Lymphatic chylomicron size is inversely related to biliary phospholipid secretion in mice

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Running head:
Chylomicron size in biliary PL-deficient and EFA-deficient mice

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Abbreviations:
CM (chylomicron), EFA (essential fatty acid), EFAD (essential fatty acid-deficient), EFAS (essential fatty acid-sufficient), HDL (high density lipoprotein), LCPUFA (long-chain polyunsaturated fatty acid), Mdr2 (multidrug resistance protein 2), PL (phospholipid), PC (phosphatidylcholine), TG (triglyceride), VLDL (very low density lipoprotein)
ABSTRACT

Background: Biliary phospholipids (PL) stimulate dietary fat absorption by facilitating intraluminal lipid solubilization and by providing surface components for chylomicron (CM) assembly. Impaired hepatic PL availability induces secretion of large VLDL, but it is unclear whether chylomicrons size depends on biliary PL availability. Biliary PL secretion is absent in $Mdr2^{-/-}$ mice, whereas it is strongly increased in essential fatty acid (EFA) deficient mice. We investigated lymphatic CM size and composition in mice with absent ($Mdr2^{-/-}$) or enhanced (EFA deficiency) biliary PL secretion and in their respective controls, under basal conditions and during enteral lipid administration. Methods: EFA deficiency was induced by feeding mice a high-fat EFA-deficient diet for 8 weeks. Lymph was collected by mesenteric lymph duct cannulation, with or without intraduodenal lipid administration. Lymph was collected in 30-minute fractions for up to 4 hours, and lymphatic lipoprotein size was determined by dynamic light scattering techniques. Lymph lipoprotein subfractions were isolated by ultracentrifugation and lipid composition was measured. Results: Lymphatic chylomicrons were significantly larger in $Mdr2^{-/-}$ mice than in $Mdr2^{+/+}$ controls, either without (+50%) or with (+25%) enteral lipid administration, and molar core-surface ratios were increased [TG-to-PL ratio: 4.4 ± 1.4 ($Mdr2^{-/-}$) vs. 2.7 ± 0.8 ($Mdr2^{+/+}$), p<0.001]. In contrast, EFA-deficient mice secreted lipoproteins into lymph that were significantly smaller than in EFA-sufficient controls (173 ± 32 vs. 236 ± 47 nm), with correspondingly decreased core-surface ratios [TG-to-PL ratio: 3.0 ± 1.0 (EFA-deficient) vs. 6.0 ± 1.9 (EFA-sufficient), p<0.001]. Chylomicron size increased during fat absorption in both EFA-deficient and EFA-sufficient mice, but the difference between the groups persisted. Conclusions: Present results strongly suggest that the availability of biliary PL is a major determinant of the size of intestinally produced lipoproteins, both under basal conditions and during lipid absorption. Altered chylomicron size may have physiological consequences for postprandial chylomicron processing.

Key words: Biliary phospholipid availability, lymphatic lipoprotein size
INTRODUCTION

Biliary phospholipids (PL) have a well-documented function in transport of dietary lipids from the intestinal lumen into lymph. Apart from their role in intraluminal lipid solubilization, biliary phospholipids have been implicated as crucial components for adequate intestinal chylomicron (CM) assembly and secretion into lymph (25). Biliary phosphatidylcholines (PC), which compose ~95% of biliary phospholipids, provide the main source for chylomicron surface coating (14; 19; 22), and the supply of bile PC to the intestine increases the synthesis of apoB48, the apolipoprotein needed for chylomicron formation (7; 16). Additionally, the high essential fatty acid (EFA) content of biliary PC may be required for maintenance of normal intestinal mucosal membrane composition and function (8; 24). Reduced availability of PC for hepatic VLDL assembly in rats has been associated with decreased VLDL secretion and with assembly of relatively large VLDL particles (26). It is well-established that fat absorption and intestinal lipoprotein secretion are strongly impaired in situations of disturbed bile formation, such as cholestasis. Studies in rats with interrupted enterohepatic circulation by means of permanent bile diversion (25) demonstrated a substantial lipid accumulation in intestinal mucosal cells. Administration of bile salts to these bile-diverted rats partially restored lymphatic lipid transfer, but only when both bile salts and biliary phospholipids were supplemented, lymphatic lipid transport was fully reinstated. Yet, a direct relationship between biliary phospholipid availability and intestinal lipoprotein size has not been established.

We recently characterized fat absorption in two mouse models with altered biliary phospholipid secretion (33). EFA deficiency increases biliary phospholipid secretion by ~80%, in conjunction with a decrease by 30-40% in dietary lipid absorption. In Mdr2<sup>−/−</sup> mice, in which biliary phospholipid secretion is absent (23), plasma appearance of enterally administered lipid is delayed and lipid accumulates in enterocytes. Quantitatively, however, lipid absorption is unaffected in Mdr2<sup>−/−</sup> mice (28).
In the present study, we investigated whether and to what extent quantitative or qualitative alterations in biliary phospholipid secretion affect chylomicron secretion into lymph. Chylomicron size and composition were measured after mesenteric lymph duct cannulation, using the aforementioned mouse models with altered intraluminal biliary phospholipid availability, i.e., \textit{Mdr2}\textsuperscript{(-/-)} mice (no biliary PL secretion), EFA-deficient mice (increased biliary PL secretion) and corresponding control mice. Our results show that the absence of biliary phospholipid secretion in mice is accompanied by production of intestinal lipoproteins of increased size and decreased phospholipid content, whereas EFA deficiency, associated with increased biliary phospholipid secretion, has the opposite effect.

