Plasma Lipid Profiling Across Species for the Identification of Optimal Animal Models of Human Dyslipidemia

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Running title: Plasma lipid profiling across species
Abstract

In an attempt to understand the applicability of various animal models to dyslipidemia in humans and to identify improved preclinical models for target discovery and validation for dyslipidemia, we measured comprehensive plasma lipid profiles in 24 models. These included five mouse strains, six other non-primate species, and four non-human primate (NHP) species, and both healthy animals and animals with metabolic disorders. Dyslipidemic humans were assessed by the same measures. Plasma lipoprotein profiles, eight major plasma lipid fractions, and fatty acid compositions within these lipid fractions were compared both qualitatively and quantitatively across the species. Given the importance of statins in decreasing plasma low-density lipoprotein cholesterol (LDL-c) for treatment of dyslipidemia in humans, the responses of these measures to simvastatin treatment were also assessed for each species and compared to dyslipidemic humans. NHPs, followed by dog, were the models that demonstrated closest overall match to dyslipidemic humans. For the subset of the dyslipidemic population with high plasma triglyceride levels, the data also pointed to hamster and db/db mouse as representative models for practical use in target validation. Most traditional models, including rabbit, ZDF rat, and the majority of mouse models, did not demonstrate overall similarity to dyslipidemic humans in this study.

Keywords: dyslipidemia; lipidomic; statins; preclinical animal models; cholesterol; low-density lipoprotein (LDL).
Nonstandard abbreviations used: LDL-c: low-density lipoprotein cholesterol; HDL-c: high-density lipoprotein cholesterol; VLDL: very low density lipoprotein; DIO: diet-induced obesity; HFD: high fat diet; NHP: non-human primate; AGM: African green monkey; TG: triglyceride; TC: total cholesterol; CE: cholesteryl ester; FC: free cholesterol; FFA: free fatty acid; DAG: diacylglycerol; PC: phosphatidylincholine; PE: phosphatidylethanolamine; LPC: lysophosphatidylcholine; CETP: cholesteryl ester transfer protein; CRP: C reactive Protein; LDLR: LDL receptor; ApoE: apolipoprotein E; DNL: de novo lipogenesis.
Introduction

Cardiovascular disease (CVD) is the leading cause of morbidity and mortality worldwide(1). Dyslipidemia has been shown to be one of the most potent risk factors for coronary heart disease (CHD)(2, 3). Dyslipidemia is characterized by elevated plasma cholesterol, especially low-density lipoprotein cholesterol (LDL-c) levels. Management of dyslipidemia is considered throughout the primary and secondary prevention of CHD(4). For the past 20 years, the statin (3-hydroxy-3-methylglutaryl coenzyme A [HMG-CoA] reductase inhibitors) class of cholesterol-lowering drugs has been used for the treatment of hypercholesterolemia, either alone or in combination with other classes of lipid-lowering drugs(5, 6). By inhibiting cholesterol synthesis and lowering LDL-c, statins have been proven to be effective in reducing cardiovascular risks in randomized, controlled clinical trials over 15 years involving more than 100,000 individuals(7-9). Although statins effectively lower cholesterol levels and reduce cardiovascular causes of death, a large portion of statin-treated patients still experience adverse coronary events. This has led to the vigorous search for new therapeutic agents for cardiovascular diseases, targeting the residual CVD risk that remains after statin treatment(10). However, the success rate of drug development for dyslipidemia beyond statins has lagged, and the major cause of failure in drug development has been lack of efficacy in the clinic (11). This has led us to address the need for better preclinical animal models at the target validation stage of drug discovery, in an effort to obtain better prediction of efficacy.

