Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 18 September 2018 doi:10.20944/preprints201809.0345.v1

Review

A newly integrated model for intestinal cholesterol absorption and efflux infers how plant sterol intake reduces circulating cholesterol levels

Takanari Nakano [1]*, Ikuo Inoue [2], and Takayuki Murakoshi [1]

[1] Department of Biochemistry, Faculty of Medicine, Saitama Medical University, Saitama, Japan; tnakano@saitama-med.ac.jp (T.N.); t_mura@saitama-med.ac.jp (T.M.)
[2] Department of Diabetes and Endocrinology, Faculty of Medicine, Saitama Medical University, Saitama, Japan; i1901018@saitama-med.ac.jp
* Correspondence: tnakano@saitama-med.ac.jp; Tel.: +81-492-76-1155

Abstract: Hypercholesterolemia accelerates atherosclerosis, and extensive research has been undertaken to ameliorate this abnormality. Plant sterols have been shown to inhibit cholesterol absorption and lower plasma cholesterol level since the 1950s. This ingredient has recently been reappraised as a food additive that can be taken daily in a preclinical period to prevent hypercholesterolemia, considering that cardiovascular-related diseases are the top cause of death globally even with clinical interventions. Intestinal cholesterol handling is still elusive, making it difficult to clarify the mechanism for plant sterol-mediated inhibition. Notably, although the small intestine absorbs cholesterol, it is also the organ that excretes it abundantly, via trans-intestinal cholesterol efflux (TICE). In this review, we show a model where the brush border membrane (BBM) of intestinal epithelial cells stands as the dividing ridge for cholesterol fluxes, making cholesterol absorption and TICE inversely correlated. With this model, we tried to explain the plant sterol-mediated inhibitory mechanism. As well as cholesterol, plant sterols diffuse into the BBM but are effluxed back to the lumen rapidly. We propose that repeated plant sterol shuttling between the BBM and lumen promotes cholesterol efflux, and plant sterol in the BBM may disturb the trafficking machineries that transport cholesterol to the cell interior.

Keywords: ATP-binding cassette G5/G8; brush border membrane; cholesterol absorption; Niemann-Pick C1-like 1; phytosterols; trans-intestinal cholesterol efflux; fecal neutral sterol excretion.

1. Introduction

Cholesterol is an essential component of cell membranes, required for proper permeability, structural integrity, membrane-mediated signaling, and so on [1]. Cholesterol is transported between tissues via the circulation; however, abnormally elevated circulating plasma cholesterol levels are atherogenic and increase the risk of cardiovascular diseases (CVDs) [2], which account for ~31% of deaths globally [3]. Cholesterol in the plasma originates from either the diet or endogenous synthesis in cells. Thus, limiting the supply pathways can reduce the level of cholesterol. To ameliorate the abnormality, statins have been widely used since the 1980s to inhibit the cholesterol synthetizing pathway. Ezetimibe has been available since the 2000s to reduce the other source, intestinal cholesterol absorption [4]. A meta-analysis showed that, with pharmacological treatment that is initiated later in life, all-cause mortality was reduced by 10% per 1.0 mmol/L reduction in low-density lipoprotein cholesterol (LDL-C) [5]. Although reductions to 2–3 mmol/L LDL-C can be achievable with intensive medication regimens, a high CVD risk still persists among patients [6].

© 2018 by the author(s). Distributed under a Creative Commons CC BY license.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 18 September 2018 doi:10.20944/preprints201809.0345.v1

Niemann-Pick C1-like 1 (NPC1L1), the pharmacological target of ezetimibe, is a protein that facilitates cholesterol absorption in the small intestine [7]. Subjects who carry a heterozygous inactivating mutation of *NPC1L1* had 53% lower CVD risk with 0.31 mmol/L lower mean LDL-C (p = 0.04) [8], suggesting that modest reductions in plasma LDL-C levels over a lifetime lead to a much larger benefit in CVD risk than pharmacological treatments that start later in life when hypercholesterolemia becomes clinically obvious.

Sterols are present in all eukaryotes. Land plants contain noncholesterol sterol analogs, called plant sterols and stanols, which are referred to as "phytosterols" hereafter. Phytosterols are chemically analogous to cholesterol, differing only in their side chain length [9], but they are more hydrophobic than cholesterol [10]. Phytosterol accounts for up to 20–25% of total dietary sterol [11]. When phytosterols are enriched in the diet, plasma LDL-C levels are reduced [12], and the effect had already been shown in the 1950s [13,14]. The LDL-C-lowering effect continues to increase up to intakes of approximately 3 g per day to an average effect of 12% [15]. This favorable effect has been attributed to the inhibition of intestinal cholesterol absorption by phytosterols [16].

Because cholesterol is hydrophobic and metabolically undegradable, it is eliminated as bile salts or in its original from the gut into the feces. In addition to hepatobiliary cholesterol removal [17], the small intestine also eliminates endogenous cholesterol, the pathway for which was known as early as 1967 [18] and is now referred to as trans-intestinal cholesterol efflux (TICE) [19]. TICE constitutes approximately a half of the total fecal neutral sterol (FNS) excretion physiologically. Although the overall mechanism is still to be elucidated, the pathway can be stimulated pharmacologically [20,21], and is thus excepted to provide a therapeutic target for hypercholesterolemia [22]. Brufau et al. [23] showed that phytosterol feeding increased FNS excretion, a surrogate marker of TICE stimulation, in mice, demonstrating another contribution for phytosterol-mediated LDL-C lowering.

Although evidence is accumulating as to the merit of phytosterol supplementation, appropriate regimens and safety concerns are not settled yet [24-26]. Understanding the inhibitory mechanism could promote the further development and utilization of phytosterols. In this review, we begin with a brief overview of the process of intestinal cholesterol absorption, because the overall intestinal cholesterol absorption process is described in detail elsewhere [27-30]. Then, we will describe our ideas on how cholesterol is handled in the small intestine, especially focusing on cholesterol dynamics at the brush border membrane (BBM), and why TICE is mechanistically associated with cholesterol absorption. We show a model that the BBM of intestinal epithelial cells stands as the dividing ridge for cholesterol fluxes, meaning that cholesterol absorption and TICE are inversely correlated. We further expand this idea to integrate the mechanisms of how phytosterols inhibit cholesterol absorption and stimulates TICE as well.

2. Intestinal cholesterol absorption

2.1. An overview of the cholesterol absorption process

The small intestine is the site where cholesterol absorption takes place. "Absorption" of cholesterol includes multiple steps for the movement and metabolism of cholesterol to its transfer from the lumen into the circulation via the thoracic duct. We have divided "absorption" into two major processes: "uptake" of cholesterol, which refers to the entry of cholesterol into intestinal epithelial cells, and "assimilation" of cholesterol, which indicates the transfer of cholesterol from the cell interior to the lymph via the basolateral membrane (Figure 1a). In the latter process, cholesterol esterification, packaging into lipoproteins, and exocytosis are included.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 18 September 2018 doi:10.20944/preprints201809.0345.v1