**MATERIALS AND METHODS**

**Animals**

Mice homozygous for disruption of the multidrug resistance gene-2 (\textit{Mdr2}\textsuperscript{(-/-)}) and wild-type \textit{Mdr2}\textsuperscript{(+/-)} mice with a free virus breed (FVB) background were obtained from the breeding colony at the Central Animal Facility, Academic Medical Center, Amsterdam, The Netherlands (9). For dietary induction of EFA deficiency in mice, male wild-type FVB mice were obtained from Harlan (Horst, The Netherlands). All mice were 8 weeks old, weighed 25 to 35 gram and were housed in a light-controlled (lights on 6 AM - 6 PM) and temperature-controlled (21ºC) facility. Mice were allowed tap water and chow \textit{ad libitum} until 2 hours before lymph duct cannulation, since overnight fasting strongly decreased the success rate of the cannulation procedure, attributable to low visibility of the mesenteric lymph duct. All cannulation procedures were performed between 8 AM and noon. The experimental protocols were approved by the Ethics Committee for Animal Experiments, Faculty of Medical Sciences, University of Groningen, The Netherlands.
Experimental diets

The diets used for induction of EFA deficiency in mice by us in present and in previous studies (17; 31-33) and by others are usually high-fat diets. In contrast, in studies characterizing the \( Mdr2^{(-/-)} \) mouse, standard laboratory low-fat chow is most frequently employed. To allow our results to be compared with previous murine studies on EFA deficiency and on biliary PL deficiency, we chose to use high-fat diet for the EFAD and EFAS mice and standard laboratory low-fat chow for the experiments with \( Mdr2^{(-/-)} \) mice. The standard laboratory low-fat chow (RMH-B, Arie Blok BV, Woerden, The Netherlands) contained 14 energy% fat. The high-fat EFA-deficient (EFAD) diet contained 34 energy% fat, and had the following fatty acid composition: 41.4 mol% palmitic acid (C16:0), 47.9 mol% stearic acid (C18:0), 7.7 mol% oleic acid (C18:1n-9) and 3 mol% linoleic acid (C18:2n-6). An isocaloric EFA-sufficient (EFAS) diet was used as control diet, containing 37 energy% fat with 32.1 mol% C16:0, 5.5 mol% C18:0, 32.2 mol% C18:1n-9 and 30.2 mol% C18:2n-6 (custom synthesis, diet numbers 4141.08 (EFAD) and 4141.07 (EFAS), Arie Blok BV, Woerden, The Netherlands). The parenteral lipid emulsion that was used for the intraduodenal bolus administration (Intralipid® 20%, Fresenius Kabi, 's Hertogenbosch, The Netherlands) was mixed with glucose 3.3% and NaCl 0.3% in a 50:50 (v:v) ratio, and a 200 µl bolus of this mixture contained 2.4 nmol PL. This amount of PL constitutes less than 0.2% of luminal biliary PL secreted by EFAD, EFAS and \( Mdr2^{(-/-)} \) mice during the 4-hour lymph collection period. Inevitably, for \( Mdr2^{(-/-)} \) mice the percentage of administrated PL as compared to biliary PL is up to 40% of bile PL, but again less than 0.2% of available bile PL in the intestinal lumen of \( Mdr2^{(+/-)} \) mice.

Experimental procedures
Induction of EFA deficiency in mice

All mice were fed standard laboratory chow from weaning. For induction of EFA deficiency, wild-type FVB mice were fed the EFA-deficient (EFAD) diet for eight weeks. A control group of FVB mice was fed the isocaloric EFA-sufficient (EFAS) diet for eight weeks. This method for induction of EFA deficiency was previously applied in mice and characterized by our group (17; 31; 33).

Mesenteric lymph duct cannulation in mice

Cannulation of the mesenteric lymph duct was performed according to procedures described by Wang et al. (29; 30). After a 2-hour fast, mice were anesthetized with halothane / NO₂ and dorsally arched over a cotton cylinder for optimal visualization of the mesenteric lymph duct. After extra-abdominal displacement of the intestine, the common mesenteric lymph duct was exposed by removal of surrounding tissues and membranes using a blunt mini-kocher. A 0.305 x 0.635 mm (id x od) silicone catheter was introduced through the abdominal wall and positioned parallel to the lymph duct. Subsequently, the catheter was carefully inserted into a small incision in the lymphatic duct and fixated by drops of tissue glue at the junction of lymph duct and catheter. A subgroup of mice subsequently received an intraduodenal 200 µl bolus of a parenteral lipid emulsion (Intralipid® 20%), mixed with glucose 3.3% and NaCl 0.3% (50:50), after collection of a 30-minute baseline lymph sample. The lipid bolus was administered by puncture of the intestinal wall with a 25 G Sterican needle (Braun, Melsungen, Germany), after previous ligation of the duodenum immediately distal to the pylorus to prevent regurgitation into the stomach. After repositioning the intestine, the abdominal incision was closed with 8-10 sutures and mice were placed in restrainer cages in a 37°C incubator. Analgesia was maintained with intraperitoneal injection of buprenorphine (Temgesic®) 0.1 mg/kg. Lymph was collected by gravity into EDTA-containing microtubes, in 30-minute fractions for up to 4 hours after cannulation of the mesenteric lymph duct.
Analytical techniques

