Plasma Lipoprotein-X Quantification on Filipin-Stained Gels: Monitoring Recombinant LCAT Treatment Ex-Vivo

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Abbreviations:

CE: cholesteryl ester; CKD: chronic kidney disease; ERT: enzyme replacement therapy; FC: free cholesterol; FED: Fish-Eye Disease; FLD: Familial LCAT Deficiency; HDL-C: HDL-cholesterol; LP-X: Lipoprotein-X; PL: phospholipid; rhLCAT: recombinant human LCAT.
ABSTRACT

Background: Familial LCAT Deficiency (FLD) patients accumulate LP-X, an abnormal, nephrotoxic lipoprotein enriched in free cholesterol (FC). The low neutral lipid content of LP-X limits the ability to detect it after separation by lipoprotein electrophoresis and staining with Sudan Black or other neutral lipid stains.

Aim: A sensitive and accurate method for quantitating LP-X would be useful to examine the relationship between plasma LP-X and renal disease progression in FLD patients and could also serve as a biomarker for monitoring recombinant human LCAT (rhLCAT) therapy.

Methods: Plasma lipoproteins were separated by agarose gel electrophoresis and cathodal migrating bands corresponding to LP-X were quantified after staining with filipin, which fluoresces with FC, but not neutral lipids. rhLCAT was incubated with FLD plasma and lipoproteins and LP-X changes were analyzed by agarose gel electrophoresis.

Results: Filipin detects synthetic LP-X quantitatively (linearity 20-200 mg/dL FC; c.v.<20%) and sensitively (LLQ < 1 mg/mL FC), enabling LP-X detection in FLD, cholestatic and even Fish-Eye Disease patients. rhLCAT incubation with FLD plasma ex-vivo reduced LP-X dose-dependently, generated HDL and decreased lipoprotein FC content.

Conclusions: Filipin staining after agarose gel electrophoresis sensitively detects LP-X in human plasma and accurately quantifies LP-X reduction after rhLCAT incubation ex-vivo.

Key words: LP-X; LCAT; Filipin; Familial LCAT Deficiency, FLD; Fish-Eye Disease, FED; Lecithin:cholesterol acyltransferase; Cholesterol; Phospholipids; HDL; Kidney.
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INTRODUCTION

Lecithin:cholesterol acyltransferase (LCAT) is the only enzyme that catalyzes cholesteryl ester (CE) production in the plasma compartment from free cholesterol and phosphatidylcholine (lecithin). It plays a key role in maturation of HDL from small, discoidal particles to large, spherical HDL particles and has been proposed to enhance cholesterol efflux by HDL (1). Familial LCAT Deficiency (FLD), an autosomal recessive disorder, is characterized by very low plasma HDL-cholesterol (HDL-C), cloudy corneas, anemia, and proteinuria that progresses to nephrotic syndrome and end-stage renal disease in the fourth or fifth decade of life (1).

A hallmark of FLD is the presence of lipoprotein-X (LP-X), an abnormal multilamellar particle that is enriched in phospholipids (PL) and free cholesterol (1-5). Accumulation of LP-X is implicated in kidney damage and chronic kidney disease (CKD) in FLD patients (6,7) and has been shown to be nephrotoxic in mice (8,9) and rats (10). Patients with fish-eye disease (FED) have partial LCAT deficiency, with some residual esterification of free cholesterol in apoB-containing lipoproteins but not HDL(1). These patients have reduced HDL-C and corneal opacities but do not develop renal disease, presumably because they do not form LP-X (11,12). Recent data using a mouse model of FLD with accelerated renal disease showed that LCAT treatment prevents plasma and tissue deposition of LP-X and the development of renal disease (9). Since the major cause of morbidity and mortality in FLD patients is end-stage renal disease, these data suggest that lowering LP-X may prevent renal disease progression in these patients.

