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Full title: **Contemporary aspects of the biology and therapeutic regulation of the microsomal triglyceride transfer protein**

Short title: **Hooper, MTP inhibition**

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Abstract

The microsomal triglyceride transfer protein (MTP), the product of the *MTTP* gene, is essential for the assembly and secretion of apolipoprotein (apo) B-containing lipoproteins, but when defective causes abetalipoproteinemia. Abetalipoproteinemia is a very rare autosomal recessive disorder characterized by the inability to produce chylomicrons or very low density lipoproteins, with the absence of apoB-containing lipoproteins in the circulation. Knowledge of the molecular basis for abetalipoproteinemia has led to the development of therapies for dyslipidemia that inhibit MTP. Partial MTP inhibition using small molecule inhibitors, such as lomitapide, can effectively lower plasma LDL-cholesterol and apoB levels, but is associated with gastrointestinal side-effects and hepatic steatosis, whose long-term sequelae remain unclear; lomitapide has accordingly only been approved as a treatment for homozygous familial hypercholesterolemia. Intestine-specific inhibitors of MTP decrease chylomicron biogenesis and improve insulin sensitivity in experimental animals and, while overcoming hepatic steatosis, may have significant gastrointestinal side-effects that could limit their use in humans. We review contemporary aspects of the biology and therapeutic regulation of MTP and their significance for lipid metabolism and cardiovascular disease.

Non-standard Abbreviations and Acronyms

ASCVD	atherosclerotic cardiovascular disease
ALT	alanine aminotransferase
apo	apolipoprotein
ASO	antisense oligonucleotide
CD1d	cluster of differentiation 1d
CETP	cholesteryl ester transfer protein
DR1	direct repeat 1
ER	endoplasmic reticulum
FH	familial hypercholesterolemia
GLP-1	glucagon-like peptide-1
HCV	hepatitis C virus
HDL	high density lipoprotein
hoFH	homozygous familial hypercholesterolemia
IDL	intermediate density lipoprotein
INR	international normalized ratio
IRE	insulin response element
LCAT	lecithin-cholesterol acyltransferase
LDL	low density lipoprotein
LDLR	LDR-receptor
Lp(a)	lipoprotein (a)
mAbs	monoclonal antibodies
miR	microRNA
MTP	microsomal triglyceride transfer protein
NKT	natural killer T
PCSK9	proprotein convertase subtilisin/kexin type 9
PDI	protein disulfide isomerase
PPAR	peroxisome proliferator-activated receptor
RXR	retinoid X receptor
SCD-1	stearoyl-CoA desaturase
SRE	steroid response element
SREBP	sterol regulatory element binding protein
VLDL	very low density lipoprotein

Introduction

Apolipoprotein (apo) B plays a central role in lipoprotein metabolism. The human apoB gene (*APOB*) is located on chromosome 2 and produces, via a unique mRNA editing process, two physiological isoforms in circulating lipoproteins, namely apoB-48 and apoB-100.[1] ApoB-48, a truncated form of apoB-100, is synthesized in the intestine and is essential for the formation and secretion of chylomicrons. ApoB-100 is synthesized in the liver and is a fundamental structural component of very low density lipoprotein (VLDL), and its metabolic products, intermediate density lipoprotein (IDL) and low density lipoprotein (LDL), and is also a ligand for the LDL-receptor. Increased plasma concentration of apoB, a direct measure of the number of circulating atherogenic lipoproteins, is an important risk factor for atherosclerotic cardiovascular disease (ASCVD), whereas a low level is cardioprotective.

A pentapartite model for human apoB-100 has been proposed, which depicts a five superdomain structure composed of alternating amphipathic α helices and amphipathic β strands, namely $\beta\alpha1$-$\beta1$-$\alpha2$-$\beta2$-$\alpha3$.[2] Several lines of evidence indicate that the amino-terminal $\beta\alpha1$ domain of apoB is important for lipoprotein assembly and stability. ApoB segments lacking the $\beta\alpha1$ domain are either unable to be secreted or are secreted poorly by experimental cell lines.[3, 4] The sequence elements involved in the physical interaction between apoB and its molecular chaperone the microsomal triglyceride transfer protein (MTP), a heterodimeric lipid transfer protein, have been located to the $\beta\alpha1$ superdomain.[5]

MTP, a product of the abetalipoproteinemia (OMIM 200100) gene (*MTTP*), is essential for the assembly and secretion of apoB-containing lipoproteins, but when defective causes abetalipoproteinemia.[6-8] Abetalipoproteinemia is a very rare autosomal recessive disorder which is characterized by the absence of MTP expression or activity; this results in the inability to produce chylomicrons or VLDL, with the absence of apoB-containing lipoproteins in the circulation and an associated decreased likelihood of ASCVD. Knowledge of the molecular bases for abetalipoproteinemia and homozygous familial hypobetalipoproteinemia, a phenotypically similar condition to abetalipoproteinemia due to *APOB* gene mutations, has led to the development of new anti-lipemic therapies that inhibit the activity of MTP and the production of apoB-100, respectively. Partial inhibition of MTP has clear potential as a pharmacological strategy to lower LDL-cholesterol and apoB levels in high risk subjects with familial hypercholesterolemia (FH). This review focuses on contemporary aspects of the biology and therapeutic regulation of MTP and their implications for lipid and lipoprotein metabolism and the prevention of ASCVD.

MTP structure and function

MTP was first described in 1984 by Wetterau and Zilversmit, who isolated a protein from bovine liver microsomes that accelerated the transfer of triglyceride, cholesteryl ester, and phospholipids, between biological membranes.[9] MTP was soon found to consist of two subunits. The first is the unique MTP subunit which in humans is 894 amino acids (97 kDa) and expressed primarily in hepatocytes and enterocytes. The second subunit is the ubiquitously expressed multifunctional protein disulfide isomerase (PDI; 55 kDa).[10] The isomerase activity of PDI is not necessary for MTP's function.[11] While it has no lipid transfer activity *per se*, PDI appears to be important for maintaining the activity and solubility of the complex. Without PDI the MTP subunit forms insoluble aggregates.[12]

MTP is a member of the large lipid transfer protein family, which includes apoB, insect apolipophorin II/I and vitellogenin, the egg yolk precursor protein.[13] Members of this family have similar amino-terminal regions consisting of a β-barrel and an α-helical domain, with divergent carboxyl-terminal lipid-binding domains reflecting the different quantities and types of lipids bound.[14] MTP may represent the ancestral lipid transfer protein, evolved to transfer lipids, while the other members of this protein family may have diverged to carry lipids rather than shuttle them intracellularly.[15, 16] MTP consists of three structural regions, an amino-terminal β-barrel domain (amino acids 22-297), central α-helical region (298-603), and carboxyl-terminal domain (604-894) (Figure 1).[5, 15] Evidence suggests that while both

the amino-terminal and central regions interact with apoB, PDI binding occurs in the central region and lipid binding and transfer occur in the carboxyl-terminal domain.