Determination of lipoprotein size and composition

Lymphatic lipoprotein size and volume distribution profiles were analyzed within 6 hours after lymph collection by dynamic light scattering techniques, using a Nicomp model 370 submicron particle analyzer (Nicomp Particle sizing Systems, Santa Barbara, CA, USA). Particle diameters were calculated from the volume distribution patterns provided by the analyzer. The lymphatic chylomicron (CM) fraction (d<1.006) was isolated after complementing collected lymph with a 1.006 g/ml NaCl solution containing 0.02% NaN₃ to a final volume of 1 ml, and subsequent centrifugation at 40 000 rpm at 4°C in an Optima TM LX table top centrifuge (Beckman Instruments, Inc., Palo Alto, CA, USA) for 15 minutes. The top layer containing the chylomicron fraction was isolated by tube slicing and the volume was recorded by weight. The remaining lymph solution was again complemented with 1.006 g/ml NaCl to a final volume of 1 ml and centrifuged at 120 000 rpm for 1 hour and 40 minutes for isolation of the very low density lipoprotein (VLDL) fraction.

Lipoprotein lipid concentrations were measured using commercially available assay kits from Roche (Mannheim, Germany) for triglycerides and total cholesterol, and from WAKO chemicals GmbH (Neuss, Germany) for phospholipids.

Calculations and statistics

All results are presented as means ± S.D. for the number of animals indicated. Data were statistically analyzed using Student’s t-test or ANOVA test with post-hoc Bonferroni correction. Level of significance was set at p<0.05. Analyses were performed using SPSS for Windows software (SPSS, Chicago, IL).

RESULTS
Lymph flow was highly variable between and within individual mice, ranging between 1.0 and 15.8 µl/min per 100 g bodyweight, but no significant differences in lymph flow were observed between experimental groups and their respective controls (data not shown). In Mdr2-Pgp-deficient (Mdr2\(^{-/-}\)) mice, biliary phospholipid secretion into the intestine is virtually absent (<0.5 nmol/min per 100 g) (23). Voshol et al. described that postprandial plasma appearance of chylomicrons is impaired in Mdr2\(^{-/-}\) mice (28). Figure 1 shows that non-fed Mdr2\(^{-/-}\) mice secreted chylomicrons of significantly greater size (+51%) into lymph than Mdr2\(^{+/+}\) controls during the first 2 hours of lymph collection (131 ± 23 vs. 87 ± 27 nm for Mdr2\(^{-/-}\) and Mdr2\(^{+/+}\) mice, respectively, p<0.001). Concentrations of triglycerides (TG), phospholipids (PL) and cholesterol were significantly lower in lymph of Mdr2\(^{-/-}\) mice than of controls (Figure 2a). The decrease in phospholipid and cholesterol content (-57% and -93%, respectively) was considerably more pronounced than that of triglyceride (-26%). In the isolated lymphatic chylomicron (Figure 2b) and very low density lipoprotein (VLDL) fractions (Figure 2c), similar differences in lipid content were detected: in the first hour of lymph collection, concentrations of phospholipid, triglyceride and cholesterol were decreased in lipoproteins of Mdr2\(^{-/-}\) mice compared to controls, but the relative triglyceride concentration slightly increased (CM fraction: 86 ± 3% vs. 78 ± 7%; VLDL fraction: 85 ± 1% vs. 80 ± 1% for Mdr2\(^{-/-}\) and Mdr2\(^{+/+}\) mice, respectively, p<0.005). The core-to-surface ratio of lymphatic lipoproteins (i.e., [TG] / [PL], Figure 2d) was increased in total lymph as well as in the isolated chylomicron and VLDL fractions of Mdr2\(^{-/-}\) mice during the first hour of lymph collection, indicating secretion of larger lipoproteins in Mdr2\(^{-/-}\) mice than in controls. In the later fractions, a similar trend was observed, but the increased core-surface ratio only reached significance in the VLDL fraction. We also assessed the effects of absence of biliary phospholipids on chylomicron formation during the active phase of lipid absorption. After collection of a 30-minute baseline sample, a 200 µl lipid bolus was administered intraduodenally to Mdr2\(^{-/-}\) and Mdr2\(^{+/+}\) mice. Similar to the non-fed state, the average diameter of lymph lipoproteins during lipid absorption was.
significantly larger in $Mdr2^{-/-}$ mice than in $Mdr2^{+/+}$ controls (192 ± 38 nm vs. 160 ± 39 nm; p<0.001). The increase in lipoprotein size after lipid administration occurred more rapidly in $Mdr2^{-/-}$ mice (Figure 3). Figure 4 shows the absolute and relative lipid concentrations in lymph, at baseline and 1 and 2 hours after intraduodenal lipid administration. Absolute phospholipid, triglyceride and cholesterol concentrations (Figure 4a) were significantly lower in $Mdr2^{-/-}$ mice than in controls, and did not increase over time. Relatively, however, lymph of $Mdr2^{-/-}$ mice contained more triglyceride and less cholesterol and phospholipid than that of $Mdr2^{+/+}$ controls (Figure 4b), suggesting the presence of larger particles. Indeed, core-to-surface ratio’s were increased in $Mdr2^{-/-}$ mice compared to controls (4.4 ± 1.4 vs. 2.7 ± 0.8, p<0.001; Figure 4c). Thus, a quantitative decrease in biliary phospholipid secretion in $Mdr2^{-/-}$ mice was associated with secretion of larger lymphatic lipoproteins.