LP-X also accumulates in patients with liver disease, particularly those that have cholestasis (13). The pathologic significance of elevated LP-X in liver disease is uncertain, but it has been shown to result in xanthoma formation in cholestatic liver disease due to macrophage uptake of LP-X in the skin (14). It is important to detect LP-X in cholestatic patients, because cholestasis can present with profoundly elevated levels of plasma cholesterol (>1000 mg/dL) that is non-responsive to statins or other conventional lipid lowering therapy. Furthermore, the high levels of LP-X in cholestasis can interfere
with numerous clinical laboratory tests because of turbidity issues and volume displacement from LP-X, which can lead to pseudohyponatremia and other lab test abnormalities (15,16).

Recently, recombinant human LCAT (rhLCAT) (ACP 501) was developed as a potential enzyme replacement therapy (ERT) therapy for FLD and for cardiovascular disease patients. It was found to be safe and well-tolerated in a Phase I clinical trial (17). Infusion into a patient with FLD also showed promising results: HDL-C rapidly increased, peaking to nearly normal levels in 8–12 hours (18). Importantly, anemia improved and renal function stabilized after long-term treatment with ACP 501 (18). This trial demonstrated that ERT with rhLCAT is a potential strategy for increasing HDL-C in FLD; however, the effect of rhLCAT on LP-X was not examined. Moreover, relatively high levels of ACP-501 were required to raise HDL to near-normal levels in this study. After completion of this study, a more potent formulation of rhLCAT, MEDI6012, with a longer half-life was developed and is now being tested in patients with cardiovascular disease in a Phase II clinical trial (19).

A validated rapid clinical method for measuring plasma LP-X levels would be useful not only for identifying patients with LP-X that could potentially benefit from rhLCAT treatment, but also for monitoring LP-X levels during rhLCAT therapy for FLD. Currently, however, there are no routinely available assays for efficient screening for LP-X in patient plasma. Agarose gel electrophoretic analysis of patient plasma has shown that LP-X particles migrate toward the cathode, i.e. with reverse electrophoretic mobility compared to other lipoproteins (13). LP-X, however, is difficult to detect using Sudan Black or other similar neutral lipid stains that are typically used to stain lipoproteins separated by gel electrophoresis. In contrast to normal plasma lipoproteins, which are enriched in neutral lipids (cholesteryl esters and triglycerides), LP-X particles are deficient in neutral lipids and instead, as noted above, are highly enriched in free cholesterol and phospholipids (1).

Filipin, a fluorescent polyene antibiotic, binds specifically to free cholesterol. In contrast to Sudan Black, it does not stain neutral lipids. It is widely used as a cytochemical fluorescent stain for free
cholesterol but also has been used to detect free cholesterol in normal lipoproteins in nondenaturing polyacrylamide gels (20).

In this paper, we demonstrate that filipin staining of agarose gels after electrophoresis is a relatively easy and sensitive method to detect and quantitate LP-X in plasma from FLD and cholestasis patients. It can also be used to monitor the effects of exogenous rhLCAT on lipoproteins. Finally, our new methodology presents a potential opportunity, at both the clinical and basic science level, to investigate the relationships between FC-enriched lipoproteins and LP-X in the pathology of FLD and cholestatic liver disease.
METHODS

Preparation of synthetic LP-X

Synthetic LP-X, which was used as a calibrator and to examine the differential staining of free cholesterol by filipin and Sudan Black, was prepared as described in Ossoli et al. (8), with minor modifications. Briefly, multilamellar LP-X particles containing 24 mole % cholesterol were formed by combining 24.4 mg L-α-lecithin (Avanti Polar Lipids, Inc., Alabaster, AL) with 4.25 mg cholesterol (Sigma Chemicals, St. Louis, MO) from their respective stock solutions in chloroform and then drying the lipid mixtures under nitrogen. Two ml of normal saline were added to the dried lipids and the mixture was vortexed and sonicated as described in Ossoli et al. to generate multilamellar particles enriched in free cholesterol and phospholipid in the typical size range of LP-X.