Traditionally, mouse models have been widely used in preclinical research and for target validation in drug discovery for dyslipidemia and atherosclerosis(12). A major difference of mouse models from humans is the absence of cholesteryl ester transport protein (CETP), a key enzyme involved in plasma cholesterol transport that transfers CE from HDL to ApoB-
containing lipoproteins such as LDL and VLDL. Rats, dogs, and pigs also have no or low plasma CETP activities, and they all display a high HDL-c and low LDL-c plasma lipoprotein distribution, similar to mice, which is associated with a low risk of CVD(13). Genetic manipulation of mice (such as ApoE<sup>−/−</sup> and LDLr<sup>−/−</sup> mice) significantly increases circulating LDL-c levels, susceptibility to atherosclerosis, and sensitivity to atherogenic diet(14-16). Expressing the human CETP gene in mice, such as the CETP<sup>+/−</sup>/LDLr<sup>−/−</sup> mouse model, results in higher plasma LDL-c and lower plasma HDL-c, which is referred to as a "human-like profile", and the animal becomes atherogenic-prone(17, 18). Animal species with naturally high CETP activity, such as rabbits and NHPs have a high LDL-c and low HDL-c profile which is associated with increased risk of CVD. Although not inherently atherosclerotic-prone, the rabbit model develops hypercholesterolemia and aortic lesions rapidly even with low amounts of dietary cholesterol, and has therefore been used extensively in studies of atherosclerosis(19). Historically, all the animal models mentioned above have been utilized to study disease mechanisms and for drug discovery for dyslipidemia and atherosclerosis. The question remains as to whether their dyslipidemic state is representative of human dyslipidemia, or whether these models show a similar response to anti-dyslipidemic pharmacological agents.

The classical guidelines for dyslipidemia and risk assessment for CVD focus mainly on plasma cholesterol levels (LDL-c and HDL-c) and to a lesser extent, on plasma triglyceride (TG) level (20). However, multiple abnormalities can lead to dyslipidemia. Recently, it has been examined with a variety of techniques in an attempt to more comprehensively understand the disease state and more reflective of the biological pathways that are relevant to specific targets (e.g., enzymes or receptors that drugs may modulate). Among these techniques, lipidomics (or lipid metabolomics) has advanced tremendously because of the ability of technology to rapidly
quantify hundreds of different molecular lipid species with different structural and functional roles (21, 22).

Here we report a comprehensive and comparative lipidomic analysis of plasma lipids in 22 commonly-used animal models across various animal species, including five different mouse strains, six other non-primate species, and five primate species including dyslipidemic humans. In addition to a comparison of classic lipid parameters (such as plasma LDL-c, HDL-c, and TG) and plasma lipoprotein profiles, we quantified the absolute and relative amounts of fatty acids in major circulating lipid fractions, under both basal and simvastatin-treated conditions. The aim of this study was to help us 1) understand the differences and similarities of various animal models to dyslipidemic human; 2) understand the applicability of different animal models to dyslipidemic humans; and 3) identify the optimal animal model(s) for target validation and drug discovery for developing treatments for dyslipidemia.
Methods

In vivo experiments

All animal experiments and protocols were reviewed and approved by the Merck Research Laboratories’ Institutional Animals Care and Use Committee.

Unless otherwise noted, animals were housed in a temperature- and humidity-controlled environment with a 12 h light/dark cycle. In all experiments, animals received ad lib food and water except for dogs. Dogs were on a weight maintenance diet which was available from 10 AM to 1PM. The gender and number of animals used for each study, the exact sources of the animals, and the diets used for each animal species are all listed in Supplemental Table 1.

Dosing: All animals were dosed with either vehicle or simvastatin for 2 weeks. For the rodent species, simvastatin was admixed into the diet by Research Diets (New Brunswick, NJ). The dose of simvastatin was 30mg/kg in all mouse models, 23 mg/kg in ZDF rats, and 20mg/kg in hamsters. For all other species, simvastatin was dosed 30 mg/kg PO except New Zealand White rabbit which was dosed at 10mg/kg. Vehicle was water in the primates, and compound was mixed into a treat (detailed in Supplemental Table 1), except for marmosets. Marmosets were dosed from a 3cc syringe, with Splenda at 5% (McNeil Nutritionals) and maple syrup added as a flavor mask.

Plasma collection: Blood samples were collected with EDTA. Samples for FPLC analysis had lipase inhibitors added.