High levels of expression of MTP are found within hepatocytes and within epithelial cells of the small intestine – the two major sites of the synthesis and secretion of triglyceride-rich lipoproteins. MTP is expressed to a lesser extent in other tissues including renal tubular epithelial cells,[17] cardiac myocytes,[18] placenta,[19] retina[20] and immune cells.[21] Cardiac expression of MTP facilitates the secretion of apoB-containing lipoproteins and likely serves to prevent lipid accumulation in the heart.[22]

MTP colocalizes with PDI and apoB in the endoplasmic reticulum (ER) and Golgi apparatus.[23, 24] MTP acts as a chaperone, stabilizing the nascent apoB polypeptide and facilitating neutral lipid transfer from the ER membrane to apoB by a shuttle mechanism. Binding of MTP to apoB and lipid transfer occur independently of each other.[25, 26] While studies of truncated apoB forms show that the first ~1000 amino acids of apoB can be secreted independent of MTP[27], the lipid transfer activity of MTP is essential for the secretion of apoB-48 and apoB-100; if MTP is absent then these underlipidated apoB peptides are targeted for proteasomal degradation (Figure 2).[28]

Regulation of MTP

The regulation of MTP has been the subject of a comprehensive review by Hussain et al.[29] The promoter region of *MTTP* contains *cis*-elements to which several transcription factors involved in the regulation of MTP expression bind. Fox and hepatic nuclear factors HNF-1 and HNF-4 are positive regulatory elements, while the sterol and insulin response element (SRE/IRE) may be negative; in cultured hepatoma cells insulin suppresses MTP via the SRE/IRE.[29] The *MTTP* promoter also contains a direct repeat-1 (DR1) element – in cells that do not express MTP, nuclear receptors NR2F1 or NR2F2 bind to this element; in cells that do express MTP, retinoid X receptor (RXR)-α binds. In the liver, MTP expression increases with a peroxisome proliferator-activated receptor (PPAR)-α agonist.[30] MTP expression is also regulated by microRNA-30c.[31] Inositol-requiring enzyme 1β, an ER stress protein, reduces intestinal MTP mRNA levels via increased posttranscriptional degradation.[32]

Diet affects *MTTP* expression; a high fat diet increases *MTTP* expression in the liver and intestine,[33] potentially through decreased binding of sterol regulating element binding proteins (SREBPs) to the MTTP promoter.[29] MTP is negatively regulated by bile acids via the nuclear receptor HNF-4.[34] Reduced leptin, through a mechanism not yet understood, decreases intestinal MTP expression.[35] MTP exhibits a diurnal rhythm, regulated by light and food and mediated by the circadian clock.[29] Rodent studies showed that *MTTP* gene transcription was high at night and low during the day; these variations might contribute to daily variation in plasma lipid and lipoprotein concentrations.[29] This could impact on hepatic cholesterol content, which in turn may relate to diurnal variation of hepatic cholesterol synthesis and proprotein convertase subtilisin kexin type 9 (PCSK9).[36] Animal studies have also demonstrated that ethanol can substantially lower hepatic and intestinal *MTTP* mRNA levels.[37]

MTP and hepatitis C

The hepatitis C virus (HCV) life cycle is closely linked to MTP. The formation and maturation of virions depends on MTP activity and apoB, mirroring the production of VLDL particles.[38] Circulating HCV is found in the apoB-containing lipoproteins. In chronic HCV, hepatic steatosis is common and associated with low total cholesterol levels and hypobetalipoproteinemia.[39] This could be due to the inhibition of MTP activity by the HCV core protein.[40] MTP mRNA levels in chronic HCV inversely correlate with the degree of hepatic steatosis.[41] The intracellular lipid accumulation caused by the virus-mediated inhibition of MTP is postulated to provide a means for HCV retention and persistence.[42]

MTTP gene variants and lipid and lipoprotein metabolism

Microsatellite analysis across the genome identified *MTTP* as one of two gene regions containing quantitative trait loci influencing cholesterol concentrations in small LDL

particles.[43] Three common polymorphisms of *MTTP* have been described that are in almost complete linkage disequilibrium: -493G>T (rs1800591), -164T>C (rs1800804) and Ile128Thr (rs3816873). The minor T allele at position -493 has been shown *in vitro* to be associated with a doubling in *MTTP* transcription compared to the wild-type G allele, as well as a shift in VLDL subfraction composition.[44] However, conflicting observations on the effect of the -493 promoter variant on plasma cholesterol concentrations have been reported in population studies. Ledmyr *et al.* report an association of the minor T allele with increased coronary heart disease risk despite a small reduction in total cholesterol,[45] while in young African American men from the CARDIA cohort the T/T genotype was associated with higher total and LDL-cholesterol.[46] Other population studies, including the Framingham Offspring Study, did not find a significant association between the -493 promoter variant and any lipid measurement.[47, 48] A lack of association with lipids and the metabolic syndrome has also been reported with respect to the -164T>C and Ile128Thr polymorphisms.[49, 50] Intriguingly, the impact of *MTTP* gene variants on cardiovascular events has not been hitherto reported nor tested in Mendelian randomization studies. *In vivo* studies suggest that while VLDL production is not different, minor allele homozygous carriers have reduced IDL and LDL production compared with subjects with the wild-type genotype.[51] *MTTP* variants may interact with other genes controlling lipid transport and their variants to bear on hepatic secretion of apoB,[52] as well as on postprandial lipemia and the responses to statin therapy in familial combined hyperlipidemia.[53] Plasma lipids are generally normal in obligate heterozygotes for abetalipoproteinemia, implying that the normal *MTTP* allele can compensate for the defective one.[54]

MTP and Cluster of Differentiation 1 (CD1)

The role of MTP extends beyond the assembly of the apoB-containing lipoproteins. CD1d-restricted T cells acquire glycolipid antigens during assembly within the ER, in part from the function of lipid transfer proteins called saposins.[55] These glycolipid antigens are presented to T cells expressing natural killer receptors and an invariant T cell receptor-α chain (NKT cells). In 2004 it was shown that MTP also associates with CD1d within the ER and, like its role in apoB assembly, facilitates the lipidation and maturation of CD1d.[21] This process utilizes MTP's phospholipid transfer activity rather than triglyceride transfer activity.[56] MTP was later shown to also regulate other CD1 molecules, namely CD1a, CD1b and CD1c, and despite its ER localization was able to regulate endogenous, as well as exogenous lipid antigen presentation.[57] In MTP deficiency, while CD1d biosynthesis, glycosylation, maturation and internalization from the cell surface were unaffected, the recycling from lysosome to plasma membrane was impaired.[58] This phenomenon is yet to be fully understood. However, it could relate to altered membrane lipid composition or structural defects in CD1d as a consequence of deficient lipid loading during biosynthesis that results in degradation, rather than recycling, of these molecules.[15, 58]

MTP is expressed in antigen-presenting cells including monocytes, B cells and bone marrow and monocyte-derived dendritic cells and its lipid transfer activity can be detected within lysates of these cells.[56] Interestingly, mice with a conditional deletion of the *Mttp* gene are unable to activate NKT cells and are resistant to invariant NKT cell-mediated hepatitis and colitis.[21] Recent studies of the immune systems in patients with abetalipoproteinemia demonstrated that CD1d was unable to load antigens and as a result NKT cell numbers were reduced and their activation impaired.[59] Owing to the small numbers of patients available it is difficult to ascertain the clinical consequences of these subtle immunological defects in abetalipoproteinemia.