Agarose gel electrophoresis

10 ul of synthetic LP-X or 10 ul EDTA-plasma was loaded on Sebia Hydragel 15/30 lipoprotein(e) agarose gels (#4134; Sebia, Inc., Norcross, GA). Purified lipoproteins (LDL, VLDL and HDL) were loaded on the same gel. Electrophoresis was for 1 hr at 100V in barbital buffer (#B5934-12VL; MilliporeSigma, St. Louis, MO) in a Titan Gel electrophoresis chamber (Helena Laboratories, Beaumont, TX). Gels were either fixed and stained with Sudan Black to detect neutral lipids, according to manufacturer’s instructions, or incubated with Filipin Stain to detect free cholesterol by a modification of the method described in reference (20). Filipin Stain was prepared fresh each time by dissolving 50 mg Filipin (Polysciences, Inc., Warrington, PA) in 2.0 ml N, N’ dimethyl formamide, and then adding 100 ml 1X PBS containing 0.1% sodium azide and mixing thoroughly. Filipin and filipin-containing solutions were shielded from light at all stages as filipin is light-sensitive. Immediately after electrophoresis, gels were washed for 5 minutes in 1X PBS plus 0.1 % sodium azide and then incubated overnight in 30 ml Filipin Stain at 4°C with gentle rocking, shielded from light. Gels, kept in the dark at all times, were then washed for 10 min at room temp, then again for 1 hour, with 100 ml Wash Solution (1X PBS). Fresh
Wash Solution was added 10 minutes before imaging. Gels were placed on the AlphaImager’s UV light transilluminator (ProteinSimple, San Jose, CA) and photographed using the ethidium bromide filter, similar to photographing an ethidium bromide-stained DNA gel. LP-X, which migrates toward the cathode in the opposite direction as normal lipoproteins, was quantified by densitometry (including the origin) and background in an equal-sized area of the gel 1 cm below the bottom of each band was subtracted using Alpha Imager software. To ensure linearity of the filipin signal, multiple exposures were taken of gels and quantitation was performed only on exposures in which band intensities did not reach saturation, as determined by the Alpha Imager software. Supplemental Figure S1 demonstrates that the UV light exposures used to visualize filipin on our gels do not result in photobleaching.

Subjects

This study was approved by the National Heart, Lung and Blood Institute, Institute Review Board (protocol 03-H-0280) and is compliant with the Declaration of Helsinki principles. All subjects provided informed consent prior to participation in the study.

Plasma collection and incubation with rhLCAT

EDTA-plasma was collected from patients (FLD, FED or cholestasis) and stored on ice or at 4°C until electrophoresis. Precision studies for LP-X in FLD patient plasma are described in Supplemental Figure S2. rhLCAT (MEDI6012) was produced as described before (21) and was kindly provided by Medimmune. Fresh, never-frozen FLD plasma was incubated at 37°C overnight with MEDI6012 (0 – 1000 ug/ml) and aliquots were analyzed for lipids (Cholesterol-E and Free Cholesterol-E, Wako Diagnostics, Mountain View, CA) and for lipoproteins and LP-X by agarose electrophoresis followed by Sudan Black or Filipin staining.

Depletion of apoB-Lps from FLD plasma
EDTA-plasma was collected as described above and incubated with dextran sulfate or LipoSep IP™ (Sun Diagnostics, New Gloucester, ME), following the methodology of Davidson et al. (22). Briefly, in the dextran sulfate method, 2.0 ul of 5% dextran sulfate (MW 500,000) and 5.0 ul of 1 M MgCl₂ were added to 100 ul FLD patient plasma. The mixture was incubated for 20 min at room temperature and then centrifuged at 3,000 g for 10 min. For depletion of apoB-100 using the LipoSep IP™ reagent, FLD patient plasma was combined with an equal volume of the LipoSep IP™ reagent in a 1.5 ml microcentrifuge tube and immediately vortexed at top speed. The mixture was incubated at room temperature for 10 minutes and then centrifuged at 12,000 g at 4°C for 10 minutes. The supernatant, consisting of apoB-depleted plasma, was used immediately for agarose gel electrophoresis, and the pellet was discarded.