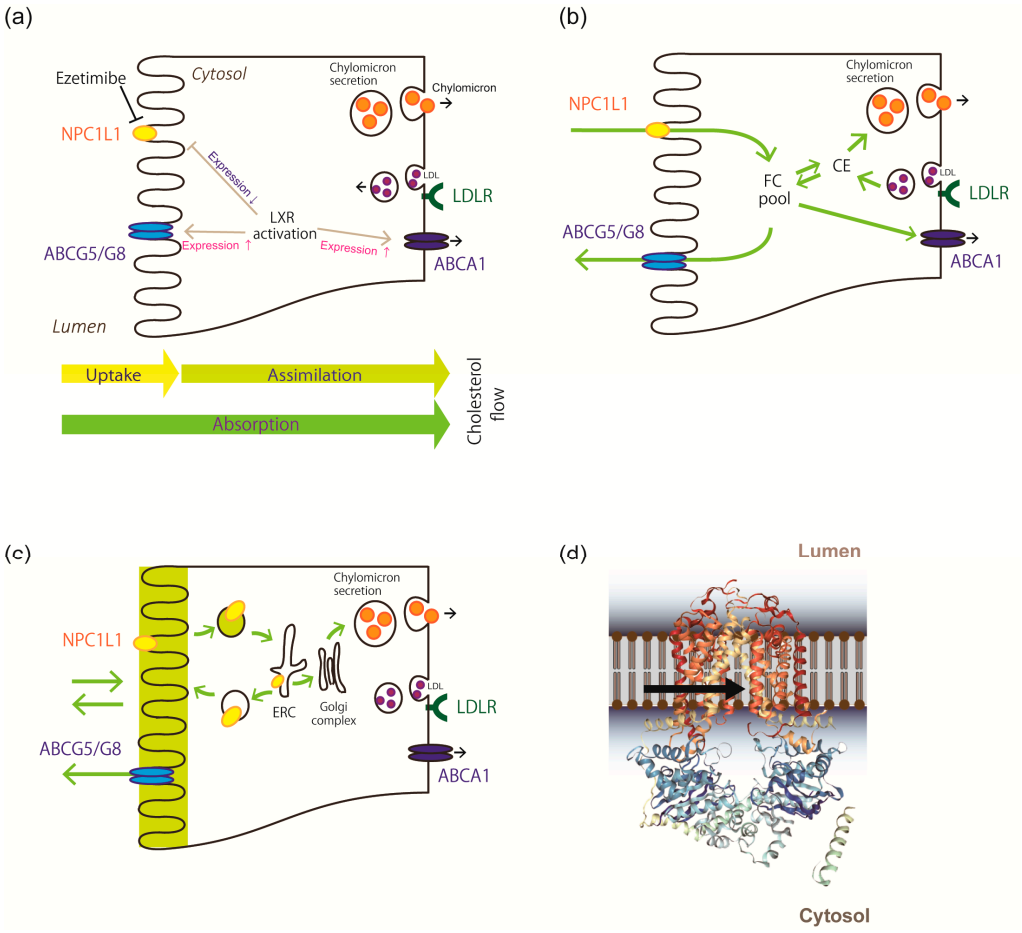

Figure 1. Overview of cholesterol trafficking in enterocytes. (**a**) Components associated with cholesterol absorption in enterocytes. Niemann-Pick C1-like 1 (NPC1L1) is the major cholesterol transporter. Enterocytes take up cholesterol as LDL via LDL receptor-mediated endocytosis from the basolateral side [31]. ATP-binding cassette (ABC) G5/G8 is an efflux transporter of sterols in the brush border membrane (BBM). Liver X receptor (LXR) modulates gene expression, the products of which are associated with the fluxes. Cholesterol in the enterocytes mainly exits as chylomicrons or very low-density lipoprotein (VLDL) [32]. Cholesterol is also transported into the serosal side to build HDL via ABCA1. Arrows indicate up-regulation. The gene expression of NPC1L1 is inhibited by LXR activation. The terms of "uptake", "assimilation", and "absorption" are defined in the text. Ezetimibe is an NPC1L1 inhibitor. (**b**) A representative illustration of how cholesterol transits in enterocytes, as depicted in previous reports (*i.e.*, [27-30]). Cholesterol in the lumen passes through the membrane via NPC1L1. ABCG5/G8 effluxes free cholesterol (FC) in the cytosolic area. CE, cholesterol ester. (**c**) A newly proposed model for cholesterol movement in enterocytes. The BBM constitutes a large reservoir for cholesterol, which comes in from and exit out to the lumen in a diffusive manner. ABCG5/G8 effluxes cholesterol to maintain the abundance using energy from ATP hydrolysis. Cholesterol in the BBM moves to the endocytic recycling compartments via NPC1L1-mediated vascular trafficking, then the Golgi complex, and packed into chylomicron to be secreted to the circulation. (**d**) Topology of ABCG5/G8 and its cholesterol handling for transport. The resolved crystal structure of ABCG5/G8 showed that it is likely to obtain substrates in the BBM at the transmembrane site (*black arrow*) [33]. The three-dimensional image of ABCG5/G8 was obtained from the Orientations of Proteins in Membranes (OPM) database; http://opm.phar.umich.edu/protein.php?search=5do7 (accessed on 2018.09.14).

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 18 September 2018 doi:10.20944/preprints201809.0345.v1

2.2. Intestinal cholesterol uptake

In addition to diet, bile and sloughed epithelial cells from the intestinal wall also supply cholesterol within the intestinal lumen, reaching 2–3 g per day in total [28]. Cholesterol solubilized into lipid micelles in the lumen penetrates an unstirred water layer in the intestinal wall and reaches the BBM, where the cholesterol reservoir is the location in cells. Unesterified cholesterol constitutes about one-third of BBM lipids (Cholesterol : phospholipid = 1:2) [34], in which cholesterol is densely packed as microvilli with a vast surface area of the epithelia. The uptake process is mediated by passive diffusion [35-37] (Figure 1c), which is the amount uptake is increased in relation to the concentration in the lumen. Passive diffusion should be natural considering the physico-chemical nature of the interaction between hydrophobic compounds, such as cholesterol, and lipid bilayer membranes [38]. Hauser et al. showed that the cholesterol incorporation capacity of the BBM was decreased by protease treatment, suggesting that it was a protein mediated process [39]. However, proteins are a major constituent of prepared BBM vesicles, accounting for two-thirds of the weight [40]. Therefore, protease treatment could tear apart the BBM vesicles and reduce the retention capacity of sterols. Moreover, because many of the proteins in the BBM constitute cholesterol-rich microdomains; thus, the disturbance can also impair the retention capacity. Furthermore, there has been no protein molecule identified that affects the uptake. Cholesterol uptake by the intestinal BBM vesicles from mice was unaffected by the deletion of genes associated with intestinal cholesterol absorption (*NPC1L1*, *SCARB1*, and *CD36*) [41,42]. These observations suggest that the uptake itself is concentration dependent and not protein mediated.

2.3. Assimilation of cholesterol in intestinal absorptive cells

Cholesterol taken up by the BBM is transferred to the endoplasmic reticulum (ER) to be esterified. The endocytic pathway organizes the mobilization from the BBM to the ER [43]. The key molecule for the process is NPC1L1 [7]. NPC1L1 resides in the BBM and internalizes upon exposure to cholesterol on the apical membrane [44,45]. Disruption of the gene encoding NPC1L1 reduces cholesterol absorption by half in mice.

Although dietary lipid triacylglycerol is absorbed almost completely, approximately half of the cholesterol in the lumen is absorbed, but with large individual variability [46]. Furthermore, absorption is a slow process. Labeled dietary cholesterol in the plasma peaked 24–48 hours after ingestion, whereas triacylglycerol levels peaked after 2 hours [47,48]. Chylomicron secretion with the subsequent meal ingestion stimulated the appearance of labeled cholesterol in the plasma, indicating that cholesterol is retained in the intestinal epithelia for a long time.

3. The small intestine excretes endogenous cholesterol

3.1. Trans-intestinal cholesterol efflux

Although cholesterol is absorbed and synthesized in the body to supply cells that require it, endogenous excess cholesterol is eliminated from the circulation via the gut, not only for balancing overall cholesterol abundance but also for physiological cholesterol turnover. TICE constitutes up to 70% of FNS excretion in mice [19] and approximately 35% of that in humans [22] in basal conditions, showing that TICE is one of the two reverse cholesterol transport pathways that excretes large amounts of endogenous cholesterol from the body.

Notably, deletion of the gene encoding *NPC1L1* increased FNS excretion [49]. Studies conducted in humans and mice have shown that the potent NPC1L1 inhibitor ezetimibe stimulates TICE by 45% in mice in studies with direct TICE measurements [50], by approximately 3–4-fold in mice as FNS excretion [20,21,51], and by 52% [52] and 67% [53] in humans as FNS excretion. With treatment, unabsorbed dietary and biliary cholesterol contributes to increased FNS excretion only partly,

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 18 September 2018 doi:10.20944/preprints201809.0345.v1

whereas FNS excretion originating from endogenous cholesterol constitutes the major part [53] (Figure 2a). Quantitative analyses with stable isotopes in mice showed that increased FNS excretion was attributable for augmenting TICE [20] (Figure 2b), whereas there were only marginal changes in the biliary cholesterol secretion rate. Indeed, NPC1L1 is not expressed in the liver of mice [7], excluding hepatic contribution to the increase.

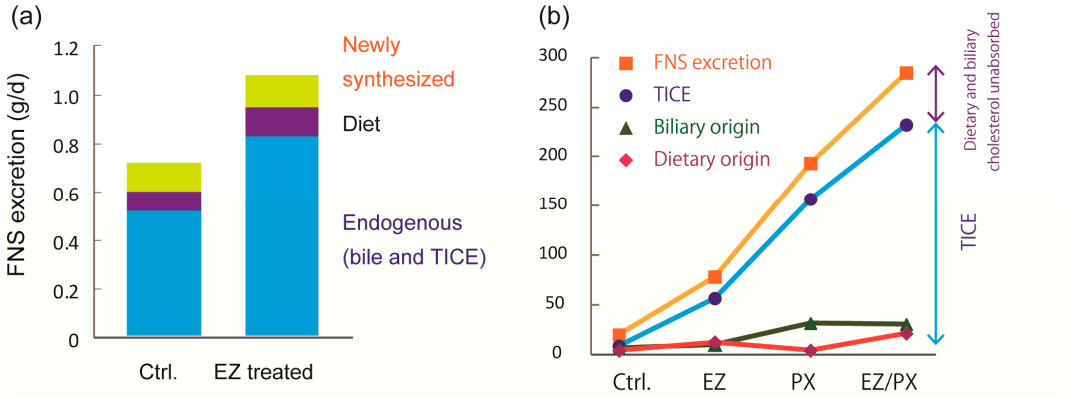

Figure 2. Stimulation of fecal neutral sterol excretion represents an increase in TICE. (**a**) Ezetimibe (EZ)-stimulated fecal neutral sterol (FNS) excretion results from an increase in endogenous cholesterol secretion into the gut lumen in humans, as determined by quantitative analysis with stable cholesterol isotopes (Data are obtained from Ref. [53]), indicating that increased FNS excretion is not attributable for the fraction of cholesterol left unabsorbed. (**b**) TICE dominates in the increase of FNS excretion in mice. Calculation of TICE in mice treated with EZ, PX20626 (PX), or both shows that the increase in FNS excretion originates from the stimulated TICE (Data are obtained from Ref. [20]).