Abetalipoproteinemia

Abetalipoproteinemia is a very rare disorder of lipoprotein metabolism affecting <1 per million people. The absence of MTP expression or activity and the inability to produce chylomicrons or VLDL is associated with a variety of clinical manifestations which affect multiple organ systems. The disorder serves as a classic model of the effects of complete inhibition of MTP in humans.

Biochemical and clinical findings

In 1992, the connection was made between abetalipoproteinemia and the absence of MTP activity in intestinal biopsies.[60] The following year, pathogenic mutations of *MTP* causing abetalipoproteinemia were described.[61, 62] The absence of MTP activity leads to triglyceride accumulation within enterocytes and hepatocytes and absent circulating apoB-containing lipoproteins; after a fat load, chylomicrons do not appear in plasma.

Overall, the clinical presentation of abetalipoproteinemia is heterogeneous. While atypical or mild cases of abetalipoproteinemia may present late and with minimal symptoms, patients usually present in childhood with failure to thrive, growth failure, and malabsorption of fat.[54] Plasma total cholesterol and triglyceride concentrations are low, while vitamin E, LDL-cholesterol and apoB are typically undetectable. While homozygous familial hypobetalipoproteinemia due to *APOB* mutations cannot be differentiated from abetalipoproteinemia based on clinical symptoms, they have different inheritance patterns. Obligate heterozygous parents carrying an *APOB* mutation will have levels of apoB and LDL-cholesterol less than half of normal, while parents of a patient with abetalipoproteinemia, a recessive disorder, usually have normal plasma lipid and lipoprotein concentrations.[63]

Hematological manifestations of abetalipoproteinemia include acanthocytosis, which could be a result of either vitamin E deficiency or an altered membrane lipid composition.[64] Other findings include low erythrocyte sedimentation rates, decreased red cell survival,[65] anemia, hyperbilirubinemia and hemolysis,[66] and increased international normalized ratio (INR) as a result of vitamin K deficiency.[67] Fat malabsorption is a central feature of abetalipoproteinemia. The severity of gastrointestinal symptoms, chiefly steatorrhea, relates to the dietary fat content, and usually decreases with improved adherence to a low fat diet.[64] Hepatic manifestations include hepatomegaly, steatosis and abnormal plasma aminotransferases.[54] Hepatic steatosis is present in about one third of abetalipoproteinemia cases, with 4 of 58 published probands having severe fibrosis.[68] Progression to cirrhosis can also occur.[69] Neuromuscular manifestations are secondary the effects of vitamin E deficiency on both the central and peripheral nervous system.[70] Ophthalmological manifestations are variable, the most prominent being atypical pigmentation of the retina and night and/or color vision may be lost.[71]

Molecular diagnosis

Sequencing of the 18 exons and intron-exon boundaries will detect mutations of *MTTP* associated with abetalipoproteinemia. The majority of mutations either lead to premature stop codons or affect splicing, but several missense mutations have also been described and studied *in vitro*. The first, Arg540His, was shown to result in defective interaction with PDI, with the mutant MTTP remaining as an insoluble aggregate.[72] Other mutations in the central α-helical region of MTP, Leu435His and Ser590Ile, exhibit negligible lipid transfer activity.[73, 74]

A 30 amino acid carboxyl-terminal truncation of MTP, Gly865*, was found to disrupt binding with PDI, suggesting that these 30 amino acids are required for the interaction of MTP with the PDI subunit.[75] Gly746Glu and Asn780Tyr, also at the carboxyl-terminus, are able to interact with PDI, but cannot transfer lipids.[74, 76] The discovery and characterization of further missense mutations will help to elucidate the structure-function relationships of MTP.

Cardiovascular consequences

In abetalipoproteinemia there is life-long reduction in LDL-cholesterol and apoB and, by implication, protection against ASCVD. *APOB* and *PCSK9* (loss-of-function) mutation carriers, who similarly have life-long low plasma LDL-cholesterol levels, clearly exhibit longevity. For example, PCSK9 nonsense mutations lower plasma LDL-cholesterol by 28% and the relative risk of coronary artery disease by 88% and missense mutations lower LDL-cholesterol by 14% and coronary artery disease risk by 47%.[77-79] In abetalipoproteinemia, where LDL-apoB is virtually absent, the risk of ASCVD is expected to be even lower. Owing

to the rarity of the severe form of the condition and premature death from other complications, there are no good data on the incidence of ASCVD; registries are recommended for longitudinal studies. Autopsy data of isolated cases testify to protection against atherosclerosis.[80] Beyond virtually absence of apoB-containing lipoproteins, reduced coagulation due to vitamin K depletion secondary to lipid malabsorption may further lower cardiovascular risk in abetalipoproteinemia.[54] On the other hand, risk may be offset by increased platelet aggregation due to dysfunctional high density lipoprotein (HDL) that is in turn related to vitamin E depletion and increased oxidizability of these particles.[81] Whether this also impacts on endothelial function remains unclear. While no studies of arterial structure and function have been reported in abetalipoproteinemia, in the related condition familial hypobetalipoproteinemia there is case-control evidence for a reduction in carotid artery wall stiffness, even in the presence of non-lipid cardiovascular risk factors.[82] Both apoB and MTP are expressed in the myocardium,[18] but autopsy studies have not provided clear evidence of an infiltrative cardiomyopathy; further studies of the myocardial consequences of abetalipoproteinemia are recommended.

Lipid and lipoprotein metabolism

By contrast to familial hypobetalipoproteinemia, plasma lipid and lipoprotein concentrations are normal in the majority of obligate heterozygotes for abetalipoproteinemia; these subjects also do not develop hepatic steatosis. It is therefore possible that the normal *MTTP* allele can compensate for the defective one in heterozygous abetalipoproteinemia or that one *MTTP* allele is sufficient to control plasma lipids.[54] However, a lesser proportion of cases have been described with elevated or low plasma lipid levels, pointing to an effect of other gene variants. Further investigation of the genetic variability in the plasma lipid phenotype in obligate heterozygotes for abetalipoproteinemia could elucidate the inter-individual variation in the therapeutic response to MTP inhibitors.