**Statistical Analysis**

Statistical analyses were performed using GraphPad Prism. To fit the allosteric curve of LP-X vs. FC concentration during MEDI6012 incubation (Figure 4C), the “Agonist vs. Response -Variable Slope, 4 parameters” equation under “Dose-Response Curve-Stimulation” in GraphPad Prism was applied to the data. The concentration of MEDI6012 required to decrease LP-X by 50% in Figure 4D was determined by analyzing the data in GraphPad Prism similar to determining the “IC50” of MEDI6012 (nonlinear regression, enzyme kinetics, [inhibitor] vs. response analysis). Unless otherwise indicated, all results are presented as the mean plus/minus 1 SD.
RESULTS

Filipin, but not Sudan Black, quantitatively stains cholesterol on LP-X

Synthetic LP-X was prepared in vitro from purified free cholesterol and phosphatidylcholine and various dilutions (0.2 - 200 mg/dL cholesterol) were subjected to electrophoresis on duplicate agarose gels, which were then stained with either Filipin to detect free cholesterol or Sudan Black to detect neutral lipids. Figure 1A shows that synthetic LP-X migrates from the origin (horizontal red line) toward the cathode, as expected, opposite to LDL, VLDL and HDL, which migrate in the traditional lipoprotein direction towards the anode. Moreover, Filipin, but not Sudan Black, stains synthetic LP-X (Figure 1A). The lowest observable amount of synthetic LP-X that could be visualized with filipin was approximately 1 mg/dL free cholesterol (Figure 1A). In Figure 1B, LP-X dilutions between 20 and 200 mg/dl were run on triplicate agarose gels and stained with filipin for a precision study. Densitometry demonstrated that the relationship is linear between 20 – 200 mg/dL LP-X cholesterol, with $R^2$ of 0.9855 (Figure 1B). The inter-assay coefficient of variation for quantifying the cholesterol content of LP-X was less than 12% across the concentration range tested.

Filipin, but not Sudan Black, sensitively detects LP-X in plasma from FLD and cholestatic patients

Fresh, never-frozen plasma from FLD patients was subjected to electrophoresis on agarose gels followed by staining with either Filipin or Sudan Black (Figures 2A, 2B). Figure 2A shows a patient (“FLD 1”) with very high levels of LP-X that was readily detectable as a broad filipin-stained band below the origin (migration toward the cathode). Only a faint band, however, could be seen after Sudan Black staining, indicating that detection of LP-X by filipin staining is much more sensitive than Sudan Black. Moreover, compared to the normolipidemic sample (healthy control; HC) there was increased filipin staining in the FLD sample in the LDL region, migrating toward the anode. As can be seen below in the other FLD samples tested, this was a common finding. We found that immunodepleting apoB-Lp’s with the LipoSep IP™ reagent (22) removed essentially all filipin-stained beta-migrating lipoproteins...
(Supplemental Figure S3), demonstrating that apoB-Lp’s are indeed FC-enriched in FLD patients and are almost exclusively responsible for filipin staining in and around the LDL region of the gel. We also frequently noted that the purified lipoprotein standards (LDL, VLDL and HDL) that are isolated by density gradient ultracentrifugation from normolipidemic samples all stain with Sudan Black but only LDL and VLDL stain well with filipin.