3. 2. ATP-binding cassette G5/G8 heterodimer plays a major role in TICE

ATP-binding cassette (ABC) G5/G8 heterodimers reside in the BBM and are responsible for the efflux cholesterol and noncholesterol sterols (Figure 1d). In addition to a counteracting action against cholesterol absorption [54,55], it has been revealed that ABCG5/G8 plays a major role in TICE. Disruption of ABCG5/G8 genes abolished TICE partly when it was stimulated, although the disruption did not affect basal TICE, indicating that ABCG5/G8 is required for efficient and enhanced TICE [20,50].

3. 3. Cholesterol absorption inhibition is inversely correlated with TICE

We reported previously that BBM-to-lumen cholesterol efflux, *i.e.*, TICE, is inversely correlated with intestinal cholesterol absorption efficiency [50] (Figure 3a). The data shown in Figure 2 revealed that increased FNS excretion mainly originate with TICE; thus, it can be assumed that increased FNS excretion was TICE. Figure 3b shows a meta-analysis of changes in FNS excretion and intestinal cholesterol absorption obtained from published papers to date, demonstrating and supporting the inverse correlation between these two parameters.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 18 September 2018 doi:10.20944/preprints201809.0345.v1

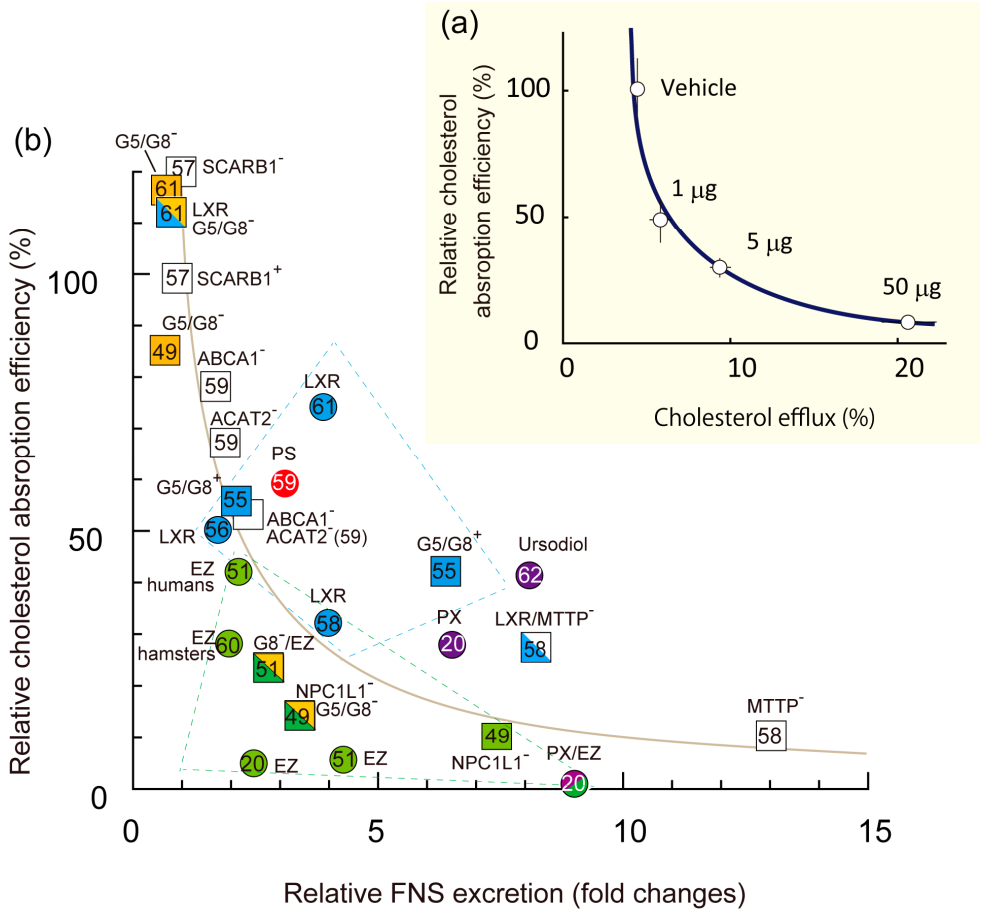

Figure 3. The relationship between intestinal cholesterol absorption and fecal neutral sterol (FNS) excretion. (**a,** *inset*) The figure was originally published in Nakano, T., et al. *PLoS ONE* 2016; 11(3): e0152207 (Ref. [50]). The original titles for X- and Y-axis are "% of DPM appearing in the lumen/DPM in intestinal segment (Efflux efficiency)" and "Relative lumen-to-circulation ^{3}H-cholesterol transit (% vs. vehicle)", respectively. The titles were changed to simplify the figure. An inverse relationship between absorption and TICE was hypothesized in the paper. The numbers (microgram) in the figure indicate the dosages of ezetimibe given to mice. (**b**) All the data presented in the figure were obtained from published papers. Cholesterol absorption efficiency (%) indicates relative ratios compared with the respective control groups, for example, wild-types for transgenic mice and vehicle administration for pharmacological treatments. *Circles*, chemical treatments; *squares*, transgenic mice or those with chemical treatments. EZ, ezetimibe, LXR, liver X receptor agonist; PX, farnesoid X receptor agonist PX20606. +, mice with overexpression of the indicated gene(s); -, mice with deletion of the indicated gene(s). *Green*; NPC1L1 was disrupted by genetic deletion or by EZ treatment; *yellow*, genes for ABCG8 (G8) or both ABCG5 and G8 (G5/G8) were deleted. *blue*, ABCG5/G8 expression levels were activated by LXR agonist or genetic modification. ACAT2, acyl-CoA acyltransferase 2; ABCA1, ATP-binding cassette A1; MTTP, microsomal triglyceride transfer protein; SR-BI, SCARB1, scavenger-receptor B type I. Mice were employed as the model unless mentioned otherwise in the plots. Letters in the symbols indicate the references from which the data were obtained. The areas shown as *blue* and *green* dotted lines indicate experiments with LXR antagonists or ABCG5/G8 overexpression and those with EZ or *NPC1L1* deletion, respectively.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 18 September 2018 doi:10.20944/preprints201809.0345.v1

3. 4. Cholesterol absorption inhibition by liver X receptor antagonism also stimulates TICE

A liver X receptor (LXR) agonist T0901317, which coordinates elimination-prone gene expression, *e.g.*, *ABCG5*, *ABCG8*, and *ABCA1*, in the small intestine [63], not only reduced intestinal cholesterol absorption but also increased FNS excretion in an ABCG5/G8-dependent manner [56,61]. The increase in FNS excretion with another LXR agonist, GW3965, was not affected in *MDR2*$^{-/-}$ mice, in which biliary cholesterol excretion is lacking, again excluding a biliary contribution to the increase [56,61].

The BBM is the site where cholesterol enters and exits for absorption and TICE, respectively. The two processes not only share the platform of the BBM but are also both functionally composed of key molecules, ABCG5/G8 and NPC1L1. So far, it has been depicted that ABCG5/G8 and NPC1L1 independently translocate cholesterol [29,64] by accepting cholesterol from the cytosol and unstirred water layer, respectively (Figure 1b). With recent evidence, we reconsidered the roles of the two transporters, as described below and depicted in Figure 1c.

3.4.1. ABCG5/G8 for cholesterol efflux at the BBM

Heterodimers composed of ABCG5 and ABCG8 are a member of the ABC transporters that excrete sterols in the liver and small intestine. Lee et al. [33] resolved the crystal structure of ABCG5/G8 in lipid bilayers, revealing that cholesterol 'vestibules' open to the inner leaflet of the plasma membrane (Figure 1d), indicating that it is the entry path of cholesterol and the transporter captures cholesterol from the BBM [65]. This observation agreed with our idea that the BBM itself is the dividing ridge for cholesterol flux, and the transporter does not necessarily receive cholesterol from the cytosol as indicated previously (Figure 1b).

3.4.2. NPC1L1 for cholesterol influx at the BBM

NPC1L1 is a polytopic transmembrane protein that is endocytosed to deliver cholesterol to the ER upon exposure of the apical membrane to cholesterol. The N-terminal domain has a cholesterol binding pocket [66]. Additionally, the protein contains a sterol-sensing domain to accommodate one molecule of cholesterol [67]. These are likely to act to sense and initiate clathrin-mediated endocytosis. NPC1L1 resides in cholesterol-rich microdomains; thus, endocytosis drags in a number of cholesterol molecules at once (Figure 1c) [68]. Indeed, NPC1L1-mediated endocytosis is also associated with the absorption of fat-soluble vitamins E and K [69-73], probably by non-specific incorporation of membrane fragments together with NPC1L1.