In the absence of chylomicrons and lipoproteins of the VLDL cascade, plasma cholesterol and triglyceride levels are markedly reduced in abetalipoproteinemia. After a fat-containing meal, plasma triglyceride concentrations generally do not increase. While total body turnover of cholesterol is normal,[83] increased urine mevalonic acid levels indicate increased cholesterol biosynthesis.[84]

Trace amounts of full-length apoB complexed with apo(a), as well as N-terminal proteolysis product fragments have been detected in abetalipoproteinemia.[85-87] Lipoproteins isolated from the VLDL and LDL density intervals have a cuboidal 'square packing' appearance in electron microscopy.[64] No tracer kinetic studies of lipoprotein metabolism have been reported in obligate heterozygotes for abetalipoproteinemia. In heterozygous familial hypobetalipoproteinemia, who classically have low plasma lipid levels, tracer studies have shown decreased hepatic secretion of VLDL apoB and subsequent production of LDL apoB, with accelerated catabolism of VLDL.[88, 89] The former mechanism is likely to be shared with the effect of both lomitapide (an MTP inhibitor) and mipomersen (an antisense oligonucleotide (ASO) directed against apoB-100), although detailed mechanistic studies have not been hitherto reported with either of this agents in humans. That plasma PCSK9 levels are normal in familial hypobetalipoproteinemia suggests that in this condition, and by implication in abetalipoproteinemia, there is no change in the catabolism of LDL particles.[90]

In homozygous familial hypobetalipoproteinemia, marked acceleration in the catabolism of VLDL particles results in a very low conversion rate to LDL, but this requires confirmation.[91] In classical abetalipoproteinemia plasma lipases are significantly decreased, consistent with enzyme down-regulation due to restriction in dietary fat and reduction in chylomicron formation.[92] Despite the defective hepatic assembly of apoB-100 in abetalipoproteinemia, small amounts of uncomplexed apo(a) may be detected in plasma,[85, 93] implying that apo(a) may be secreted by the liver into the circulation without prior intrahepatic coupling with apoB-100, with implications for furthering our understanding of the metabolic regulation of lipoprotein(a) (Lp(a)). The findings also suggest that MTP inhibitors may lower plasma Lp(a) by decreasing the intrahepatic assembly of Lp(a), but that the effect on plasma Lp(a) may be offset by independent secretion of apo(a) and its coupling with LDL

apoB outside the hepatocyte membrane. Further kinetic studies of Lp(a) metabolism in subjects with abetalipoproteinemia and familial hypobetalipoproteinemia are warranted.

HDL is the major lipoprotein in plasma in abetalipoproteinemia. The plasma concentration of HDL is on average decreased by 50%, with reduced HDL3, and HDL2 present at normal concentrations.[64, 94] HDL is enriched in apoE and is able to deliver cholesterol to tissues via the LDL-receptor pathway.[95] Low plasma lecithin-cholesterol acyltransferase (LCAT) activity in abetalipoproteinemia is considered secondary to reduction in acceptor lipoproteins in plasma and not of major significance.[96] The low circulating levels of apoA-I may result from either reduced synthesis[97] or a combination of reduced production and increased fractional catabolic rate.[98] Reduction in apoA-I secretion in abetalipoproteinemia could be due to decreased formation of chylomicrons, noting that the plasma concentrations of other enterocyte-derived apolipoproteins (apo A-IV, C-I, C-II) are similarly low; another possibility is reduction in cholesterol substrate availability in the liver that decreases the expression of apoA-I, although the rate of whole body cholesterol turnover is apparently normal in abetalipoproteinemia. The accelerated catabolism of apoA-I, chiefly in LpAI:AII particles, is most likely related to alteration in the HDL particle morphology referred to earlier, noting that the increase in HDL size in abetalipoproteinemia is likely to delay particle catabolism. The maintenance of HDL-apoA-I turnover is critical for delivering cholesterol to cells and is closely related to the turnover of apoE in abetalipoproteinemia, and underscores in general the tighter metabolic coupling between the apoA and apoE systems than between the apoA and apoB systems. Whether the reduction in HDL-cholesterol noted with MTP inhibitors involves similar mechanisms to those in abetalipoproteinemia has not been reported and warrants further investigation.

Treatment

Regular follow-up to monitor growth in children and gastrointestinal, hepatic and neurological complications is recommended in all patients with abetalipoproteinemia.[63] A low-fat diet, with total fat <30% of caloric intake and minimal consumption of long-chain fatty acids, eliminates steatorrhea. Medium chain triglycerides, which are not packed into chylomicrons, have been suggested as a form of delivering calories, but their use and safety has not been established.[63, 81] Early treatment with high oral doses of vitamin E and A can mitigate neuropathy and retinopathy.[7, 99-102] As vitamin E transport strongly relies on apoB-containing lipoproteins, high doses of vitamin E (100 to 300 mg/kg/day orally) are required in order to circumvent the chylomicron pathway, perhaps by incorporation into HDL.[54, 63, 64] Erythrocyte and platelet vitamin E levels provide the best assessment of tissue vitamin E status.[103] Supplementation with vitamins A, D and K are also recommended.

Murine models

Knockout of the *Mttp* gene in mice is lethal to the embryo, while heterozygous knockout is associated with reduced LDL-cholesterol, a 28% reduction in apoB-100 and cytosolic fat accumulation in the liver of adult animals.[104] Liver-specific inactivation of MTP ablates the synthesis and secretion of VLDL and the subsequent production of LDL-apoB-100.[105, 106] The mice develop moderate hepatic steatosis, with VLDL-sized lipid-staining particles observed within the ER and Golgi apparatus.[106] Pulse-chase experiments confirm inhibition of apoB secretion from hepatocytes.[105]

Mice with a conditional *Mttp* deletion in villus enterocytes manifested steatorrhea, growth arrest and reduced cholesterol absorption, consistent with the human abetalipoproteinemia phenotype.[107] Chylomicron secretion was reduced with an 80% decrease in apoB-48 secretion from primary enterocytes. Mice with the intestine-specific knockout of *Mttp* had increased hepatic lipogenesis and VLDL secretion presumably to compensate for the defective secretion of chylomicrons.

In a cardiac-specific *Mttp* knockout, triglyceride stores in the heart were elevated.[108] It is not known whether cardiac lipid stores are increased either in human patients with abetalipoproteinemia or by the use of synthetic MTP inhibitors.

A recent study in *Ldlr*^{-/-} mice fed a chow diet showed that an MTP inhibitor (BMS 212122) decreased the development of atherosclerotic plaques over a period of two weeks, with significantly decreased cholesterol content in monocyte-derived cells and reduction in inflammation, as well as increased collagen deposition in the arterial wall.[109]

MTP as a therapeutic target

Given that a genetic mutation, or experimental disruption, of MTP results in reduction or ablation of the secretion of apoB-containing lipoproteins, pharmacological inhibition of MTP may be a useful therapeutic option for the treatment of hyperlipidemia and mitigation in the associated risk of ASCVD (Figure 2). In 1998, Wetterau and colleagues described a small molecule inhibitor of MTP that could effectively inhibit secretion of apoB-containing lipoproteins in rodents and normalize hypercholesterolemia in the Watanabe-heritable hyperlipidemic rabbits,[110] an experimental model for LDL-receptor defective homozygous FH (hoFH). However, it was noted that hamsters treated with the compound showed reversible hepatic fat accumulation, as well as fat accumulation in enterocytes. These observations foreshadowed the side effects that have plagued human clinical trials of MTP inhibitors. Despite this, MTP inhibitors may still play a role in the treatment of severe forms of hyperlipidemia.