Figure 2B shows the results from two cousins with FLD. The patient on the left (“FLD 2”) had more LP-X by filipin staining, and also more advanced renal disease, than the patient shown on the right (“FLD3”). However, only an extremely faint LP-X band migrating in the cathodal direction could be observed for both FLD2 and FLD3 after staining with Sudan Black, again showing the increased sensitivity for LP-X detection by filipin staining. As can be seen in patient FLD2, we sometimes observe in FLD patients a filipin-stained band at the origin, which may be large LP-X that cannot migrate into the gel.

Figure 2C demonstrates that filipin staining is so sensitive that it can even detect very low levels of LP-X in an FED patient. FED patients have low HDL-C and corneal opacities but do not develop renal disease. Until now, LP-X has not been reported in FED patients, and the absence of LP-X was thought to be the explanation for why they do not develop renal disease (1). The increased sensitivity of filipin staining appears to allow the detection of even extremely low levels of LP-X that are below the threshold for the development of renal dysfunction. Precision studies for LP-X in FLD patient plasma are described in Supplemental Figure S2.

Figure 2D shows LP-X in a patient with cholestasis from Graft-Versus-Host (“GVH”) disease. In general, we have observed that cholestatic LP-X is present in plasma at much higher levels than in FLD plasma, as determined by Filipin staining, and as in this case can often be detected by Sudan Black staining as well.

**Freezing and thawing of samples interferes with LP-X detection**
In Figure 3, the stability of LP-X detection to freezing and thawing was investigated. Synthetic LP-X (lanes 1-4) or plasma from an FLD patient (lanes 5-8) were either maintained at 4°C without freezing or freeze-thawed 1, 2 or 3 times. Synthetic LP-X maintained at 4°C (lane 1, filipin-stained gel) migrated as a smear towards the cathode, but after just a single freeze-thaw (lane 2) synthetic LP-X was no longer detectable by filipin staining. Similarly, in FLD plasma, a cathode-migrating LP-X band seen by filipin staining in the non-frozen sample (lane 5, filipin-stained gel) could not be observed after a single freeze-thaw (lane 6, filipin-stained gel). In addition, the filipin intensity at the origin was also diminished after just a single freeze-thaw. The overall level of filipin staining of all lipoprotein fractions was decreased by further freeze-thawing (lanes 7 and 8, filipin-stained gel). Consistent with our previous results, neither synthetic LP-X nor backwards-running LP-X in FLD patient plasma was stained by Sudan Black (lanes 1-8, Sudan-stained gel). Sudan-stained FLD lipoproteins with LDL mobility were somewhat diminished after the first freeze-thaw but not by subsequent freeze-thaws (lanes 5-8, Sudan-stained gel). The filipin-stained gels clearly demonstrate that freezing and thawing interferes with LP-X detection.

**Incubation of FLD plasma with rhLCAT decreases LP-X in a dose-dependent manner**

To investigate the ex-vivo effects of rhLCAT (MEDI6012) on LP-X in plasma, increasing doses of MEDI6012 were incubated with plasma from an FLD patient (“FLD 4”). Figure 4A shows that free cholesterol decreased and cholesteryl esters increased with increasing doses of MEDI6012. Consistently, MEDI6012 decreased LP-X and increased HDL on agarose gels stained with Filipin and Sudan Black, respectively, for the proband (Figure 4B). Figure 4C demonstrates a strong correlation between LP-X and free cholesterol at different doses of MEDI6012, with both free cholesterol and LP-X diminishing with increased doses of MEDI6012. Interestingly, the relationship between FC and LP-X during the course of MEDI6012 incubation was not linear but instead appeared to be allosteric (Figure 4C), with decreased efficiency of FC esterification occurring after a ~ 4-fold drop in LP-X, from ~8000 to ~2000 (corresponding to 10 ug/mL MEDI6012). This allosteric relationship suggests that structural changes to LP-X occurring past this point may affect the efficiency of FC esterification. Figure 4D
indicates that the dose of MEDI6012 required to decrease LP-X by half in this in vitro assay is approximately 3.7 ug/mL.
DISCUSSION

The main advance in this study was the development of a sensitive, reliable filipin gel-based assay for the detection and quantitation of plasma LP-X. Using this assay, we also made several potentially important findings related to lipoprotein abnormalities in FLD and have explored the feasibility of using LP-X as a biomarker for monitoring rhLCAT therapy.