Human measurements: The human simvastatin study was originally reported by Chen et al. (23).

Plasma measurements

FPLC analysis
The generation of lipoprotein profiles was performed as previously reported(24). In brief, plasma lipoprotein was separated by chromatography using a Superose-6 size exclusion column (GE LifeSciences, Inc.) on an Ultimate 3000 Series HPLC system (Dionex Corporation). Total cholesterol levels in the column effluent were continuously measured using in-line mixture with an enzymatic and colorimetric cholesterol detection reagent (Total Cholesterol E, Wako USA), followed by spectrophotometric detection of the reaction products at 600-nm absorbance. VLDL, LDL-c and HDL-c were eluted from the column. The concentration for each lipoprotein fraction was calculated by multiplying the ratio of the corresponding peak area to total peak area by the total cholesterol concentration in the sample. Plasma total cholesterol level was measured with a 1:1 mixture of Cholesterol E reagent and diluted plasma using a plate reader. Cholesterol standards were provided in the kit at 200mg/dl and were serially diluted to provide a standard curve.

Biochemical analysis of circulating lipids

Plasma triglyceride levels were measured by biochemical analysis in non-human primate species. In brief, triglycerides GPO-PAP reagents (Roche Diagnostics, Indianapolis, IN) were used on a Roche Diagnostics Modular Analytics P Clinical Chemistry Analyzer to determine levels of triglycerides. Assays were carried out following all recommended procedures for instrument operation, calibration, quality control and assay guidelines. The instrument was calibrated with Calibrator for automated systems (Roche Diagnostics, Indianapolis, IN) and controls were Precipath U (Roche Diagnostics) for triglycerides.

Measurements of fatty acid composition in circulating major lipid fractions

The concentration and complete fatty acid composition of plasma cholesteryl ester (CE), TG, diacylglycerol (DAG), free fatty acid (FFA), phosophatidylcholine (PC),
phosphatidylethanolamine (PE); and lysophosphatidylcholine (LPC) were determined using TrueMass® Lipomic Panel by Lipomics, Technologies (West Sacramento, CA). Lipid fractions were isolated and methylated, fatty acids were separated and quantified by gas chromatography, and absolute masses and percentage of each fatty acid of the total within each lipid fraction were calculated as previously reported(25).

**Plasma CRP measurements**

Plasma CRP levels in all species were measured by Rules Based Medicine (Austin, TX), using fully automated, bead-based multiplex sandwich immunofluorescence assays. The HumanMap antigen panel was used to determine plasma CRP levels in human, non-human primates, and pigs. The RodentMap antigen panel was used to determine plasma levels in dog, rabbit, and all rodent species.

CRP was also measured using the MSD 96-well multi-array human CRP assay kit obtained from Meso Scale Diagnostics, LLC (Gaithersburg, MA) for all non-human primate species. Plasma samples were diluted 200 fold and added 10ul/well. Human CRP protein was used for standard curves.

**Simvastatin Measurements**

Simvastatin levels in plasma were determined by LC-MS/MS following acetonitrile protein precipitation. Standards were prepared from 1 to 8000ng/mL. Internal standard L-000050672 (20uL at 0.5ug/mL) was spiked into samples prior to being extracted with 500uL of acetonitrile for protein precipitation. Samples were centrifuged at 4000rpm for 5 min before supernatant plate transfer. A Waters Atlantis T3 (3um, 2.1x30mm) column was used with gradient chromatography (Acetonitrile, 0.1% Formic Acid/Water, 0.1% Formic Acid). Negative-ion
mode mass spectrometry transition monitored for Simvastatin was 435.3 -> 319.1. The levels of simvastatin are shown in the Supplemental Table 2.