4. Altered cholesterol flux in the small intestine with phytosterol

4.1. Cholesterol pool and flux in the small intestine

A number of studies have been conducted to determine cholesterol flux and synthesis in the intestinal tract, and Magot et al. [75] explained this relationship as an open one-compartment model (Figure 4a). The intestinal tract, especially the upper small intestine, is the second greatest organ to synthesize cholesterol [76,77]. In addition to cholesterol absorbed from the lumen, newly synthesized cholesterol remains in the villi, with a slow translocation to the lymph or the lumen (approximately 4% per hour [74]), providing a large pool of cholesterol in the mucosa [77-79]. The cholesterol pool mainly consists of free cholesterol [75], indicating that cholesterol in the membrane, especially the BBM, constitutes the major part (Figure 1d). In principle, the ultimate absorption efficiency will be determined by the direction of flux that the pool of cholesterol goes to and how fast it moves. The lymph flow determines the amount of cholesterol to move from the pool into the circulation [22]. This means that the movement of cholesterol of dietary origin, or absorption, is driven by meal intakes,

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 18 September 2018 doi:10.20944/preprints201809.0345.v1

especially fatty diets [79,80]. Cholesterol in the BBM can also translocate to the lumen, namely TICE, which should be increased when absorption is limited.

4.2. Proposed mechanisms for the inhibitory effect to date

Several ideas have been postulated for the mechanism of phytosterol-mediated cholesterol absorption inhibition so far [14]. Solubilization of sterols in the lumen is an indispensable process for the absorption. β-Sitosterol reduces cholesterol solubilization in mixed micelles *in vitro* [81-83]. Ikeda et al. showed that the presence of β-sitosterol limited the cholesterol transfer efficiency from micelles to the BBM in a series of *in vitro* experiments [16]. These effects of phytosterols are described as the micellar solubilization hypothesis [84].

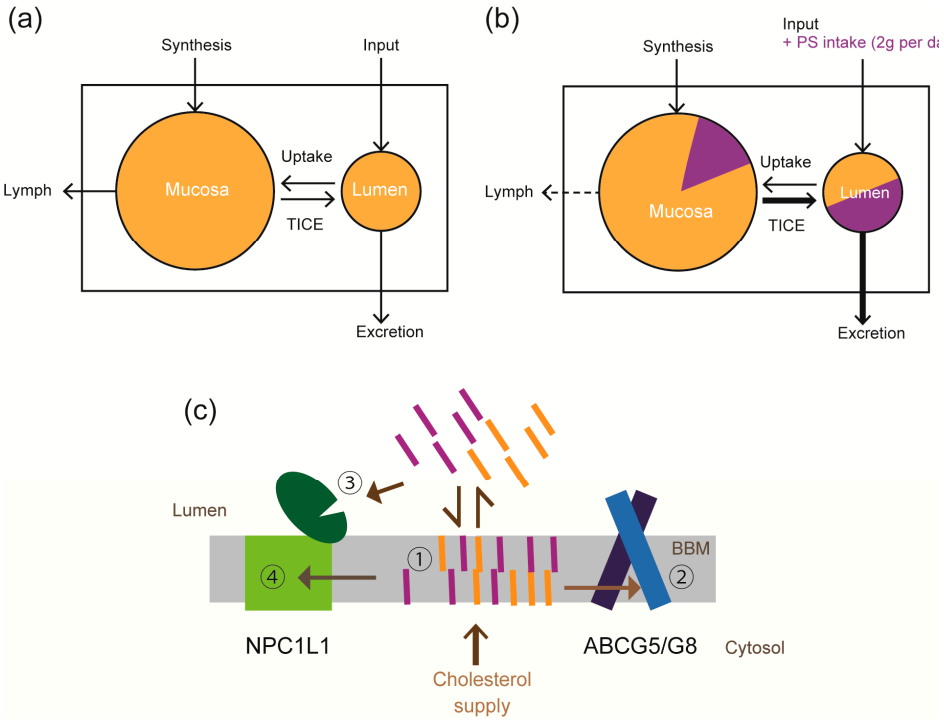

Figure 4. Phytosterol-mediated cholesterol absorption inhibition. (**a**) An open one-compartment model for intestinal cholesterol flux. The rectangle represents the small intestine. "Lumen" and "Mucosa" in white indicate cholesterol pools in the intestinal lumen and in the intestinal epithelia, respectively. "Input" and "Excretion" indicate cholesterol supplied to the intestinal lumen (bile, diet, and intestinal cell sloughing), and that excreted into feces, respectively. "Synthesis" indicates cholesterol biosynthesis in the small intestine that supplies to the mucosal cholesterol pool. "Lymph" indicates cholesterol transport from the mucosal pool to the lymphatic fluid and eventually the circulation. The model was originally presented by Magot et al. [32], simplified by Perrodin et al. [74], and reworked by us. (**b**) Changes in cholesterol flux with phytosterol intake. Two grams of phytosterol (PS) dominates the luminal sterols and is incorporated into the brush border membrane (Mucosa). PS stimulates TICE, thus increasing total fecal sterol excretion. These increased pathways are shown in *bold arrows*. PS is indicated in *purple*. (**c**) Possible effective sites of phytosterols for cholesterol absorption inhibition. The possible sites are indicated as (1) to (4) in the figure. (1), cholesterol (*yellow bars*) and phytosterols (*purple bars*) are taken up by the brush border membrane (BBM), where the sterol capacity is limited. (2), ABCG5/G8 accepts both substrates. ABCG5/G8 might be activated by phytosterols and efflux more cholesterol concomitantly. (3), phytosterols might dilute cholesterol at the surface of the mucosa, reducing the chance to access to the binding site in the N-terminal domain of NPC1L1. (4), phytosterols can be mixed with cholesterol in the BBM, reducing the abundance of cholesterol in it and preventing the sterol-sensing domain (SSD) of NPC1L1 from

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 18 September 2018 doi:10.20944/preprints201809.0345.v1

sensing an increase in cholesterol. Phytosterols may also compete with cholesterol for the binding site of the SSD.

However, there has been no evidence that demonstrates inhibition *in vivo* [81,85]. Adding phytosterols to feed did not reduce the amount of cholesterol solubilized in the luminal contents in rats with statistically significance [85]. In fact, even with approximately 30–40% limited solubility in mixed micelles [16], the small intestine seemed to have a vast capacity to take up several hundred mg of cholesterol in a meal. Furthermore, it can take a couple of days to transit unabsorbed cholesterol through the intestinal tract [86]. Thus, the small intestine could compensate for the limited cholesterol availability from mixed micelles in the presence of phytosterols. The micellar solubilization hypothesis presupposes the presence of phytosterol in the lumen, or ingestion of cholesterol together with phytosterols to exert the effect. On the other hand, even when a phytosterol-supplemented meal was taken once a day, a similar LDL-C lowering effect was obtained compared with the same amount of phytosterol (for example, 2.5 g in ref [87]) taken three times a day at meals [87-89], implying that the presence of phytosterol in the lumen is not necessarily required for the effect. Finally, we showed that TICE counteracts cholesterol absorption, as shown in Figure 3. Phytosterol stimulates TICE [23]; thus, this augmentation of efflux should be involved in the effect.

4.3. Phytosterol transition to the BBM

Cholesterol and phytosterols have a similar physicochemical nature, thus it is likely that they behave alike during the movement between the unstirred water layer at the luminal surface and the lipid bilayer of the BBM [81]. Indeed, cholesterol and phytosterols were incorporated into the BBM to almost the same extent, when the incorporations were measured at an initial period after the exposure in mice [39,90]. After incorporation, phytosterols are effluxed from the tissue rapidly according to their hydrophobicity [50,90]. Accordingly, phytosterols will be taken up again, resulting in repeated shuttling between the lumen and the BBM in the small intestinal tract (Figure 4b).

4.4. Possible sites where phytosterols compete with the absorption process of cholesterol

Cholesterol and phytosterols are both incorporated into the BBM, but the capacity of the BBM to accommodate sterols is limited, possibly resulting in the induction of sterol efflux from the BBM to the lumen. Thus, the BBM is a possible site where phytosterols and cholesterol complete (Figure 4c, *site number 1*). We showed that phytosterol perfusion in intestinal segments increased cholesterol efflux from the epithelia [91]. On the contrary, such an effect was not observed when the same amount of cholesterol was perfused.

Driven by ATP hydrolysis, ABCG5/G8 effluxes sterols back into the lumen. Interactions with the other molecules can increase the hydrolyzing activity [92,93]. Rapid efflux of phytosterols by ABCG5/G8 implies a possibility that phytosterols stimulate ATP hydrolysis by ABCG5/G8 [91]. Thus, an increase in ABCG5/G8 activity in the presence of phytosterols might promote cholesterol efflux concomitantly (Figure 4c, *site number 2*). However, comparable phytosterol-mediated fractional cholesterol absorption inhibition was observed even in mice lacking ABCG5/G8 [23,94], negating ABCG5/G8-mediated cholesterol efflux as the inhibitory mechanism, although a phytosterol-induced increase in FNS excretion was reduced by one-third in mice [23].

Endocytosis by NPC1L1, a process to facilitate cholesterol absorption, takes place when excess cholesterol is exposed in tissues or cells [44,45,95]. This means that excess cholesterol is sensed by NPC1L1 or NPC1L1-containing complexes. As analogous compounds to cholesterol, the presence of phytosterols may impair this sensing, leading to the attenuation of endocytosis, although there are no available data on this possibility at present. NPC1L1 has a cholesterol binding pocket in its N-terminal domain (Figure 4c, *site number 3*). The binding site is relatively cholesterol specific and was not disturbed by adding β-sitosterol [66]; therefore, it is unlikely to be a possible competitive site. The

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 18 September 2018 doi:10.20944/preprints201809.0345.v1

sterol-sensing domain (Figure 4c, *site number 4*) is also a possible competitive site, which needs to be elucidated in future studies.