As is readily apparent, the positive staining of synthetic LP-X with filipin but absence of staining with Sudan Black indicates that detecting endogenous plasma LP-X with filipin should be more sensitive compared to Sudan Black. It varies depending on the lipoprotein class, but normal lipoproteins typically contain neutral lipids as their major component (23) and hence readily stain with Sudan Black. In contrast, LP-X contains less than 10% neutral lipids (1,24). This probably accounts for our past inability to detect LP-X in many of our FLD patients when stained with Sudan Black. It is likely that only in those patients with very high levels of LP-X is it possible to detect LP-X with Sudan Black. The increased sensitivity of our method also probably accounts for the detection of LP-X in our one FED patient. Additional FED patients need to be examined, but it may be that low levels of LP-X are often present in FED patients but below the level at which nephrotoxicity is observed.

Regardless of the staining method used, a key to detecting LP-X by electrophoresis is its “backward” or cathodal migration. Normally, lipoproteins contain a net negative charge, most likely because of their protein content, and hence migrate toward the anode. We observed that not only endogenous LP-X from our FLD patients but also synthetic LP-X migrated toward the cathode. The synthetic LP-X was made with phosphatidylcholine, a zwitterion, and thus has no net charge. Endogenous LP-X is relatively devoid of proteins and based on its lipid composition (1) does not appear to be enriched in cationic lipids. The backward migration of LP-X is, therefore, most likely due to electroendosmosis (25,26) which causes the bulk movement of buffer ions that non-specifically sweeps neutrally charged particles toward the cathode. Besides charge, agarose gel electrophoresis also separates lipoproteins based on their size. Large lipoproteins move slower relative to smaller particles, which
explains why chylomicrons typically get trapped at the origin (application point). We also observed filipin-stained bands in fasted samples at the origin in some of our FLD patients. It is known that LP-X can be quite heterogeneous in size (24,26) and thus some LP-X particles can perhaps be too large to enter the gel. Since the filipin-enriched bands migrating at the origin were still present after immunodepletion of apoB-Lps, and synthetic LP-X also contained a subpopulation of filipin-stained particles that remained at the origin, the filipin-stained material remaining at the origin in patient plasma is most likely LP-X. Size heterogeneity could also account for the fuzzy bands and smearing in the migration position for LP-X that we observed in our FLD patients or with our synthetic LP-X particles. Finally, cholestatic patient plasma had significant Sudan Black staining of backwards-running LP-X. Whether the exact migration position of LP-X has any disease relevance is not known at this time but can be examined in the future with our assay.

Another observation that we made based on filipin staining is that there is enrichment of free cholesterol in FLD patients not only in LP-X but also in and around the “beta” region where LDL typically migrates on agarose gels and sometimes almost as far as VLDL. Supplemental Figure S3 demonstrates that immunodepleting apoB-Lps removes almost all filipin staining in this “broad-beta” region, confirming that these FC-enriched bands do in fact correspond to apoB-Lp’s. This is consistent with the known chemical composition of apoB-containing lipoproteins from FLD patients, which have higher levels of free cholesterol (6). VLDL has also been reported to migrate as beta-lipoproteins in FLD (6). Whether these abnormal lipoprotein particles may also impact on the pathogenesis of FLD is not known, but cholesterol enrichment of lipoproteins in SR-BI-KO mice has been shown to lead to abnormal red cell shape changes, which also occurs in FLD, and to enrichment of cholesterol in platelets and their hypercoagulability (27). Although LP-X is relatively devoid of proteins, they have been reported to bind apoC-class proteins (1,24), which have a negative charge (28). Supplemental Figure S4 demonstrates that adding purified apoC-II to synthetic LP-X in vitro decreases cathodal LP-X migration on agarose gels. In fact, at very high ratios of apoC-II to synthetic LP-X, a subpopulation of LP-X particles can be seen to migrate towards the anode, with mobility in the LDL/VLDL region. Incorporation of negatively charged
lipids, such as free fatty acids, into LP-X particles can also cause the particles to migrate towards the anode (29). Thus, some of the filipin-stained particles migrating with beta mobility, particularly those with LP-X-like instability to freezing and thawing, might also be LP-X.