**Statistical Analyses**

Separate studies were carried out for each animal model and the design of the studies varied with species. The Gottingen mini-pig model had only a chow diet control group with no simvastatin treated group. Rhesus DIO, marmoset and dog models had two groups, a vehicle treated group and a simvastatin treated group, with two sets of lipid measurements (pre-treatment and post-treatment) per group. African Green monkey, rhesus with metabolic syndrome, diabetic rhesus, and cynomolgus each had a single group of animals, with each animal measured under vehicle treatment and simvastatin treatment. Rabbit and all rodent models included a vehicle treated group and a simvastatin treated group, with one set of lipid measurements per group.

**Basal lipid levels and percents**

Means and standard deviations of basal levels of lipids and lipid composition were calculated using vehicle-treated animals and placebo-treated human subjects. The exception was pigs, for which levels in untreated animals were used.

**Comparison of eight major circulating lipid fractions in various animal models to dyslipidemic humans**

For each animal model, the mean of a lipid fraction (CE, TG, DAG, FFA, PC, PE, LPC or FC) was calculated and differences from dyslipidemic human were subtracted from the mean of the same lipid fraction in dyslipidemic human. For a given lipid fraction \( j \), these differences were then weighted (multiplied) by the square root of the proportion that fraction represented out of total lipids in humans. To describe the calculation in more details, we use \( x_{ij} \) to represent the
amount of \( j \) for a given animal model \((i)\), and \( x_{hj} \) to represent the amount of \( j \) for dyslipidemic human \((h)\). The sum (over lipid fractions) of the squared weighted differences was calculated for each model as below:

\[
 d_i = \sqrt{\sum_{j=1}^{n} p_j (x_{ij} - x_{hj})^2}
\]

\( p_j \) represents the calculated proportion of lipid fraction \( j \) over total lipid for humans:

\[
 p_j = \frac{x_{hj}}{\sum k x_{hk}}
\]

Using \( p_j \) as the weighing factor, the weighted Euclidean distance of each lipid fraction in each animal model from human was calculated as below:

\[
 \sqrt{p_j (x_{ij} - x_{hj})}
\]

The animal models were then sorted by distance and the values from the last step are shown in row \( i \) and column \( j \) of the heat map shown in Fig. 3.

*Estimation of simvastatin effects on lipid levels*

For animals that have separate groups for different conditions, unpaired \( t \)-test was used to calculate the significance of simvastatin effect between groups. For models that had the same group of animals (African Green monkey, rhesus with metabolic syndrome, diabetic rhesus, and cynomolgus) for repeat measurements, statistical analysis was done using paired \( t \)-test between conditions.

*Estimation of simvastatin effects on fatty acid composition*

All analyses were performed after applying a \( \log_{10}(x+1) \) scale transformation to measured fatty acid values. The specific statistical analysis model varied with study, due to differences in design, but the primary result of each analysis was an estimate of the simvastatin effect on each fatty acid endpoint for the tested species. The effect was expressed as the log of the ratio of fatty acid values with simvastatin to values under control conditions. \( P \)-values and confidence
intervals for the effect were not multiplicity-adjusted because of the exploratory nature of the analyses.

*Distance of the simvastatin effect profile of each animal species to dyslipidemic humans*

The vector of simvastatin effects (log ratios) on a set of fatty acids for a given animal species was the effect profile for that species. To compare the effect profile of each animal species to dyslipidemic humans, three distance measures were used. a) Euclidean distance between the vectors of log ratios. b) A variance-weighted Euclidean distance applied to the log ratios. This weighted the contribution of a particular fatty acid category to the distance calculation inversely proportional to the estimated variance of the difference in simvastatin effects for that category. Therefore categories with high variability were down-weighted. This metric was an approximation to the Mahalanobis distance between two effect profiles, where the off-diagonal elements of the covariance matrix were ignored. c) Uncentered correlation-based distance (1-Cosine distance). This measured the distance between effect vectors by one minus the cosine of the angle between the vectors. Unlike (a) and (b), (c) assessed only the similarity of the *pattern* of simvastatin effects across fatty acid categories, ignoring any differences between animal species based on the magnitude or scale of those effects. Each of these metrics measured a different aspect of the difference between animal models and dyslipidemic humans.
Results

Basal plasma lipid levels and lipid distributions in dyslipidemic human and various animal models

The LDL-c, TC, and TG levels of the dyslipidemic humans we studied here were borderline high (154±7 mg/dL, 226±6 mg/dL, and 154±15 mg/dL respectively) (Table 1)(26). This group had a fairly balanced lipid profile, with HDL-c being about 30% of LDL-c (48±4 mg/dL). A representative FPLC trace of a plasma lipid profile is shown in Fig. 1. VLDL was not measured, but the normal range is typically ≤ 30 mg/dL in populations with triglyceride levels under 150 mg/dL(26).