To assess the effects of increased biliary phospholipid secretion, we measured lymphatic lipoprotein size and composition in mice after dietary induction of EFA deficiency. Previously, we characterized the effects of EFA deficiency on fat absorption and biliary phospholipid secretion (33). EFA-deficient (EFAD) mice have a dietary lipid malabsorption ranging between 60-70% of the amount ingested, combined with a 70% increased bile flow and an 83% increased biliary phospholipid excretion. Biliary phospholipid acyl chains of EFA-deficient mice contained significantly less essential fatty acids and their long-chain metabolites (i.e., C18:2n-6, C18:3n-6 and C20:4n-6), and more non-essential fatty acids (C16:1n-7, C18:1n-7, C18:1n-9) than EFA-sufficient controls (Figure 5a). Figure 5b shows that at baseline, before enteral lipid administration, lymphatic chylomicrons of EFA-deficient mice were significantly smaller (-32%) than those of EFA-sufficient controls. This decreased particle size in EFA-deficient mice persisted during the active phase of fat absorption, i.e., for two hours after lipid bolus administration (173 ± 32 nm vs. 236 ± 47 nm; p<0.001). Lymph of EFA-deficient mice contained, both absolutely and relatively, more phospholipid and less
triglyceride and cholesterol than lymph of EFA-sufficient controls (Figure 6a and 6b). This was associated with a lower core-to-surface ratio in EFA-deficient mice (3.0 ± 1.0 vs. 6.0 ± 1.9 for EFA-deficient and EFA-sufficient mice, respectively; p<0.001, Figure 6c), indicating secretion of smaller lymphatic lipoproteins.

**DISCUSSION**

Biliary phospholipids (PL) facilitate efficient transport of dietary lipids from the intestinal lumen into lymph, primarily by providing the surface coat for chylomicrons, but also by stimulating apoB48 synthesis and maintaining adequate enterocyte membrane composition. Several conditions can alter biliary phospholipid secretion; essential fatty acid (EFA) deficiency in mice is associated with decreased EFA contents of biliary phospholipids and profoundly increased bile flow, whereas Mdr2-Pgp-deficiency is associated with virtual absence of biliary phospholipid secretion. We previously demonstrated that in both murine models, postprandial plasma appearance of enterally administered lipids is decreased. In EFA-deficient mice, this is combined with decreased net intestinal fat absorption compared to EFA-sufficient controls, as determined by fecal fat balance. In Mdr2(-/-) mice, however, net intestinal fat absorption is only marginally affected, indicating that despite postprandial hypolipidemia, dietary fat is eventually almost quantitatively absorbed during deficiency of biliary phospholipid. In the present study, we applied these two in vivo models to investigate the relationship between biliary phospholipid secretion rate and the size and composition of lipoproteins produced by the intestine.

Our data indicate that in the absence of biliary phospholipid secretion, significantly larger lipoproteins are secreted into lymph. The secretion of large lymphatic lipoproteins could be deduced from several independent observations: from particle size determination by dynamic light scattering techniques, from the relatively increased triglyceride and decreased phospholipid and cholesterol concentrations in lymph of Mdr2(-/-) mice, and from the calculated
lymphatic lipoprotein core-to-surface ratio. Our results on altered intestinal chylomicron formation during biliary phospholipid scarcity in mice are in line with those of Ahn et al., who reported on decreased lymphatic phospholipid and triglyceride output and an increased lymphatic TG-to-PL ratio in zinc-deficient rats, possibly due to the limited supply of biliary phospholipids to the enterocytes during zinc deficiency (1). The lipoproteins secreted by Mdr2(-/-) mice were continuously larger during active fat absorption, but strikingly, in non-fasted mice, the size difference was only significant during the first two hours of lymph cannulation. Since mice had access to chow ad libitum in the night prior to the lymph cannulation experiments, this phenomenon could refer to a delay in intestinal chylomicron formation or transport into lymph (and subsequently into the plasma compartment) in Mdr2(-/-) mice, as previously postulated (28). Possibly, the large chylomicrons assembled during intraluminal phospholipid deficiency enter the lymph more slowly than during sufficient intestinal phospholipid availability. This speculation, combined with the fact that there is no quantitative lipid malabsorption in Mdr2(-/-) mice, supports the concept that the amount of intraluminal bile phospholipid is important for the rate of, but not for the net intestinal absorption of dietary lipid.

Remarkably, cholesterol was virtually absent in intestinal lipoproteins secreted by Mdr2(-/-) mice, possibly related to the fact that biliary cholesterol secretion is strongly reduced in these animals. Voshol et al. previously reported on reduced intestinal cholesterol absorption in Mdr2(-/-) mice (27; 28), which was postulated to result from increased intestinal de novo cholesterol synthesis combined with accelerated enterocyte desquamation due to exposure to detergent lipid-free bile. However, Kruit et al. recently reported that fractional cholesterol absorption is unimpaired in Mdr2(-/-) mice (13). The application of different methods for quantifying cholesterol absorption, i.e., the plasma dual isotope method and the fecal dual isotope method, respectively, probably explains the discrepancy between these studies. The reduction in plasma high-density lipoprotein (HDL) cholesterol levels noted in both reports is
conceivably related to altered chylomicron composition and secretion in \textit{Mdr2}^{-/-} mice, since a major part of HDL is thought to be derived from excess chylomicron surface material (phospholipid and cholesterol), shed during the lipolysis process.