Several synthetic MTP inhibitors (BMS-201038, CP-346086, and BAY-13-9953) were shown to dose-dependently lower plasma LDL-cholesterol and apoB in experimental animals and humans, but development programs were discontinued owing to gastrointestinal side-effects and hepatic steatosis; we have reviewed these early trials elsewhere.[111] The remainder of this section focuses on lomitapide, a small molecule MTP inhibitor that has been approved by the US Food and Drug Administration (FDA) and European Medicines Agency (EMA) Committee for Medicinal Products for Human Use (CHMP) as adjunctive therapy to diet and statins for patients with hoFH aged ≥18 years. In this rare, but serious autosomal dominant condition, patients have deficient or defective hepatic LDL-receptors and plasma LDL-cholesterol concentrations typically ranging from 10 to 25 mmol/L and develop premature onset CHD in childhood, almost none achieve plasma LDL-cholesterol targets (<2.0 mmol/L) on conventional cholesterol-lowering therapy, and many require lipoprotein apheresis.[112]

Lomitapide (Juxtapid, Lojuxta)

FH and primary hypercholesterolemia

Lomitapide (BMS-201038 at that time) was initially evaluated open-label in six hoFH patients (5 receptor-negative, 1 receptor-defective) on a low fat diet and reported to reduce plasma LDL-cholesterol by 50.9% (mean LDL-cholesterol at baseline 15.9 mmol/L) and apoB of 55.6% at the highest dose tested (1.0 mg/kg body weight/day) after 4 weeks; there was a significant increase in plasma aminotransferase levels and hepatic fat content, but these returned to normal in all patients 14 weeks after cessation of therapy.[113]

In a subsequent randomized, double-blind trial, the effect of lomitapide (AEGR-733 at that time; dose 5 mg, 7.5 mg and 10 mg/day) alone and in combination with ezetimibe (10 mg/day) was studied over 12 weeks in 85 patients with primary hypercholesterolemia (mean baseline LDL-cholesterol 4.4 mmol/L) on a low fat diet.[114] There was a dose-dependent reduction in LDL-cholesterol and apoB with lomitapide, which at the highest dose averaged 30% and 24% from baseline, respectively, increasing significantly to 46% and 37% with the addition of ezetimibe; plasma triglyceride, HDL-cholesterol and Lp(a) levels fell by 10%, 6% and 17% with lomitapide, with no statistically significant changes in combination with ezetimibe. Twenty percent of patients either stopped or were taken off lomitapide, mainly due to elevation in hepatic aminotransferase levels, which returned to normal two weeks after cessation of therapy; gastrointestinal symptoms were frequent but mild.

Most recently, lomitapide was studied in an open-label phase 3 trial, using a dose-ranging regimen (maximum 60 mg/day over 26 weeks), in 29 patients with hoFH (defined genetically or phenotypically; mean LDL-cholesterol 8.7 mmol/L) on best standard of care, including lipoprotein apheresis.[115] Plasma LDL-cholesterol, apoB and triglyceride levels fell

by 50%, 49% and 45% from baseline at week 26 on a median dose of lomitapide of 40 mg/day. Twenty three subjects completed a 78 week safety phase, with LDL-cholesterol, apoB and triglycerides remaining 38%, 43% and 31% below baseline; 55% and 33% of subjects attained LDL-cholesterol treatment goals less than 2.6 mmol/L and less than 1.8 mmol/L, respectively.[116, 117] Lp(a) levels fell significantly by 19% at week 26, but returned to baseline levels by week 78. Plasma HDL-cholesterol and apoA-I levels also dropped significantly by 12% and 14%, respectively, after 26 weeks, but approached baseline levels by week 78. Of 18 subjects treated with lipoprotein apheresis, three stopped this treatment after reaching LDL-cholesterol of <4.0 mmol/L, while in three others the interval between procedures was increased. Plasma aminotransferases increased at least five-fold in 4 patients, resolving after dose reduction.[115] Gastrointestinal symptoms (diarrhea, nausea, dyspepsia, abdominal discomfort) were common with lomitapide, accounting for three withdrawals from the study. No steatorrhea was reported, plasma levels of fat soluble vitamins and essential fatty acids remaining within the reference interval. Hepatic fat content, assessed by proton spectroscopy, increased from 1.0% at baseline to 8.6% (0-33.6%) at week 26, remaining stable to 78 weeks (8.3%, 0-19%). The efficacy and safety finding of the above investigation have been confirmed in 19 patients who continue to participate in a long-term extension study, published only in abstract form.[118] The long-term effect of hepatic steatosis on insulin resistance and cellular injury remains unclear, but other data suggest that it may be more closely related to hepatic accumulation of toxic saturated fatty acids rather than triglycerides per se,[119] which requires further investigation in the context of lomitapide.

Hypertriglyceridemia
Sacks *et al*. recently described the long-term use of lomitapide in a woman with lipoprotein lipase deficiency and severe hypertriglyceridemia.[120] She had suffered multiple episodes of pancreatitis from the age of 15 years, despite maximal dietary fat restriction and standard lipid-lowering pharmacotherapies. Lomitapide (40 mg/day) lowered her plasma triglyceride levels by 83% from a baseline of 35 mmol/L, presumably by inhibition the enterocytic and hepatic secretion of chylomicrons and VLDLs, respectively; she was free of chronic abdominal pain and pancreatitis during 13 years of treatment. However, fatty liver progressed to steatohepatitis and eventually to fibrosis, underscoring the long-term sequelae of lomitapide, at least in the setting of marked hypertriglyceridemia. No studies have been reported on the effect of lomitapide in patients with familial combined hyperlipidemia or familial hypertriglyceridemia; these disorders are *per se* associated with insulin resistance and hepatic steatosis, however, and the use of moderate-to-high doses of MTP inhibition may be contraindicated.

Mechanism of action on lipid and lipoprotein metabolism
The mechanism of action of MTP inhibition on human lipid and lipoprotein metabolism has not been fully investigated. In a tracer study of six patients with hoFH (5 with LDL-receptor deficiency) lomitapide decreased LDL apoB-100 production by 70%, accounting for the fall in LDL-cholesterol.[113] The inverse correlation between reduction of plasma LDL-cholesterol and accumulation of hepatic fat supports a dose-dependent effect. On the basis of other data, lomitapide may decrease the hepatic secretion of apoB-100 and its transfer down the VLDL-IDL-LDL cascade.[121] An effect on direct hepatic secretion of LDL apoB-100 cannot be ruled out, although such a defect has not been demonstrated in familial hypobetalipoproteinemia due to *APOB* mutations.