4.5. Phytosterol intake stimulates TICE

Intake of phytosterol stimulates FNS excretion, in which TICE is attributed for the increase in mice. Even with an extensive and long research history for phytosterol-mediated LDL-C lowering, no study has been addressed to this notable effect except for that by Brufau et al. [23]. They raised phytosterol content in the diet up to 8%, thereby observing a gradual increase in cholesterol absorption inhibition and 85% maximum reduction. On the other hand, 1% phytosterol diet increased FNS excretion by more than 3-fold, but there was no such dose-dependent effect in it with a 1–8% phytosterol diet.

The subjects who carry a heterozygous inactivating mutation of NPC1L1 had much lower CVD risk even with a weak reduction of LDL-C [8]. A number of studies have demonstrated that impairment of NPC1L1 stimulates FNS excretion [20,21,49,51-53]. The increased FNS excretion should promote the renewal of circulating cholesterol-rich lipoproteins, especially LDL, through the life [96,97]. LDL undergoes a variety of modifications in the body [98,99]. The modified products potently induce atherosclerosis [100]. Accordingly, the accelerated renewal or clearance of atherogenic lipoproteins may play a role in the favorable effect.

The abovementioned inconsistency in the efficacies between the two parameters with phytosterol-supplemented feed in mice [23] suggests a possibility that phytosterol-supplemented diet stimulates FNS excretion at a low dosage that does not provide apparent LDL-C lowering. If so, not only phytosterol-enriched foods [89,101], but also just encouraging to consume phytosterol-rich ingredients, such as nuts, seeds, vegetable oils, could be comparable to the partially NPC1L1-inactive conditions [8]. Further studies are needed to elucidate the possibility.

5. Concluding remarks

In this review, we proposed a model that the BBM stands as the dividing ridge of cholesterol fluxes, influx and efflux. An inverse correlation was found in two parameters in the meta-analysis shown in Figure 3b, supporting the above model. However, the correlation appears not to be so decisive. The inhibitions were superior when NPC1L1 activity was impaired. In contrast, FNS excretion was superior when LXR was agonized or ABCG5/G8 was overexpressed (Figure 3b, see the boxes in the dashed lines). These findings indicated that other factors are involved to provide the balancing. For example, increased cholesterol synthesis with atorvastatin treatment augmented FNS excretion without reducing cholesterol absorption [102].

Although there may be limitations in the model we propose, we show that it is obvious that the BBM is providing the location for balancing the fluxes. Cholesterol absorption inhibition and TICE have been independently investigated as therapeutic targets of hypercholesterolemia. Because the two phenomena are mechanistically interrelated, they should be considered together when targeting the small intestine for treatment, and of course by phytosterols as well.

Author Contributions: T.N. wrote the paper. I.I. and T.M. reviewed and edited the paper. All authors read and approved the final manuscript.

Funding: This study was supported by JSPS KAKENHI (25504013 and 16K00864).

Acknowledgments: The authors thank Ms. Sawako Sato and Ms. Yuka Nakano for secretarial assistance.

Conflicts of Interest: The authors declare no competing interest.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 18 September 2018 doi:10.20944/preprints201809.0345.v1

References

1. Desmond, E.; Gribaldo, S. Phylogenomics of sterol synthesis: Insights into the origin, evolution, and diversity of a key eukaryotic feature. *Genome Biol. Evol.* **2009**, *1*, 364-381. doi:10.1093/gbe/evp036

2. Navarese, E.P.; Robinson, J.G.; Kowalewski, M.; Kołodziejczak, M.; Andreotti, F.; Bliden, K.; Tantry, U.; Kubica, J.; Raggi, P.; Gurbel, P.A. Association between baseline LDL-C level and total and cardiovascular mortality after LDL-C lowering: A systematic review and meta-analysis. *JAMA* **2018**, *319*, 1566-1579. doi:10.1001/jama.2018.2525

3. WHO. Cardiovascular diseases (CVDs). World Health Organization **2018**. Available online: http://www.who.int/cardiovascular_diseases/en/ (accessed on 2018.09.14)

4. Rader, D.J. Future outlook: Changing perspectives on best practice. *Am. J. Manag. Care.* **2002**, 8, S40-44.

5. Cholesterol Treatment Trialists' (CTT) Collaboration. Efficacy and safety of more intensive lowering of LDL cholesterol: A meta-analysis of data from 170 000 participants in 26 randomised trials. *Lancet* **2010**, *376*, 1670-1681. doi:10.1016/S0140-6736(10)61350-5

6. LaRosa, J.C.; Grundy, S.M.; Waters, D.D.; Shear, C.; Barter, P.; Fruchart, J.-C.; Gotto, A.M.; Greten, H.; Kastelein, J.J.P.; Shepherd, J., et al. Intensive lipid lowering with atorvastatin in patients with stable coronary disease. *N. Engl. J. Med.* **2005**, *352*, 1425-1435. doi:10.1056/NEJMoa050461

7. Altmann, S.W.; Davis, H.R., Jr.; Zhu, L.J.; Yao, X.; Hoos, L.M.; Tetzloff, G.; Iyer, S.P.; Maguire, M.; Golovko, A.; Zeng, M., et al. Niemann-Pick C1 like 1 protein is critical for intestinal cholesterol absorption. *Science* **2004**, *303*, 1201-1204.

8. The Myocardial Infarction Genetics Consortium Investigators. Inactivating mutations in NPC1L1 and protection from coronary heart disease. *N. Engl. J. Med.* **2014**, *371*, 2072-2082. doi:10.1056/NEJMoa1405386

9. Sudhop, T.; Lütjohann, D.; von Bergmann, K. Sterol transporters: Targets of natural sterols and new lipid lowering drugs. *Pharmacol. Ther.* **2005**, *105*, 333-341. doi:10.1016/j.pharmthera.2004.10.011

10. Matsuoka, K.; Nakazawa, T.; Nakamura, A.; Honda, C.; Endo, K.; Tsukada, M. Study of thermodynamic parameters for solubilization of plant sterol and stanol in bile salt micelles. *Chem. Phys. Lipids* **2008**, *154*, 87-93. doi:10.1016/j.chemphyslip.2008.05.002

11. Tso, P.; Fujimoto, K. The absorption and transport of lipids by the small intestine. *Brain Res. Bull.* **1991**, *27*, 477-482.

12. O'Neill, F.H.; Sanders, T.A.B.; Thompson, G.R. Comparison of efficacy of plant stanol ester and sterol ester: Short-term and longer-term studies. *Am. J. Cardiol.* **2005**, *96*, 29-36. doi:10.1016/j.amjcard.2005.03.017

13. Peterson, D.W. Effect of soybean sterols in the diet on plasma and liver cholesterol in chicks. *Proc. Soc. Exp. Biol. Med.* **1951**, *78*, 143-147.

14. Smet, E.D.; Mensink, R.P.; Plat, J. Effects of plant sterols and stanols on intestinal cholesterol metabolism: Suggested mechanisms from past to present. *Mol. Nutr. Food Res.* **2012**, *56*, 1058-1072. doi:10.1002/mnfr.201100722

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 18 September 2018 doi:10.20944/preprints201809.0345.v1

15. Ras, R.T.; Geleijnse, J.M.; Trautwein, E.A. LDL-cholesterol-lowering effect of plant sterols and stanols across different dose ranges: A meta-analysis of randomised controlled studies. *Br. J. Nutr.* **2014**, *112*, 214-219. doi:10.1017/S0007114514000750

16. Ikeda, I.; Tanaka, K.; Sugano, M.; Vahouny, G.V.; Gallo, L.L. Inhibition of cholesterol absorption in rats by plant sterols. *J. Lipid Res.* **1988**, *29*, 1573-1582.

17. Nijstad, N.; Gautier, T.; Briand, F.; Rader, D.J.; Tietge, U.J.F. Biliary sterol secretion is required for functional in vivo reverse cholesterol transport in mice. *Gastroenterology* **2011**, *140*, 1043-1051. doi:10.1053/j.gastro.2010.11.055

18. Simmonds, W.J.; Hofmann, A.F.; Theodor, E. Absorption of cholesterol from a micellar solution: Intestinal perfusion studies in man. *J. Clin. Invest.* **1967**, *46*, 874-890.