A limitation of our assay is that frozen plasma samples do not appear to be suitable for analysis. Unlike normal lipoproteins that have a micellar-like structure of surface phospholipids and free cholesterol, with a neutral lipid core, LP-X is thought to be a multi-lamellar vesicle made of phospholipid and free cholesterol. It is thought to have an aqueous core made up of non-specifically trapped plasma proteins. It is therefore likely that freezing of LP-X causes a disruption of its structure when ice crystals form in the aqueous core. If so, it may be possible to preserve LP-X to freezing by adding a cryoprotectant as has been done before for preserving lipoproteins (30,31). We also observed, however, a general decrease in total filipin staining after freezing and thawing. Possibly the lipids have reorganized after freezing, decreasing the ability of filipin to interact with cholesterol, which may not be prevented even in the presence of cryoprotectant.

Because of the necessity of having a fresh sample and the rarity of FLD, we have only analyzed a limited number of samples by our new method. More studies will be needed to determine if quantitation of the cholesterol content of LP-X, which we showed is possible with our assay, can be used to predict risk for renal disease. In the case of cholestasis, the filipin-gel staining method could be useful to determine the extent of cholesterol on LP-X versus other lipoproteins, which may be predictive of their response to lipid-lowering therapy. The ex-vivo recombinant LCAT spiking studies into FLD plasma are promising and suggest that this method may also be useful in determining the response to rhLCAT therapy in FLD patients. Interestingly, the dose that caused almost the complete dissolution of LP-X is in the physiologic range and is similar to what was used in phase 2a clinical trials of rhLCAT (19). The generation of cholesteryl esters by the added rhLCAT in our study must cause a spontaneous reorganization of the LP-X particles. We even observed the in vitro formation of HDL, which again must occur after rhLCAT forms cholesteryl esters, allowing the residual apoA-I in the plasma from FLD patients to form HDL-like particles.
Finally, a variety of methods have been used to detect and quantify plasma LP-X including lipoprotein precipitation (32-39), NMR (40-42), and LP-X calculation from standard clinical lipid measurements (43,44). Our detailed validation of the agarose gel electrophoresis/filipin staining method described in this manuscript may provide a means to independently validate these and other such LP-X quantification assays.