The reduction in plasma triglycerides with lomitapide is consistent with inhibition of hepatic secretion of VLDL apoB-100, but the contributory role of decreased enterocytic secretion of apoB-48 remains untested under postabsorptive and postprandial conditions. Animal data suggest that the impact of hepatic steatosis on insulin resistance is related to fatty acid lipotoxicity rather than to accumulation of triglycerides[119, 122] and may depend on the fatty acid partitioning enzyme, stearoyl-CoA desaturase (SCD)-1;[119] the effect of MTP

inhibition on hepatic fatty acid metabolism, insulin resistance and SCD-I activity merits further research. One human study has suggested that lomitapide decreases plasma triglycerides less than LDL-cholesterol, but employed lower drug doses and did not investigate postprandial triglycerides that are more responsive than fasting levels to MTP inhibition.[123] However, whether low dose lomitapide has an inhibitory effect on the direct hepatic secretion of LDL apoB remains unclear. The significant fall in plasma HDL-cholesterol with lomitapide was sustained in the recently reported long-term study, and, as in abetalipoproteinemia, could be due to decreased secretion of apoA-I by the enterocyte and liver and/or to increased catabolism of apoA-I; the therapeutic low fat diet aimed at decreasing chylomicron biogenesis can also decrease the expression and secretion of apoA-I.[124] Whether MTP inhibition affects the turnover of apoE and its coupling with apoA-I remains to be investigated, as does the impact on apo A-IV, C-I, C-II and C-III kinetics.

Reduction in plasma Lp(a) concentration could be due to decreased hepatic secretion of apoB-100 and its coupling with apo(a) within the hepatocyte with on its extracellular membrane.[125, 126] The Lp(a) lowering effect appears to be lost over time, the reason for which remains unclear, but may be due to a compensatory increase in hepatic secretion of apo(a), a phenomenon that may not be seen with mipomersen[127] and requires further research.

The postulated mechanism of action of lomitapide on apoB-100 transport differs from statins and ezetimibe, both of which increase the fractional catabolism of apoB-100 containing lipoproteins.[128] This provides a rationale for the additive effects noted above. MTP inhibition is also more likely to lower plasma LDL-cholesterol in hoFH with LDL-receptor deficiency than PCSK9 inhibitors, that *a priori* accelerate the catabolism of LDL particles.[129] This offers a synergistic mechanism of action for combination therapy in patients with defective LDL-receptors and requires verification.

LDL apheresis acutely lowers LDL apoB-100 and delivery of neutral lipids to the liver, but does not affect hepatic secretion rate of apoB-100 nor the fractional catabolism of LDL apoB-100.[130] Lomitapide is therefore likely to have a comparable effect on LDL-cholesterol in hoFH patients on or off lipoprotein apheresis.[115] Future studies should explore how genetic variants and hormonal factors bear on the effect of lomitapide on the wider spectrum of disordered lipoprotein metabolism in FH.

Other strategies for inhibiting MTP
Entero-specific

Intestinal-specific inhibition of MTP aims to decrease the secretion of apoB-48 and therefore reduce triglyceride-rich lipoprotein secretion from enterocytes. Theoretically, this approach should not have a direct effect on plasma LDL-cholesterol, but may indirectly reduce cholesterol supply to the liver in chylomicrons with possible upregulation of the LDL-receptor. These mechanisms, as well as the impact on HDL particle turnover, will require investigation. Other potential effects of intestinal MTP inhibition include improved insulin sensitivity and the suppression of food intake.[131, 132] Entero-specific MTP inhibition therapies are being investigated not only for the treatment of hypercholesterolemia but also severe hypertriglyceridemia and type 2 diabetes; they may initially have a clinical role for treating primary chylomicronemia and preventing recurrent acute pancreatitis, and eventually also for regulating the postprandial accumulation of atherogenic chylomicron remnant particles.[133]

JTT-130

JTT-130 (Japan Tobacco) is an orally administered compound that inhibits MTP's triglyceride transfer activity in enterocytes, thus avoiding the elevated liver aminotransferases and steatosis seen with hepatic MTP inhibition.[134] In hyperlipidemic hamsters, two weeks of treatment with JTT-130 decreased plasma triglyceride and cholesterol and reduced triglyceride content of the liver without causing hepatotoxicity. Studies in Sprague-Dawley rats showed that treatment with JTT-130 reduced food intake and gastric emptying and increased plasma concentrations of gut peptide YY and glucagon-like peptide-1 (GLP-1).[131] Triglyceride, free fatty acids and cholesterol were elevated in the

luminal contents of the intestine, which may trigger the suppression of food intake in response to the hydrolysis of triglycerides. Further studies in rats showed that when fed a high fat (35%) diet, JTT-130 treatment was associated with reduced body weight and plasma glucose and insulin during intraperitoneal glucose tolerance tests compared to controls.[135]

In Zucker diabetic fatty rats, JTT-130 treatment suppressed lipid absorption and food intake, ameliorating impaired glucose and lipid metabolism.[132] Recently, JTT-130 was used in combination with pioglitazone to improve glycemic control, enhance insulin sensitivity and reduce plasma triglycerides in this rat model, suggesting that this combination therapy could be useful in the treatment of type 2 diabetes.[136] A phase 2 clinical trial of JTT-130 for the treatment of obese type 2 diabetic subjects has been completed (NCT00929539), but no results have been published.

SLx-4090

Like JTT-130, SLx-4090 (Surface Logix) is an intestine-specific MTP inhibitor. Mice treated with SLx-4090 showed a reduction in serum LDL-cholesterol and triglyceride and and increase in HDL-cholesterol concentration, with evidence of weight loss despite a high fat diet.[137] The fall in LDL-cholesterol may be a consequence on reduced hepatic delivery of cholesterol substrate in chylomicrons and requires further mechanistic study. The increase in HDL-cholesterol probably involves reduction in the catabolism of apoA-I due to remodeling of HDL particles as a consequence of fall in plasma triglyceride levels. These data, as well as observations in the Caco-2 cell line, suggest that specific inhibition of chylomicron formation with SLx-4090 has no influence on the enterocytic secretion of apoA-I. In a phase 2a trial in 24 patients with dyslipidemia, SLx-4090 reduced postprandial triglycerides and LDL-cholesterol and, importantly, was well tolerated, with no effect on liver function tests.[138] SLx-4090 is currently in phase 2 trials for the treatment of familial chylomicronemia (NCT01675154).