The intestinal requirement of phospholipid for production of chylomicrons may be comparable to that of the liver for the assembly and secretion of VLDL. Verkade \textit{et al.} demonstrated in choline-deprived rats that during hepatic phosphatidylcholine scarcity, fewer but larger VLDL particles are secreted from the liver (26; 35). We recently observed that EFA-deficient mice secrete larger VLDL particles from the liver than controls (31), possibly secondary to hepatic phosphatidylcholine depletion resulting from increased biliary phospholipid secretion rates.

Animal models for increased biliary phospholipid secretion are relatively rare. Since the profoundly augmented biliary phospholipid secretion rate in EFA-deficient mice affects hepatic lipoprotein size and is associated with dietary fat malabsorption, we considered the EFA-deficient mouse an intriguing model to further study the potentially organ-specific effects of phospholipid availability on intestinal lipoprotein production. Our data demonstrate that EFA deficiency in mice is associated with lymphatic secretion of considerably smaller chylomicrons compared to EFA-sufficient controls, as determined by three different particle size estimation techniques. Since EFA-deficient mice secrete larger hepatic VLDL particles than EFA-sufficient controls, secretion of smaller lipoproteins apparently is not an intrinsic feature of EFA deficiency. Rather, EFA deficiency differentially affects lipoprotein size in liver and intestine, with phospholipid availability as the major determinant of lipoprotein size.

Our data are in accordance with studies by Amate \textit{et al.}, who demonstrated that dietary EFA-rich phospholipid administration to piglets resulted in production of lymphatic lipoproteins with significantly smaller diameters compared to piglets fed EFA-rich triglycerides (2).
Our present data do not allow conclusions on quantitative lipid absorption due to the highly variable lymph flow rates in these studies. Possibly, continuous enteral lipid administration could overcome this limitation of our murine lymph cannulation model in future experiments. Although the \( \text{Mdr2}^{(-/-)} \) and EFA-deficient mouse models correspond well regarding postprandial hypolipidemia, they profoundly differ with respect to overall intestinal lipid absorption as determined by fat balance. In EFA-deficient mice, lymphatic TG concentrations were reduced with almost 70%, combined with an abundant lymphatic PL output. In \( \text{Mdr2}^{(+/-)} \) mice, in accordance with the profoundly impaired biliary PL availability, lymphatic PL content was twofold lower than lymphatic TG content, supporting the concept that the difference in bile PL (and cholesterol) contents between these two models strongly affect lymphatic dietary fat output.

In \( \text{Mdr2}^{(-/-)} \) mice, plasma lipoprotein appearance is delayed, but net intestinal lipid absorption is unaffected as determined by 72 h fat balance (28). Thus, lack of biliary PL seems to impair the kinetics of dietary fat absorption, but compensatory mechanisms apparently exist that account for still almost quantitative fat uptake, such as recruitment of more distal parts of the small intestine for absorption, or use of endogenously synthesized PL in enterocytes. EFA deficiency in mice, on the other hand, induces fat malabsorption both in \( \text{Mdr2}^{(+/-)} \) and \( \text{Mdr2}^{(-/-)} \) mice, i.e., irrespective of the presence of absence of biliary phospholipid secretion (33). This suggests that during EFA deficiency, intracellular events involved in chylomicron formation or secretion by the enterocytes, with EFA-depleted intracellular and plasma membranes, are defective and apparently cannot be compensated for, resulting in fat malabsorption. EFA depletion of biliary phospholipid does not seem to affect net dietary fat absorption. For, although EFA deficiency in mice (33), as in rats (3; 4), profoundly decreases EFA acyl chains of bile PL, fat absorption is equally impaired in EFA-deficient \( \text{Mdr2}^{(+/-)} \) and \( \text{Mdr2}^{(-/-)} \) mice. In the present study we chose not to investigate EFA-deficient \( \text{Mdr2}^{(-/-)} \) mice, since these
animals do not differ in their absence of biliary phospholipid secretion compared with EFA-sufficient $Mdr2^{+/+}$ mice.

Baseline lymphatic chylomicrons from EFA-sufficient were significantly larger than those from $Mdr2^{+/+}$ mice (Figures 1, 3 and 5b), which can be attributed to the fact that the EFAD and EFAS diets were high-fat diets (34 energy% fat) and the $Mdr2^{+/+}$ and $Mdr2^{-/-}$ mice were fed standard chow (14 energy% fat).

Hayashi and Tso demonstrated that the number of secreted intestinal lipoproteins remains relatively constant during active lipid absorption, while lipoprotein size increases (10). Obviously, in Mdr2-deficiency, an increased TG-to-PL ratio is a highly favorable means to maintain triglyceride packaging into chylomicrons during lack of surface coat material. In addition, up to 20% of lipoprotein phospholipid is thought to be derived from de novo synthesis (19), and it could be speculated that in $Mdr2^{-/-}$ mice, intestinal phospholipids also partially compensate for the lack of biliary phospholipids for chylomicron surface coating.

