syndrome, NAFLD and an increase in obesity [39–42]. Postprandial hyperlipidaemia and hyperglycaemia are emerging risk factors for CVD [40,43]. It is possible that postprandial hyperlipidaemia and hyperglycaemia might contribute to CVD in people taking lipid-lowering medication or those with a normal lipid profile. Currently, undertaking an OLTT is not part of a routine clinical investigation and their use is restricted to research studies. The presence of polymorphisms in intestinal transporters, suggesting altered function, and the link with insulin resistance suggests the need to screen such individuals with OLTTs [33]. Ezetimibe might be particularly useful in treating people with an exaggerated postprandial lipaemic response. There is an urgent need for OLTT standardization, particularly if this test is considered for use in clinical practice. We speculate that ezetimibe might reduce postprandial hyperlipidaemia, and the use of OLTT might provide a tool for testing this hypothesis. An OLTT might also have potential for identifying those people who would especially benefit from lifestyle modifications focusing on dietary

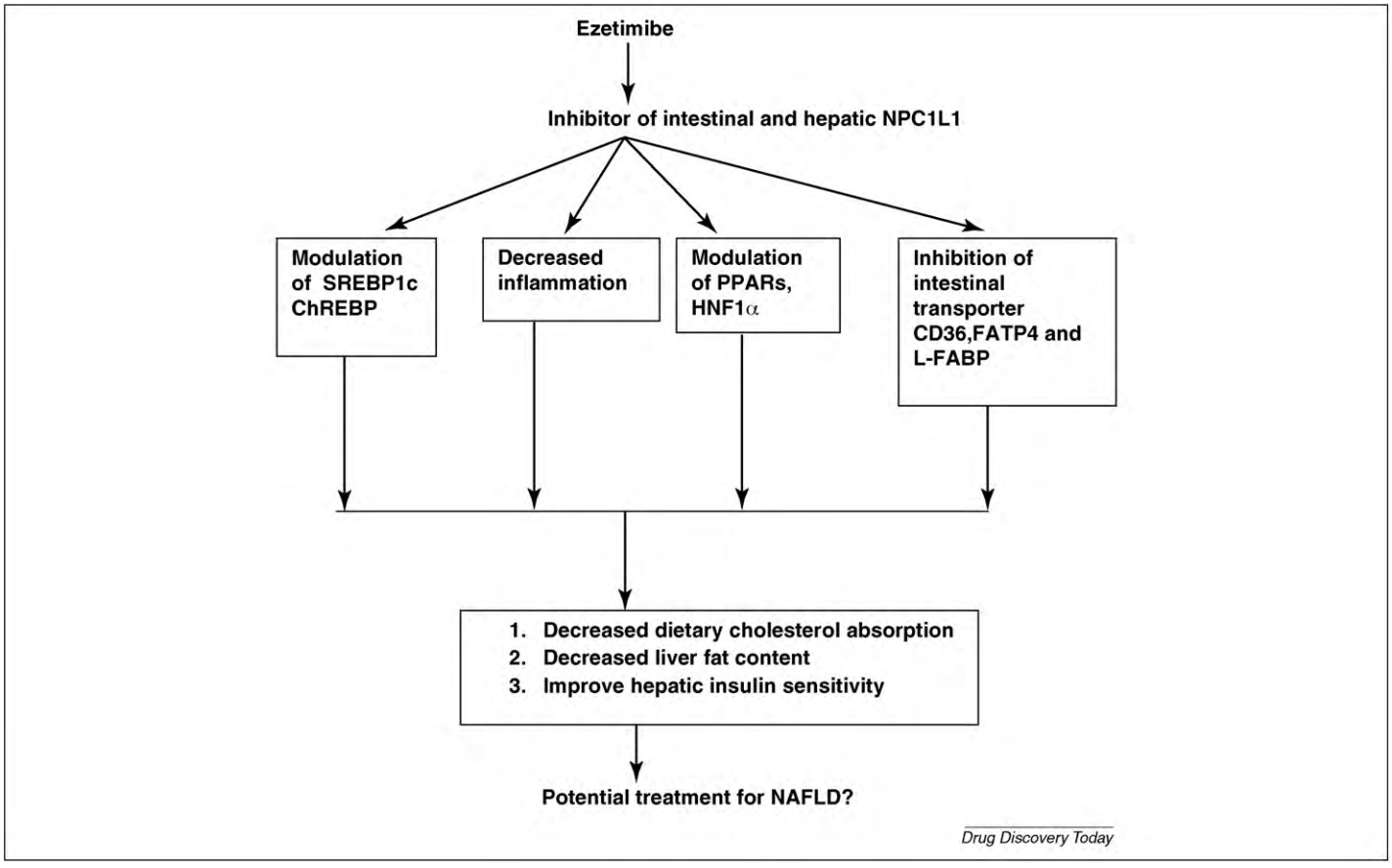

FIGURE 2

Schematic figure showing how NPC1L1 might modulate different factors that have a direct and indirect effect on insulin sensitivity and lipid metabolism to potentially ameliorate NAFLD. Abbreviations: PPAR, peroxisome proliferator-activated receptor; HNFs, hepatic nuclear factors; SREBP-1c, sterol regulatory element binding protein-1; ChREBP, carbohydrate responsive element-binding protein.

manipulation, as well as lipid-lowering medication to decrease CVD risks.

Concluding remarks

NAFLD is the hepatic component of the metabolic syndrome and is emerging as a risk factor of CVD. Ezetimibe treatment is associated with improvement in liver enzymes, a reduction in hepatic fat and improvement in hepatic insulin sensitivity. We recommend that there is a need for urgent clinical trial testing of the effects of ezetimibe in NAFLD. Importantly, the focus for future research should be to clarify the effect of hepatic and intestinal functioning of NPC1L1 on hepatic insulin sensitivity and lipid accumulation. Clinically, we suggest that it is important to determine the utility of a standardized OLTT in risk stratification to enable better targeted treatment of postprandial hyperlipidaemia in people with NAFLD at risk of CVD.

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***Mohamed H. Ahmed*[1]**
***Christopher D. Byrne*[2,3,*]**
[1]Chemical Pathology Department, Southampton University Hospital NHS Trust, Southampton SO16 6YD, UK
[2]Endocrinology & Metabolism Unit, DOHaD Division, University of Southampton and Southampton University Hospitals Trust, Southampton SO16 6YD, UK
[3]Current address: Institute for Developmental Sciences (IDS Building), MP887 (University of Southampton), Southampton General Hospital, Southampton SO16 6YD, UK.

**Corresponding author:*
email: cdtb@southampton.ac.uk