19. van der Velde, A.E.; Brufau, G.; Groen, A.K. Transintestinal cholesterol efflux. *Curr. Opin. Lipidol.* **2010**, *21*, 167-171. doi:10.1097/MOL.0b013e3283395e45

20. de Boer, J.F.; Schonewille, M.; Boesjes, M.; Wolters, H.; Bloks, V.W.; Bos, T.; van Dijk, T.H.; Jurdzinski, A.; Boverhof, R.; Wolters, J.C., et al. Intestinal farnesoid X receptor controls transintestinal cholesterol excretion in mice. *Gastroenterology* **2017**, *152*, 1126-1138. doi:10.1053/j.gastro.2016.12.037

21. Jakulj, L.; Vissers, M.N.; van Roomen, C.P.; van der Veen, J.N.; Vrins, C.L.J.; Kunne, C.; Stellaard, F.; Kastelein, J.J.P.; Groen, A.K. Ezetimibe stimulates faecal neutral sterol excretion depending on *abcg8* function in mice. *FEBS Lett.* **2010**, *584*, 3625-3628. doi:10.1016/j.febslet.2010.07.035

22. Reeskamp, L.F.; Meessen, E.C.E.; Groen, A.K. Transintestinal cholesterol excretion in humans. *Curr. Opin. Lipidol.* **2018**, *29*, 10-17. doi:10.1097/mol.0000000000000473

23. Brufau, G.; Kuipers, F.; Lin, Y.; Trautwein, E.A.; Groen, A.K. A reappraisal of the mechanism by which plant sterols promote neutral sterol loss in mice. *PLoS ONE* **2011**, *6*, e21576. doi:10.1371/journal.pone.0021576

24. Fumeron, F.; Bard, J.-M.; Lecerf, J.-M. Interindividual variability in the cholesterol-lowering effect of supplementation with plant sterols or stanols. *Nutr. Rev.* **2017**, *75*, 134-145. doi:10.1093/nutrit/nuw059

25. Weingärtner, O.; Baber, R.; Teupser, D. Plant sterols in food: No consensus in guidelines. *Biochem. Biophys. Res. Comm.* **2014**, *446*, 811-813. doi:10.1016/j.bbrc.2014.01.147

26. Alemany, L.; Barbera, R.; Alegría, A.; Laparra, J.M. Plant sterols from foods in inflammation and risk of cardiovascular disease: A real threat? *Food Chem. Toxicol.* **2014**, *69*, 140-149. doi:10.1016/j.fct.2014.03.038

27. Dawson, P.A.; Rudel, L.L. Intestinal cholesterol absorption. *Curr. Opin. Lipidol.* **1999**, *10*, 315-320.

28. Wang, D.Q. New concepts of mechanisms of intestinal cholesterol absorption. *Ann. Hepatol.* **2003**, *2*, 113-121.

29. Brown, J.M.; Yu, L. Opposing gatekeepers of apical sterol transport: Niemann-Pick C1-like 1 (NPC1L1) and ATP-binding cassette transporters G5 and G8 (ABCG5/ABCG8). *Immunol. Endocr. Metab. Agents Med. Chem.* **2009**, *9*, 18-29. doi:10.2174/187152209788009797

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 18 September 2018 doi:10.20944/preprints201809.0345.v1

30. Kidambi, S.; Patel, S.B. Cholesterol and non-cholesterol sterol transporters: ABCG5, ABCG8 and NPC1L1: A review. *Xenobiotica* **2008**, *38*, 1119-1139. doi:10.1080/00498250802007930

31. Thomson, A.B.; Schoeller, C.; Keelan, M.; Smith, L.; Clandinin, M.T. Lipid absorption: Passing through the unstirred layers, brush-border membrane, and beyond. *Can. J. Physiol. Pharmacol.* **1993**, *71*, 531-555.

32. Magot, T.; Verneau, C.; Lutton, C.; Chevallier, F. Origin and fate of cholesterol in rat plasma lipoproteins *in vivo*. I. Qualitative analysis. *Ann. Nutr. Metab.* **1985**, *29*, 147-159. doi:10.1159/000176958

33. Lee, J.-Y.; Kinch, L.N.; Borek, D.M.; Wang, J.; Wang, J.; Urbatsch, I.L.; Xie, X.-S.; Grishin, N.V.; Cohen, J.C.; Otwinowski, Z., et al. Crystal structure of the human sterol transporter ABCG5/ABCG8. *Nature* **2016**, *533*, 561-564. doi:10.1038/nature17666

34. Christon, R.; Meslin, J.C.; Thevenoux, J.; Linard, A.; Leger, C.L.; Delpal, S. Effects of a low dietary linoleic acid level on intestinal morphology and enterocyte brush border membrane lipid composition. *Reprod. Nutr. Dev.* **1991**, *31*, 691-701.

35. Westergaard, H.; Dietschy, J.M. The mechanism whereby bile acid micelles increase the rate of fatty acid and cholesterol uptake into the intestinal mucosal cell. *J. Clin. Invest.* **1976**, *58*, 97-108.

36. Chijiiwa, K.; Linscheer, W.G. Distribution and monomer activity of cholesterol in micellar bile salt: Effect of cholesterol level. *Am. J. Physiol. – Gastrointest. Liver Physiol.* **1987**, *252*, G309-G314. doi:10.1152/ajpgi.1987.252.3.G309

37. Sylvén, C.; Borgström, B. Absorption and lymphatic transport of cholesterol in the rat. *J. Lipid Res*. **1968**, *9*, 596-601.

38. Fujikawa, M.; Nakao, K.; Shimizu, R.; Akamatsu, M. QSAR study on permeability of hydrophobic compounds with artificial membranes. *Bioorg. Med. Chem.* **2007**, *15*, 3756-3767. doi:10.1016/j.bmc.2007.03.040

39. Compassi, S.; Werder, M.; Weber, F.E.; Boffelli, D.; Hauser, H.; Schulthess, G. Comparison of cholesterol and sitosterol uptake in different brush border membrane models. *Biochemistry* **1997**, *36*, 6643-6652. doi:10.1021/bi9620666

40. Hauser, H.; Howell, K.; Dawson, R.M.; Bowyer, D.E. Rabbit small intestinal brush border membrane preparation and lipid composition. *Biochim. Biophys. Acta* **1980**, *602*, 567-577.

41. Knöpfel, M.; Davies, J.P.; Duong, P.T.; Kværnø, L.; Carreira, E.M.; Phillips, M.C.; Ioannou, Y.A.; Hauser, H. Multiple plasma membrane receptors but not NPC1L1 mediate high-affinity, ezetimibe-sensitive cholesterol uptake into the intestinal brush border membrane. *Biochim. Biophys. Acta (BBA) – Mol. Cell Biol. Lipids* **2007**, *1771*, 1140-1147. doi:10.1016/j.bbalip.2007.05.011

42. Nguyen, D.V.; Drover, V.A.; Knopfel, M.; Dhanasekaran, P.; Hauser, H.; Phillips, M.C. Influence of class B scavenger receptors on cholesterol flux across the brush border membrane and intestinal absorption. *J. Lipid Res.* **2009**, *50*, 2235-2244. doi:10.1194/jlr.M900036-JLR200

43. Field, F.J.; Watt, K.; Mathur, S.N. Ezetimibe interferes with cholesterol trafficking from the plasma membrane to the endoplasmic reticulum in Caco-2 cells. *J. Lipid Res*. **2007**, *48*, 1735-1745.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 18 September 2018 doi:10.20944/preprints201809.0345.v1

44. Skov, M.; Tønnesen, C.K.; Hansen, G.H.; Danielsen, E.M. Dietary cholesterol induces trafficking of intestinal Niemann-Pick type C1 like 1 from the brush border to endosomes. *Am. J. Physiol. – Gastrointest. Liver Physiol.* **2010**, *300*, G33-G40.

45. Xie, C.; Zhou, Z.-S.; Li, N.; Bian, Y.; Wang, Y.-J.; Wang, L.-J.; Li, B.-L.; Song, B.-L. Ezetimibe blocks the internalization of NPC1L1 and cholesterol in mouse small intestine. *J. Lipid Res.* **2012**, *53*, 2092-2101. doi:10.1194/jlr.M027359

46. Wilson, M.D.; Rudel, L.L. Review of cholesterol absorption with emphasis on dietary and biliary cholesterol. *J. Lipid Res.* **1994**, *35*, 943-955.

47. Dubois, C.; Armand, M.; Ferezou, J.; Beaumier, G.; Portugal, H.; Pauli, A.; Bernard, P.; Becue, T.; Lafont, H.; Lairon, D. Postprandial appearance of dietary deuterated cholesterol in the chylomicron fraction and whole plasma in healthy subjects. *Am. J. Clin. Nutr.* **1996**, *64*, 47-52.

48. Samuel, P.; Crouse, J.R.; Ahrens, E.H. Evaluation of an isotope ratio method for measurement of cholesterol absorption in man. *J. Lipid Res.* **1978**, *19*, 82-93.