Dirlotapide

In canines, this MTP inhibitor is specific to intestinal MTP, and has been approved by the FDA for the treatment of obesity in dogs. Over 3 months of treatment with oral doses ranging from 0.15 to 0.5 mg/kg/day, treated dogs lost 19% of body weight, compared to a 10% gain in control animals.[139] This effect is attributed to an anorectic effect as well as reduced fat absorption.[140] A modified version of dirlotapide with improved gut selectivity, PF-02575799, has advanced into human phase I trials for the treatment of obesity.[140]

RNA-based therapies

RNAi

MicroRNAs (miR) are short non-coding RNAs that can negatively regulate gene expression by interacting with mRNA to either prevent protein translation or cause the mRNA to be degraded.[141] Recently, the Hussain laboratory showed that miR-30c interacts with MTP mRNA, leading to a reduction in MTP protein activity and apoB secretion.[31] Transduction of mice with a miR-30c lentivirus led to mainly hepatic expression of miR-30c, with levels about four-fold higher than normal. Overexpression of miR-30c reduced plasma non-HDL cholesterol, while an anti-miR-30c increased non-HDL cholesterol; no effects were seen on fasting plasma triglycerides. Importantly, *Apoe* knockout mice expressing miR-30c had smaller atherosclerotic plaques compared to controls.

A possible advantage to this approach is that miR-30C, in addition to reducing apoB secretion, reduces lipid synthesis independently of MTP by targeting lysophosphatidylglycerol acyltransferase 1 (LPGAT1) mRNA. Hepatic overexpression of miR-30c therefore does not cause fatty liver. However, the administration and delivery vehicle for miR-30c therapy may prove challenging, as to be effective miR-30c expression would probably need to be increased in the small intestine as well as liver.[142] In addition, miR-30c has a potential tumor suppressor role; downregulation of its expression has been associated with poorer survival in ovarian and breast cancer, but better survival in malignant

mesothelioma.[143-145] Potential long-term consequences of miR overexpression need to be carefully considered.

Antisense oligonucleotides (hepato-specific MTP inhibition)

ASOs can be used for the targeted inhibition of mRNA, and mipomersen, an ASO specific for apoB-100, has been approved by the FDA for the treatment of hoFH.[146] Experiments performed in mice showed that administration of an MTP ASO (ISIS 144477) could reduce hepatic triglyceride secretion and serum lipid concentrations.[147] However, ASOs preferentially distribute to the liver, and hepatic MTP mRNA inhibition also resulted in greater accumulation of hepatic triglyceride and elevation in hepatic aminotransferases compared with apoB ASO therapy. This difference appears to be due to a compensatory decrease in hepatic *de novo* lipogenesis which occurs with apoB ASO.

Conclusion: future therapeutic perspective

MTP is a key protein necessary for the assembly and secretion of apoB-containing lipoproteins by the liver and small intestine. Absence of MTP activity results in abetalipoproteinemia, a rare, but potentially serious metabolic disorder with absent or very low LDL-cholesterol often presenting with hepatic steatosis, failure to thrive, and very low concentrations of the fat-soluble vitamins; the disorder is considered to protect against the development of ASCVD. The molecular defects in abetalipoproteinemia and familial hypobetalipoproteinemia have informed the development of MTP inhibitors and apoB-100 ASOs, respectively, for the treatment of severe hyperlipidemia. Partial MTP inhibition using small molecule inhibitors, such as lomitapide, can effectively lower plasma cholesterol, but is associated with gastrointestinal side effects and hepatic steatosis; steatosis is mild-to-moderate and stabilizes over time, but its long-term sequelae, such as hepatic insulin resistance, fibrosis, cirrhosis and cancer, remain to be determined.

In hoFH patients, who from a young age have a very high risk of cardiovascular events and death and in whom diet, statins and other conventional lipid-regulating drugs are either not effective or partially effective, lomitapide could be a useful additional therapy for achieving targets plasma levels of LDL-cholesterol. The most efficacious and safe approach to target hepatic steatosis due to lomitapide is lifestyle modifications, including weight loss and exercise. FH is associated with extended metabolic defects in lipoprotein metabolism, such as hepatic oversecretion of VLDL and increased production of LDL, which can be specifically targeted by MTP inhibitors.[113, 148] This is a unique mechanism of this agent that has hitherto not been confirmed with either mipomersen or PCSK9 monoclonal antibodies (mAbs). Further mechanistic studies of the integrated effect of lomitapide and entero-specific MTP inhibitors on lipoprotein metabolism in humans are warranted.

Lomitapide has an orphan indication for hoFH. The precise definition of this disorder remains under debate, however, noting recent data testifying to the genetic and clinical heterogeneity of severe forms of FH.[112, 149] For use in US via the REMS program, a clinical definition is required, without the need for molecular testing. Until the definition of hoFH can be refined and extended, lomitapide will retain orphan drug indication, but its high cost will remain a drawback for payers. It is possible that its indication could be extended to patients with refractory heterozygous FH, as well as in combination with ezetimibe for high risk hypercholesterolemic patients who cannot tolerate statins.[114] While cardiovascular outcome trials with MTP inhibitors are most unlikely to ever be done, efficacy could be assessed using surrogate cardiovascular endpoints and prospective cardiovascular outcome data from FH registries.[150]

The efficacy, utility and cost-effectiveness of lomitapide will need to be evaluated if indications are widened to cover these other conditions, but owing to its gastrointestinal and hepatic adverse event profile may be superseded by PCSK9 mAbs.[151] The balance between side-effects and benefits of lomitapide is likely to be less acceptable to lower risk patients than to those with hoFH. Lomitapide is likely to be more acceptable than mipomersen as it is administered orally and not by injection. However, by contrast to mipomersen and PCSK9 mAbs, it is metabolized by CYP3A4 and is susceptible to drug interactions, including statins

and warfarin. On the other hand, lomitapide may lower the need and cost of lipoprotein apheresis in hoFH. The lack of a sustained reduction in plasma Lp(a) and the fall in HDL-cholesterol with lomitapide would also favour use of mipomersen or PCSK9 mAbs, but whether these small metabolic differences are clinically meaningful remains unclear. Head-to-head comparisons of these new agents are unlikely to be done, but testing the efficacy of combination therapy could be possible in severer forms of FH. Cholesteryl ester transfer protein (CETP) inhibition may also have a future role in the management of FH, but its efficacy remains to be demonstrated by ongoing clinical trials (NCT01841684, NCT01524289). The place of lomitapide and other new agents in the management of hoFH was recently reviewed by two expert groups.[117, 152]

Further studies are needed to determine the clinical utility RNA-based and other therapies targeting MTP that could be useful for the treatment of dyslipidemia, diabetes and/or obesity. These alternative strategies may be able to avoid the development of fatty liver by either concomitantly reducing hepatic lipogenesis or by specifically inhibiting MTP in the intestine, although gastrointestinal side-effects may continue to limit their clinical use.