Not only biliary but also enterocytic phospholipids are markedly EFA-depleted during EFA deficiency (11; 12; 18). The rapid intestinal cellular turnover rate of enterocytes, which is even faster during EFA deficiency (5), renders the membranes of the intestinal mucosa particularly sensitive to altered intraluminal fatty acid availability. Structural membrane modifications, such as an increased degree of phospholipid fatty acid saturation, affect the fluidity of intestinal membranes and may subsequently result in functional alterations of membrane enzymes and transporters, thus impairing intracellular events involved in chylomicron formation or secretion.

The deviant size and composition of lipoproteins secreted during altered biliary phospholipid secretion (like in EFA deficiency or biliary PL deficiency) may not only affect the rate of plasma appearance of dietary triglycerides, but also intravascular chylomicron metabolism. Smaller chylomicrons have a lower affinity for lipoprotein lipase (LPL) than large ones, and there is competition between small chylomicrons and VLDL for available LPL on the capillary
endothelium (34). Quarford (20) and Chajek-Shaul (6) et al. demonstrated that plasma clearance of large chylomicrons, or chylomycin remnants, is substantially more rapid than that of small particles. Similar findings were described by Martins et al., (15), who reported that particle size and number are major determinants of plasma lipoprotein clearance rates. Altered acyl chain composition on the chylomycin surface (derived from EFA-depleted biliary phospholipids) also affects chylomycin clearance (21); chylomicrons with saturated phospholipids are cleared more slowly than chylomicrons with a high content of PUFA in surface phospholipids. Thus, by affecting lipoprotein size and clearance rates, biliary phospholipids may affect plasma and hepatic lipid levels (31). Notwithstanding these circumstantial indications, it should be realized that our study does not allow firm conclusions on whether chylomycin metabolism indeed is affected during altered biliary phospholipid secretion (like EFA deficiency or biliary PL deficiency).

Our data are compatible with the concept that the size of intestinal chylomicrons, secreted into lymph under basal conditions or during active lipid absorption, is inversely related to the quantity of biliary phospholipid secretion, and that altered lymphatic lipoprotein size is not necessarily related to net dietary fat absorption as determined by fat balance. For clinical implications, it might be of interest to know that for cholestatic patients, in contrast to lack of bile salts in the intestinal lumen, intraluminal phospholipid deficiency per se does not necessarily have negative repercussions on dietary fat absorption. On the other hand, a possible consequence of cholestasis, that is, EFA deficiency, does compromise fat absorption. To specify the clinical effects of small lymphatic lipoproteins synthesized during increased bile phospholipid secretion in EFA deficiency, additional studies would be required, yet prevention or treatment of EFA deficiency in cholestatic patients could contribute to improved dietary fat absorption and subsequent metabolism.
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FIGURE LEGENDS

Figure 1
Lymphatic lipoprotein size (nm), measured by dynamic light scattering, in lymph of non-fasted Mdr2-Pgp-deficient ($Mdr2^{-/-}$) mice (gray circles) and $Mdr2^{+/+}$ mice (black circles). Lipoprotein size was measured in 30-minute fractions of collected mesenteric lymph, and lymph was collected for 3 hours. Data represent means ± SD of 6-10 mice per group. *$p<0.05$ for differences between $Mdr2^{-/-}$ and $Mdr2^{+/+}$ mice.

Figure 2a
Absolute (mM) and relative (%) concentrations of phospholipid (PL), triglyceride (TG) and cholesterol (chol) in lymph of $Mdr2^{-/-}$ mice (gray bars) and $Mdr2^{+/+}$ mice (black bars) in the first hour of lymph collection and after the first hour. Data represent means ± SD of 6-10 mice per group. *$p<0.05$, #$p<0.005$, ##$p<0.001$ for differences between $Mdr2^{-/-}$ and $Mdr2^{+/+}$ mice.

Figure 2b
Absolute (mM) and relative (%) concentrations of phospholipid (PL), triglyceride (TG) and cholesterol (chol) in the isolated lymphatic chylomicron (CM) fraction of $Mdr2^{-/-}$ mice (gray bars) and $Mdr2^{+/+}$ controls (black bars) in the first hour of lymph collection and after the first hour. Data represent means ± SD of 6-10 mice per group. **$p<0.01$, #p$<0.005$, ##p$<0.001$ for differences between $Mdr2^{-/-}$ and $Mdr2^{+/+}$ mice.

Figure 2c
Absolute (mM) and relative (%) concentrations of phospholipid (PL), triglyceride (TG) and cholesterol (chol) in the isolated lymphatic very low density lipoprotein (VLDL) fraction of $Mdr2^{-/-}$ mice (gray bars) and $Mdr2^{+/+}$ controls (black bars) in the first hour of lymph collection and after the first hour. Data represent means ± SD of 6-10 mice per group. **$p<0.01$, #p$<0.005$, ##p$<0.001$ for differences between $Mdr2^{-/-}$ and $Mdr2^{+/+}$ mice.
#p<0.005, ##p<0.001 for differences between $Mdr2^{-/-}$ and $Mdr2^{+/+}$ mice.