49. Tang, W.; Ma, Y.; Jia, L.; Ioannou, Y.A.; Davies, J.P.; Yu, L. Genetic inactivation of NPC1L1 protects against sitosterolemia in mice lacking ABCG5/ABCG8. *J. Lipid Res.* **2009**, *50*, 293-300. doi:10.1194/jlr.M800439-JLR200

50. Nakano, T.; Inoue, I.; Takenaka, Y.; Ono, H.; Katayama, S.; Awata, T.; Murakoshi, T. Ezetimibe promotes brush border membrane-to-lumen cholesterol efflux in the small intestine. *PLoS ONE* **2016**, *11*, e0152207. doi:10.1371/journal.pone.0152207

51. Jakulj, L.; van Dijk, T.H.; de Boer, J.F.; Kootte, R.S.; Schonewille, M.; Paalvast, Y.; Boer, T.; Bloks, V.W.; Boverhof, R.; Nieuwdorp, M., et al. Transintestinal cholesterol transport is active in mice and humans and controls ezetimibe-induced fecal neutral sterol excretion. *Cell Metab.* **2016**, *24*, 783-794. doi:10.1016/j.cmet.2016.10.001

52. Briand, F.; Naik, S.U.; Fuki, I.; Millar, J.S.; Macphee, C.; Walker, M.; Billheimer, J.; Rothblat, G.; Rader, D.J. Both the peroxisome proliferator-activated receptor delta agonist, GW0742, and ezetimibe promote reverse cholesterol transport in mice by reducing intestinal reabsorption of HDL-derived cholesterol. *Clin. Transl. Sci.* **2009**, *2*, 127-133.

53. Lin, X.; Racette, S.B.; Ma, L.; Wallendorf, M.; Ostlund, R.E. Ezetimibe increases endogenous cholesterol excretion in humans. *Arterioscler. Thromb. Vasc. Biol.* **2017**, *37*, 990-996. doi:10.1161/atvbaha.117.309119

54. Bhattacharyya, A.K.; Connor, W.E.; Lin, D.S.; McMurry, M.M.; Shulman, R.S. Sluggish sitosterol turnover and hepatic failure to excrete sitosterol into bile cause expansion of body pool of sitosterol in patients with sitosterolemia and xanthomatosis. *Arterioscler. Thromb. Vasc. Biol.* **1991**, *11*, 1287-1294. doi:10.1161/01.atv.11.5.1287

55. Yu, L.; Li-Hawkins, J.; Hammer, R.E.; Berge, K.E.; Horton, J.D.; Cohen, J.C.; Hobbs, H.H. Overexpression of ABCG5 and ABCG8 promotes biliary cholesterol secretion and reduces fractional absorption of dietary cholesterol. *J. Clin. Invest.* **2002**, *110*, 671-680.

56. Kruit, J.K.; Plösch, T.; Havinga, R.; Boverhof, R.; Groot, P.H.E.; Groen, A.K.; Kuipers, F. Increased fecal neutral sterol loss upon liver X receptor activation is independent of biliary sterol secretion in mice. *Gastroenterology* **2005**, *128*, 147-156. doi:10.1053/j.gastro.2004.10.006

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 18 September 2018 doi:10.20944/preprints201809.0345.v1

554 57. Bura, K.S.; Lord, C.; Marshall, S.; McDaniel, A.; Thomas, G.; Warrier, M.; Zhang, J.; Davis,
555 M.A.; Sawyer, J.K.; Shah, R., et al. Intestinal SR-BI does not impact cholesterol absorption or
556 transintestinal cholesterol efflux in mice. *J. Lipid Res.* **2013**, *54*, 1567-1577. doi:10.1194/jlr.M034454

557 58. Xie, Y.; Kennedy, S.; Sidhu, R.; Luo, J.; Ory, D.S.; Davidson, N.O. Liver X receptor agonist
558 modulation of cholesterol efflux in mice with intestine-specific deletion of microsomal triglyceride
559 transfer protein. *Arterioscler. Thromb. Vasc. Biol.* **2012**, *32*, 1624-1631. doi:10.1161/atvbaha.112.246066

560 59. Turley, S.D.; Valasek, M.A.; Repa, J.J.; Dietschy, J.M. Multiple mechanisms limit the
561 accumulation of unesterified cholesterol in the small intestine of mice deficient in both ACAT2 and
562 ABCA1. *Am. J. Physiol. – Gastrointest. Liver Physiol.* **2010**, *299*, G1012-G1022.
563 doi:10.1152/ajpgi.00190.2010

564 60. Valasek, M.A.; Repa, J.J.; Quan, G.; Dietschy, J.M.; Turley, S.D. Inhibiting intestinal NPC1L1
565 activity prevents diet-induced increase in biliary cholesterol in Golden Syrian hamsters. *Am. J.*
566 *Physiol. – Gastrointest. Liver Physiol.* **2008**, *295*, G813-G822. doi:10.1152/ajpgi.90372.2008

567 61. Yu, L.; York, J.; von Bergmann, K.; Lutjohann, D.; Cohen, J.C.; Hobbs, H.H. Stimulation of
568 cholesterol excretion by the liver X receptor agonist requires ATP-binding cassette transporters G5
569 and G8. *J. Biol. Chem.* **2003**, *278*, 15565-15570. doi:10.1074/jbc.M301311200

570 62. Wang, Y.; Liu, X.; Pijut, S.S.; Li, J.; Horn, J.; Bradford, E.M.; Leggas, M.; Barrett, T.A.; Graf, G.A.
571 The combination of ezetimibe and ursodiol promotes fecal sterol excretion and reveals a G5G8-
572 independent pathway for cholesterol elimination. *J. Lipid Res.* **2015**, *56*, 810-820.
573 doi:10.1194/jlr.M053454

574 63. Bonamassa, B.; Moschetta, A. Atherosclerosis: Lessons from LXR and the intestine. *Trends*
575 *Endocrinol. Metab.* **2013**, *24*, 120-128. doi:10.1016/j.tem.2012.10.004

576 64. Afonso, M.S.; Machado, R.M.; Lavrador, M.; Quintao, E.C.R.; Moore, K.; Lottenberg, A.
577 Molecular pathways underlying cholesterol homeostasis. *Nutrients* **2018**, *10*, 760.

578 65. Neumann, J.; Rose-Sperling, D.; Hellmich, U.A. Diverse relations between ABC transporters
579 and lipids: An overview. *Biochim. Biophys. Acta (BBA) – Biomembranes* **2017**, *1859*, 605-618.
580 doi:10.1016/j.bbamem.2016.09.023

581 66. Zhang, J.-H.; Ge, L.; Qi, W.; Zhang, L.; Miao, H.-H.; Li, B.-L.; Yang, M.; Song, B.-L. The N-
582 terminal domain of NPC1L1 protein binds cholesterol and plays essential roles in cholesterol
583 uptake. *J. Biol. Chem.* **2011**, *286*, 25088-25097. doi:10.1074/jbc.M111.244475

584 67. Radhakrishnan, A.; Sun, L.-P.; Kwon, H.J.; Brown, M.S.; Goldstein, J.L. Direct binding of
585 cholesterol to the purified membrane region of SCAP: Mechanism for a sterol-sensing domain. *Mol.*
586 *Cell* **2004**, *15*, 259-268. doi:10.1016/j.molcel.2004.06.019

587 68. Ge, L.; Qi, W.; Wang, L.-J.; Miao, H.-H.; Qu, Y.-X.; Li, B.-L.; Song, B.-L. Flotillins play an
588 essential role in Niemann-Pick C1-like 1-mediated cholesterol uptake. *Proc. Natl. Acad. Sci.* **2011**,
589 *108*, 551-556. doi:10.1073/pnas.1014434108

590 69. Kamishikiryo, J.; Haraguchi, M.; Nakashima, S.; Tasaka, Y.; Narahara, H.; Sugihara, N.;
591 Nakamura, T.; Morita, T. N-terminal domain of the cholesterol transporter Niemann–Pick C1-Like 1
592 (NPC1L1) is essential for α-tocopherol transport. *Biochem. Biophys. Res. Commun.* **2017**, *486*, 476-480.
593 doi:10.1016/j.bbrc.2017.03.065

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 18 September 2018 doi:10.20944/preprints201809.0345.v1

70. Abuasal, B.; Sylvester, P.W.; Kaddoumi, A. Intestinal absorption of γ-tocotrienol is mediated by Niemann-Pick C1-like 1: In situ rat intestinal perfusion studies. *Drug Metab. Dispos.* **2010**, *38*, 939-45. doi:10.1002/bdd.1790

71. Narushima, K.; Takada, T.; Yamanashi, Y.; Suzuki, H. Niemann-Pick C1-like 1 mediates α-tocopherol transport. *Mol. Pharmacol.* **2008**, *74*, 42-9. doi:10.1124/mol.107.043034

72. Takada, T.; Yamanashi, Y.; Konishi, K.; Yamamoto, T.; Toyoda, Y.; Masuo, Y.; Yamamoto, H.; Suzuki, H. NPC1L1 is a key regulator of intestinal vitamin K absorption and a modulator of warfarin therapy. *Sci. Transl. Med.* **2015**, *7*, 275ra23. doi:10.1126/scitranslmed.3010329

73. Reboul, E.; Borel, P. Proteins involved in uptake, intracellular transport and basolateral secretion of fat-soluble vitamins and carotenoids by mammalian enterocytes. *Prog. Lipid Res.* **2011**, *50*, 388-402. doi:10.1016/j.plipres.2011.07.001

74. Perrodin, M.; Serougne, C.; Lutton, C. In vivo cholesterol synthesis by the rat digestive tract. II. A study of turnover. *Reprod. Nutr. Dev.* **1985**, *25*, 659-669.