Disclosures
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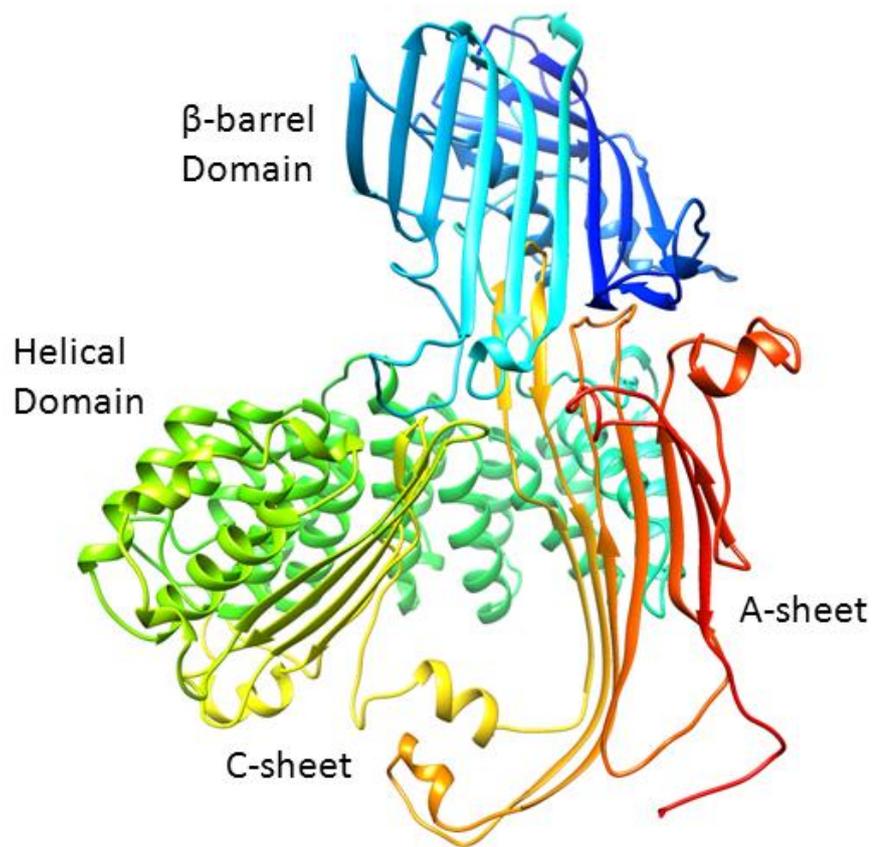
Figure 1. Model of the 97 kDa subunit of the MTP complex (M subunit) based on lamprey lipovitellin. Ribbon diagram colored to show the different domains. N-terminus, blue; β-barrel domain, blue-cyan; helical domain, green; C-sheet, green-yellow and A-sheet, yellow-red. Image courtesy of Professor C. James McKnight (Boston University).
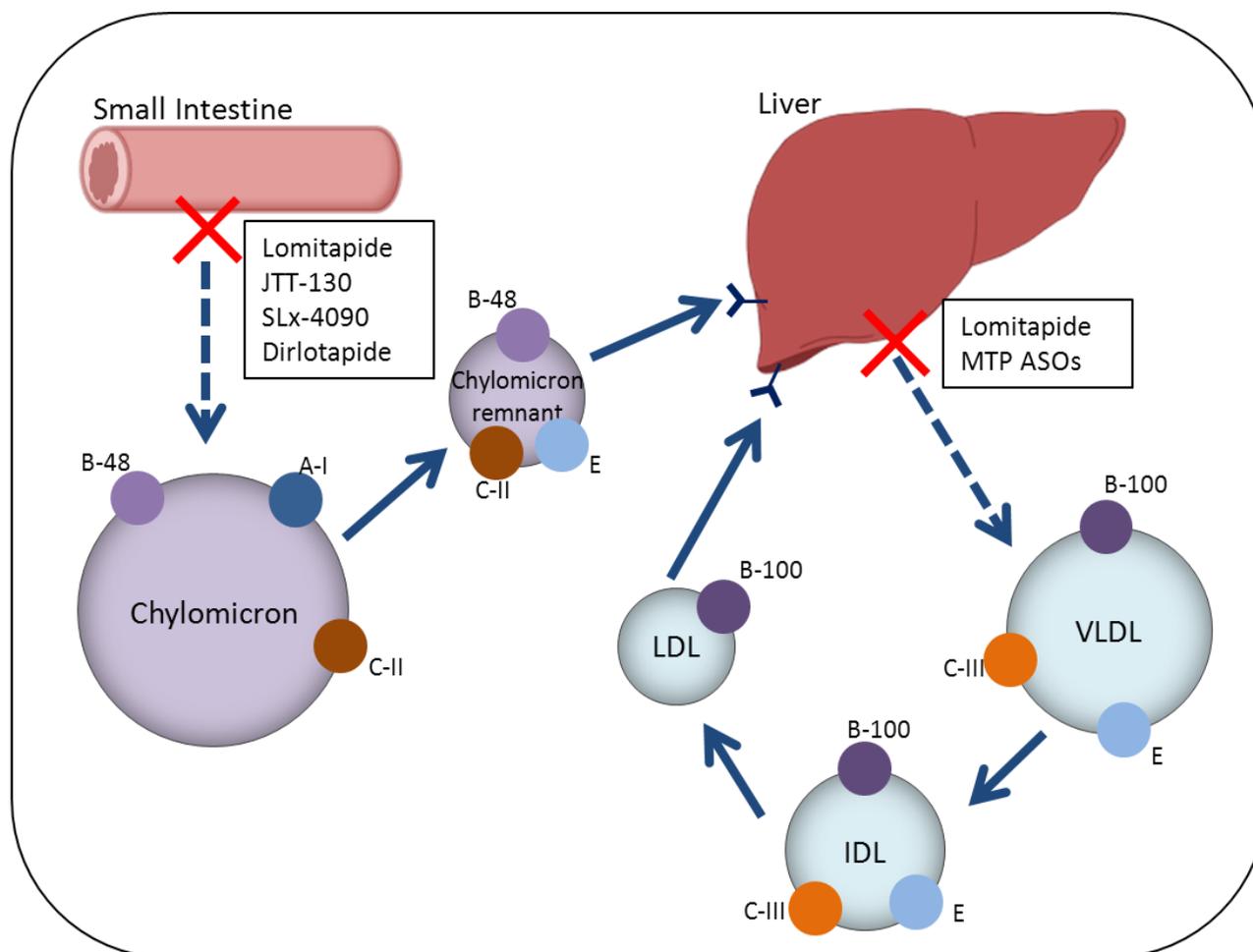
Figure 2. Mechanism of action of MTP inhibitors. These compounds inhibit MTP in the enterocyte and/or hepatocyte to reduce the secretion of chylomicrons and VLDL particles, respectively.
ASOs, antisense oligonucleotides; A-I, apolipoprotein A-I; B-48, apolipoprotein B-48; B-100, apolipoprotein B-100; C-II, apolipoprotein C-II; C-III, apolipoprotein C-III; E, apolipoprotein E