**Figure 2d**
Core-to-surface ratio, estimated by the ratio of triglyceride (TG) and phospholipid (PL) concentration (mM) in total lymph and in the isolated lymphatic chylomicron (CM) and very low density lipoprotein (VLDL) fractions of $Mdr2^{-/-}$ mice (gray bars) and $Mdr2^{+/+}$ mice (black bars) in the first hour of lymph collection and after the first hour. Data represent means ± SD of 6-10 mice per group. *p<0.05, #p<0.005 for differences between $Mdr2^{-/-}$ and $Mdr2^{+/+}$ mice.

**Figure 3**
Lymphatic lipoprotein size (nm), determined by dynamic light scattering, in lymph of $Mdr2^{-/-}$ mice (gray circles) and $Mdr2^{+/+}$ controls (black circles) during active lipid absorption. Mesenteric lymph was collected and lipoprotein size was measured in 30-minute fractions, prior to, and for 3 hours after intraduodenal lipid bolus administration. Data represent means ± SD of 5-9 mice per group. *p<0.05 for differences between $Mdr2^{-/-}$ and $Mdr2^{+/+}$ mice.

**Figure 4a**
Concentrations of phospholipid (PL), triglyceride (TG) and cholesterol (chol) in mM, in lymph of $Mdr2^{-/-}$ mice (gray bars) and $Mdr2^{+/+}$ controls (black bars) at baseline, and at one and two hours after intraduodenal lipid administration. Data represent means ± SD of 5-9 mice per group. *p<0.05, #p<0.005 for differences between $Mdr2^{-/-}$ and $Mdr2^{+/+}$ mice.

**Figure 4b**
Relative concentrations of phospholipid (PL), triglyceride (TG) and cholesterol (chol), expressed as % of total lipid, in lymph of $Mdr2^{-/-}$ mice (gray bars) and $Mdr2^{+/+}$ controls (black bars) at baseline, and at one and two hours after intraduodenal lipid administration. Data
represent means ± SD of 5-9 mice per group. *p<0.05, #p<0.005 for differences between Mdr2\(^{-/-}\) and Mdr2\(^{+/+}\) mice.

**Figure 4c**
Core-to-surface ratio, estimated by the ratio of triglyceride (TG) and phospholipid (PL) concentrations (mM) in lymph of Mdr2\(^{-/-}\) mice (gray bars) and Mdr2\(^{+/+}\) controls (black bars). Data represent means ± SD of 5-9 mice per group. *p<0.001 for differences between Mdr2\(^{-/-}\) and Mdr2\(^{+/+}\) mice.

**Figure 5a**
Relative fatty acid composition of biliary phospholipids from EFA-deficient (EFAD, open bars) and EFA-sufficient (EFAS, black bars) mice. Individual concentrations of palmitic acid (C16:0), palmitoleic acid (C16:1n-7), stearic acid (C18:0), dihomo-gamma-linolenic acid (C18:3n-6), linoleic acid (C18:2n-6), oleic acid (C18:1n-9) and arachidonic acid (C20:4n-6) are expressed as molar percentages of total fatty acids. Data represent means ± SD of 7 mice per group. *p<0.001 for differences between EFAD and EFAS mice.

**Figure 5b**
Lymphatic lipoprotein size (nm), determined by dynamic light scattering, in lymph of EFA-deficient (EFAD) mice (open circles) and EFA-sufficient (EFAS) controls (black circles), during active lipid absorption. Mesenteric lymph was collected and lipoprotein size was measured in 30-minute fractions, prior to, and for 3.5 hours after intraduodenal lipid bolus administration. Data represent means ± SD of 6-9 mice per group. *p<0.05 for differences between EFAD and EFAS mice.

**Figure 6a**
Concentrations of phospholipid (PL), triglyceride (TG) and cholesterol (chol) in mM, in lymph of EFA-deficient (EFAD, white bars) mice and EFA-sufficient (EFAS, black bars) controls at baseline, and at one and two hours after intraduodenal lipid administration. Data represent means ± SD of 6-9 mice per group. *p<0.05, #p<0.005 for differences between EFAD and EFAS mice.

Figure 6b
Relative concentrations of phospholipid (PL), triglyceride (TG) and cholesterol (chol), expressed as % of total lipid, in lymph of EFA-deficient (EFAD, white bars) mice and EFA-sufficient (EFAS, black bars) controls at baseline, and at one and two hours after intraduodenal lipid administration. Data represent means ± SD of 6-9 mice per group. *p<0.05, #p<0.005 for differences between EFAD and EFAS mice.

Figure 6c
Core-to-surface ratio, estimated by the ratio of triglyceride (TG) concentration (mM) and phospholipid (PL) concentration (mM) in lymph of EFA-deficient (EFAD, white bars) mice and EFA-sufficient (EFAS, black bars) controls. Data represent means ± SD of 6-9 mice per group. *p<0.001 for differences between EFAD and EFAS mice.
Figure 1

Lymph lipoprotein size no lipid bolus

- **Mdr2** $^{++}$
- **Mdr2** $^{-/-}$

Figure 2a

Lipid composition and relative lipid composition in lymph 0-60 min

- PL, TG, chol

Figure 2b

Lipid composition and relative lipid composition in lymph CM 0-60 min

- PL, TG, chol

Figure 2c

Lipid composition and relative lipid composition in lymph VLDL 0-60 min

- PL, TG, chol
Figure 2d

core-surface ratio (0-60 min)

```
[TG] / [PL]
0 2 4 6 8 10

total lymph CM VLDL

* Mdr2^{+/+} # Mdr2^{/-}
```

core-surface ratio (>60 min)

```
[TG] / [PL]
0 2 4 6 8 10

total lymph CM VLDL

* Mdr2^{+/+} # Mdr2^{/-}
```