In conclusion, filipin staining of agarose gels can be used to detect and quantitate LP-X from FLD plasma and cholestasis. This novel methodology addresses an important deficiency in the measurement of LP-X and has several potential clinical and basic science applications.
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Figure 1: Filipin staining of synthetic LP-X after agarose gel electrophoresis. (A) Filipin, but not Sudan Black, stains synthetic LP-X. 10 μl of sequential dilutions of synthetic LP-X, corresponding to 200, 80, 40, 20, 10, 4, 2, 0.8, 0.4 and 0.2 mg/dL free cholesterol, were loaded into lanes 1-10, respectively, of 2 identical gels and gels were run at 100V for 1 hour. The gels were stained with either Filipin (left) or Sudan Black (right). 10 μl of LDL, VLDL, HDL and healthy control (HC) plasma were included as standards loaded on the left side of each gel and the red line indicates the position of the origin (application point). LP-X can be seen as fluorescent material migrating towards the cathode (“backwards-running” compared to LDL, VLDL and HDL) by Filipin staining but it is undetectable on gels stained with Sudan Black. (B) LP-X fluorescence on agarose gels is proportional to the amount of LP-X free cholesterol loaded on the gels. Three identical gels, similar to Figure 1A but with 200, 133, 100, 56, 40, 26.7, and 20 mg/dL free cholesterol, were run and stained with Filipin as above. LP-X fluorescence for each sample was quantitated by densitometric analysis and the average and SD for each point was graphed vs. the amount of LP-X free cholesterol loaded. Linear regression analysis using Prism revealed linearity over an order of magnitude, with $R^2 = 0.9855$. 
Filipin staining of LP-X in FLD and FED patients (A) FLD patient (FLD1) with highly elevated LP-X by Filipin staining but not Sudan staining. (B) Two cousins with FLD. patient to the left (“FLD2”) has more advanced renal disease and more LP-X than the patient to the right (“FLD3”) (C) An FED patient with very low levels of LP-X (lane D). A long exposure was taken and the brightness was adjusted to visualize backwards-running LP-X. For comparison, a non-FLD patient with triglycerides of 445 mg/dL (lane A) and a Type II patient (lane B) are shown. (D) Plasma from a patient with cholestasis due to graft-vs.-host disease (“GVH”). For comparison, a non-FLD patient with low HDL is shown. Electrophoresis and staining was as described in the legend to Figure 1.
**Fig. 3:** LP-X is unstable to freezing and thawing. Synthetic LP-X (lanes 1-4) or FLD plasma (lanes 5-8) were either maintained at 4°C (lanes 1 and 5) or freeze-thawed 1X, 2X or 3X (lanes 2, 3 and 4 for synthetic LP-X and lanes 6, 7 and 8 for FLD, respectively). Samples were frozen at -70°C for at least 16 hours before each thaw. Electrophoresis and staining was as described in the legend to Figure 1.
Figure 4

4. MEDI6012 Incubation With FLD Patient Plasma. (A) Lipids of FLD patient plasma treated with MEDI6012 ex vivo. FLD plasma was incubated with increasing doses (0, 0.75, 1.5, 3, 10, 30, 100, and 1000) µg MEDI6012 per ml plasma at 37°C for 16 hours and total and free cholesterol were measured. CE was calculated as the difference between total and free cholesterol. Green lines and w lines with blue-circled dots represent total and free cholesterol, respectively, in mg/dL. Orange and red lines represent percent free cholesterol and CE remaining after MEDI6012 incubation, plasma levels at 0 mg/ml MEDI6012 as 100%. (B) LP-X disappears and HDL appears after MEDI6012 incubation with FLD patient plasma. Lanes 1-9 represent doses 0, 0.75, 1.5, 3, 10, 30, 100, and 1000, respectively, µg/ml MEDI6012. The two 4°C samples represent patient plasma drawn fresh the day of electrophoresis (left “4°C sample”) and patient plasma from the same blood draw as MEDI6012 samples, left overnight at 4°C as a control, without MEDI6012 (right “4°C sample”) as the MEDI6012 samples from the same blood draw were incubating overnight at 37°C in lanes 1-9. Electrophoresis and staining with Filipin (left) or Sudan Black (right) was as described in the legend to Figure 1. (C) Plasma LP-X correlates with levels of FC in plasma after MEDI6012 incubation. Lipoprotein X in lanes 1-9 of Figure 4B (corresponding to 0 – 1000 µg/mL MEDI6012 of the filipin-stained gel) were quantitated by densitometry and plotted against the corresponding plasma cholesterol for each sample after MEDI6012 incubation. The “0 µg/ml” dose of MEDI6012 had the highest levels of LP-X and free cholesterol in plasma and the “1000 µg/ml” dose had the lowest LP-X and free cholesterol levels. (D) The concentration of MEDI6012 required to decrease LP-X halfway between the highest and lowest LP-X values (similar to an “IC50” analysis) was determined using GraphPad Prism.