75. Magot, T.; Champarnaud, G.; Anfreville, R.; Lutton, C.; Chevallier, F. Origin and fate of cholesterol in rat plasma lipoproteins in vivo. II. Modelling of cholesterol absorption and its release into plasma lipoproteins. *Ann. Nutr. Metab.* **1985**, *29*, 160-174. doi:10.1159/000176959

76. Feingold, K.R.; Wiley, M.H.; Moser, A.H.; Lau, D.T.; Lear, S.R.; Siperstein, M.D. De novo sterologenesis in intact primates. *J. Lab. Clin. Med.* **1982**, *100*, 405-410.

77. Perrodin, M.; Lutton, C. In vivo cholesterol synthesis by the rat digestive tract. I. A topological study. *Reprod. Nutr. Dev.* **1985**, *25*, 647-657.

78. Karavia, E.A.; Papachristou, N.I.; Sakellaropoulos, G.C.; Xepapadaki, E.; Papamichail, E.; Petropoulou, P.-I.; Papakosta, E.P.; Constantinou, C.; Habeos, I.; Papachristou, D.J., et al. Scavenger receptor class B type I regulates plasma apolipoprotein E levels and dietary lipid deposition to the liver. *Biochemistry* **2015**, *54*, 5605-5616. doi:10.1021/acs.biochem.5b00700

79. Borgstrom, B.; Radner, S.; Werner, B. Lymphatic transport of cholesterol in the human being. Effect of dietary cholesterol. *Scand. J. Clin. Lab. Invest.* **1970**, *26*, 227-235.

80. Beaumier-Gallon, G.; Dubois, C.; Senft, M.; Vergnes, M.-F.; Pauli, A.-M.; Portugal, H.; Lairon, D. Dietary cholesterol is secreted in intestinally derived chylomicrons during several subsequent postprandial phases in healthy humans. *Am. J. Clin. Nutr.* **2001**, *73*, 870-877.

81. Ikeda, I.; Sugano, M. Some aspects of mechanism of inhibition of cholesterol absorption by β-sitosterol. *Biochim. Biophys. Acta.* **1983**, *732*, 651-658.

82. Slota, T.; Kozlov, N.A.; Ammon, H.V. Comparison of cholesterol and β-sitosterol: Effects on jejunal fluid secretion induced by oleate, and absorption from mixed micellar solutions. *Gut* **1983**, *24*, 653-658.

83. Brown, A.W.; Hang, J.; Dussault, P.H.; Carr, T.P. Phytosterol ester constituents affect micellar cholesterol solubility in model bile. *Lipids* **2010**, *45*, 855-862. doi:10.1007/s11745-010-3456-6

84. Ikeda, I. Factors affecting intestinal absorption of cholesterol and plant sterols and stanols. *J. Oleo. Sci.* **2015**, *64*, 9-18. 10.5650/jos.ess14221

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 18 September 2018 doi:10.20944/preprints201809.0345.v1

632 85. Li, Q.; Yin, R.-X.; Wei, X.-L.; Yan, T.-T.; Aung, L.H.H.; Wu, D.-F.; Wu, J.-Z.; Lin, W.-X.; Liu, C.-
633 W.; Pan, S.-L. ATP-binding cassette transporter G5 and G8 polymorphisms and several
634 environmental factors with serum lipid levels. *PLoS ONE* **2012**, *7*, e37972.
635 doi:10.1371/journal.pone.0037972

636 86. Bosner, M.S.; Ostlund, R.E.; Osofisan, O.; Grosklos, J.; Fritschle, C.; Lange, L.G. Assessment of
637 percent cholesterol absorption in humans with stable isotopes. *J. Lipid Res.* **1993**, *34*, 1047-1053.

638 87. Plat, J.; van Onselen, E.N.; van Heugten, M.M.; Mensink, R.P. Effects on serum lipids,
639 lipoproteins and fat soluble antioxidant concentrations of consumption frequency of margarines
640 and shortenings enriched with plant stanol esters. *Eur. J. Clin. Nutr.* **2000**, *54*, 671-677.

641 88. Matvienko, O.A.; Lewis, D.S.; Swanson, M.; Arndt, B.; Rainwater, D.L.; Stewart, J.; Alekel, D.L.
642 A single daily dose of soybean phytosterols in ground beef decreases serum total cholesterol and
643 LDL cholesterol in young, mildly hypercholesterolemic men. *Am. J. Clin. Nutr.* **2002**, *76*, 57-64.
644 doi:10.1093/ajcn/76.1.57

645 89. Rudkowska, I.; AbuMweis, S.S.; Nicolle, C.; Jones, P.J. Cholesterol-lowering efficacy of plant
646 sterols in low-fat yogurt consumed as a snack or with a meal. *J. Am. Coll. Nutr.* **2008**, *27*, 588-595.

647 90. Igel, M.; Giesa, U.; Lütjohann, D.; von Bergmann, K. Comparison of the intestinal uptake of
648 cholesterol, plant sterols, and stanols in mice. *J. Lipid Res.* **2003**, *44*, 533-538. doi:10.1194/jlr.M200393-
649 JLR200

650 91. Nakano, T.; Inoue, I.; Takenaka, Y.; Ikegami, Y.; Kotani, N.; Shimada, A.; Noda, M.; Murakoshi,
651 T. Luminal plant sterol promotes brush border membrane-to-lumen cholesterol efflux in the small
652 intestine. *J. Clin. Biochem. Nutr.* (in press) doi:10.3164/jcbn.17-116

653 92. Johnson, B.J.H.; Lee, J.-Y.; Pickert, A.; Urbatsch, I.L. Bile acids stimulate ATP hydrolysis in the
654 purified cholesterol transporter ABCG5/G8. *Biochemistry* **2010**, *49*, 3403-3411. doi:10.1021/bi902064g

655 93. Carlos, V.; Edwin, V.; E., V.K.; Raoul, F.; Jurgen, S.; K., G.A. The sterol transporting
656 heterodimer ABCG5/ABCG8 requires bile salts to mediate cholesterol efflux. *FEBS Lett.* **2007**, *581*,
657 4616-4620. doi:10.1016/j.febslet.2007.08.052

658 94. Plösch, T.; Kruit, J.K.; Bloks, V.W.; Huijkman, N.C.A.; Havinga, R.; Duchateau, G.S.M.J.E.; Lin,
659 Y.; Kuipers, F. Reduction of cholesterol absorption by dietary plant sterols and stanols in mice is
660 independent of the ABCG5/8 transporter. *J. Nutr.* **2006**, *136*, 2135-2140.

661 95. Chang, T.-Y.; Chang, C. Ezetimibe blocks internalization of the NPC1L1/cholesterol complex.
662 *Cell Metab.* **2008**, *7*, 469-471. doi:10.1016/j.cmet.2008.05.001

663 96. Engelking, L.J.; McFarlane, M.R.; Li, C.K.; Liang, G. Blockade of cholesterol absorption by
664 ezetimibe reveals a complex homeostatic network in enterocytes. *J. Lipid Res.* **2012**, *53*, 1359-1368.
665 doi:10.1194/jlr.M027599

666 97. Telford, D.E.; Sutherland, B.G.; Edwards, J.Y.; Andrews, J.D.; Barrett, P.H.R.; Huff, M.W. The
667 molecular mechanisms underlying the reduction of LDL apoB-100 by ezetimibe plus simvastatin. *J.*
668 *Lipid Res.* **2007**, *48*, 699-708. doi:10.1194/jlr.M600439-JLR200

669 98. Zmyslowski, A.; Szterk, A. Current knowledge on the mechanism of atherosclerosis and pro-
670 atherosclerotic properties of oxysterols. *Lipids Health Dis*. **2017**, *16*, 188. doi:10.1186/s12944-017-0579-
671 2

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 18 September 2018 doi:10.20944/preprints201809.0345.v1

672 99. Hartley, A.; Haskard, D.; Khamis, R. Oxidized LDL and anti-oxidized LDL antibodies in
673 atherosclerosis – novel insights and future directions in diagnosis and therapy. *Trends Cardiovasc.*
674 *Med.* **2018**. (in press) doi:10.1016/j.tcm.2018.05.010

675 100. Que, X.; Hung, M.Y.; Yeang, C.; Gonen, A.; Prohaska, T.A.; Sun, X.; Diehl, C.; Maatta, A.;
676 Gaddis, D.E.; Bowden, K., et al. Oxidized phospholipids are proinflammatory and proatherogenic
677 in hypercholesterolaemic mice. *Nature* **2018**. *558*, 301-306. doi:10.1038/s41586-018-0198-8

678 101. Jakulj, L.; Trip, M.D.; Sudhop, T.; von Bergmann, K.; Kastelein, J.J.P.; Vissers, M.N. Inhibition
679 of cholesterol absorption by the combination of dietary plant sterols and ezetimibe: Effects on
680 plasma lipid levels. *J. Lipid Res.* **2005**, *46*, 2692-2698. doi:10.1194/jlr.M500260-JLR200

681 102. Schonewille, M.; Freark de Boer, J.; Mele, L.; Wolters, H.; Bloks, V.W.; Wolters, J.C.;
682 Kuivenhoven, J.A.; Tietge, U.J.F.; Brufau, G.; Groen, A.K. Statins increase hepatic cholesterol
683 synthesis and stimulate fecal cholesterol elimination in mice. *J. Lipid Res.* **2016**, *57*, 1455-1464.
684 doi:10.1194/jlr.M067488