Figure 3

lymph lipoprotein size after lipid bolus

```
particle size (nm)

time (min)

lipid bolus

-30 0 30 60 90 120 150 180

Mdr2^{+/+} Mdr2^{/-}

* *
```
Figure 4a and 4b

**Lipid Composition Lymph**

1. **Baseline**
   - **Mdr2**<sup>+/+</sup>
   - **Mdr2**<sup>-/-</sup>

2. **1 hour after lipid bolus**
   - **Mdr2**<sup>+/+</sup>
   - **Mdr2**<sup>-/-</sup>

3. **2 hours after lipid bolus**
   - **Mdr2**<sup>+/+</sup>
   - **Mdr2**<sup>-/-</sup>

**Relative Lipid Composition Lymph**

1. **Baseline**
   - **Mdr2**<sup>+/+</sup>
   - **Mdr2**<sup>-/-</sup>

2. **1 hour after lipid bolus**
   - **Mdr2**<sup>+/+</sup>
   - **Mdr2**<sup>-/-</sup>

3. **2 hours after lipid bolus**
   - **Mdr2**<sup>+/+</sup>
   - **Mdr2**<sup>-/-</sup>
Table 4a:

<table>
<thead>
<tr>
<th>hours after lipid bolus</th>
<th>TG (mM)</th>
<th>PL (mM)</th>
<th>Chol (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mdr2(+/+)</td>
<td>Mdr2(-/-)</td>
<td>Mdr2(+/+)</td>
</tr>
<tr>
<td>0</td>
<td>7.2 ± 1.5</td>
<td>5.8 ± 1.0 *</td>
<td>2.7 ± 0.7</td>
</tr>
<tr>
<td>1</td>
<td>8.9 ± 2.3</td>
<td>6.6 ± 1.8 *</td>
<td>3.6 ± 1.2</td>
</tr>
<tr>
<td>2</td>
<td>11.2 ± 2.6</td>
<td>7.2 ± 3.4 *</td>
<td>4.1 ± 1.0</td>
</tr>
</tbody>
</table>

Table 4b:

<table>
<thead>
<tr>
<th>hours after lipid bolus</th>
<th>TG (%)</th>
<th>PL (%)</th>
<th>Chol (%)</th>
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<tbody>
<tr>
<td></td>
<td>Mdr2(+/+)</td>
<td>Mdr2(-/-)</td>
<td>Mdr2(+/+)</td>
</tr>
<tr>
<td>0</td>
<td>61.2 ± 4.1</td>
<td>81.9 ± 1.6 #</td>
<td>23.3 ± 3.9</td>
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<tr>
<td>1</td>
<td>62.7 ± 7.6</td>
<td>77.1 ± 3.9 #</td>
<td>25.3 ± 6.0</td>
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<tr>
<td>2</td>
<td>66.1 ± 3.8</td>
<td>80.3 ± 5.3 #</td>
<td>24.3 ± 2.9</td>
</tr>
</tbody>
</table>

Figure 4c
Figure 5a

**Biliary fatty acids in EFAD and EFAS mice**

![Graph showing biliary fatty acids in EFAD and EFAS mice](#)

**Figure 5b**

**Lymph lipoprotein size after lipid bolus**

![Graph showing lymph lipoprotein size after lipid bolus](#)
Figure 6a and 6b
Table 6b

<table>
<thead>
<tr>
<th>hours after lipid bolus</th>
<th>TG (mM)</th>
<th>PL (mM)</th>
<th>Chol (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EFAS</td>
<td>EFAD</td>
<td>EFAS</td>
<td>EFAD</td>
</tr>
<tr>
<td>0</td>
<td>12.2 ± 2.6</td>
<td>6.8 ± 1.0*</td>
<td>2.1 ± 0.8</td>
</tr>
<tr>
<td>1</td>
<td>14.6 ± 2.9</td>
<td>9.5 ± 2.0*</td>
<td>2.5 ± 0.6</td>
</tr>
<tr>
<td>2</td>
<td>18.9 ± 2.8</td>
<td>15.0 ± 3.7*</td>
<td>3.9 ± 1.0</td>
</tr>
</tbody>
</table>

Table 6c

<table>
<thead>
<tr>
<th>hours after lipid bolus</th>
<th>TG (%)</th>
<th>PL (%)</th>
<th>Chol (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EFAS</td>
<td>EFAD</td>
<td>EFAS</td>
<td>EFAD</td>
</tr>
<tr>
<td>0</td>
<td>75.4 ± 5.7</td>
<td>60.5 ± 3.3*</td>
<td>12.1 ± 3.1</td>
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<tr>
<td>1</td>
<td>76.7 ± 5.1</td>
<td>67.8 ± 3.7*</td>
<td>13.4 ± 3.4</td>
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<tr>
<td>2</td>
<td>76.1 ± 2.9</td>
<td>71.1 ± 4.6*</td>
<td>15.6 ± 2.4</td>
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</table>

Figure 6c

![core-surface ratio graph]