Based on plasma lipid parameters and lipoprotein traces, several animal models carried the majority of plasma cholesterol on non-HDL lipoproteins (i.e. VLDL and LDL) similar to dyslipidemic humans: NHPs, rabbits on cholesterol diet, ApoE⁻/⁻ mice on chow or cholesterol diet, LDLr⁻/⁻ mice on chow or cholesterol diet, and CETP⁺/⁻/LDLr⁻/⁻ mice on chow or cholesterol diet (Table 1 and Fig. 1). Among these animal models, the greatest similarities to humans were observed in NHPs. The African green monkey (AGM) and cynomolgus studied here were young and healthy animals, and their plasma TC, LDL-c, HDL-c, and TG levels all fell into the normal range of healthy humans (Table 1)(26). Diseased NHP models, including dyslipidemic AGM, rhesus with metabolic syndrome, diabetic rhesus and rhesus with DIO (diet-induced obesity) all had characteristics of dyslipidemia in human populations, with either high TC, LDL-c or both, or high TG levels, while HDL-c remains stable (Table 1). The exception among NHP models was the marmoset, which while healthy animals, exhibited abnormally high TG with big variations (Table 1 and Fig. 1).
New Zealand white rabbits on chow diet had extremely low TC, VLDL-c, LDL-c and HDL-c levels (29±1 mg/dL, 4±0.3 mg/dL, 8±0.4 mg/dL, and 18±1 mg/dL respectively). Upon the challenge of 0.5% cholesterol in their diet, the plasma TC, VLDL-c, and LDL-c levels all dramatically increased to 811±48 mg/dL, 439±26 mg/dL, and 317±24 mg/dL respectively, with the majority of plasma cholesterol carried in the VLDL fraction, while HDL-c remained low (Table 1 and Fig. 1). ApoE⁻/⁻ mice had high TC (412±21 mg/dL), VLDL-c (226±17 mg/dL), LDL-c (178±13 mg/dL) and very low HDL-c (8±1 mg/dL) when on normal chow diet; a high-fat Western diet significantly increased TC (629 mg/dL), LDL-c (402 mg/dL), and TG (154±31 mg/dL), with minimal change in VLDL-c and HDL-c (205 and 22 mg/dL respectively). The LDLr⁻/⁻ mouse is considered a model of human familial hypercholesterolemia (FH) caused by mutations of LDLR. Unlike the ApoE⁻/⁻ mouse model, LDLr⁻/⁻ mice had only mildly elevated TC (250±8 mg/dL), LDL-c (168±5 mg/dL), and TG (124±8 mg/dL), and they had normal VLDL-c and HDL-c (13±1 and 69±3 mg/dL respectively) on chow diet. However on a low-fat Western diet, they developed extremely high TC (1677±98 mg/dL), VLDL-c (904±47 mg/dL), and LDL-c (761±57 mg/dL), as well as high TG (404±35 mg/dL), while HDL-c decreased dramatically (Table 1). The CETP⁺/⁻/LDLR⁻/⁻ heterozygous mouse is a more recently developed model that was intended to mimic human lipoprotein distribution by introducing a single copy of the CETP gene along with deletion of LDLr(17). On normal chow these mice had TC, VLDL-c, LDL-c, HDL-c, and TG within the lower range of normal human, and a cholesterol diet pushed their TC and LDL-c levels to borderline high (Table 1). All three mouse models above had significantly decreased HDL-c levels compared to wild-type C57BL/6 mice.

In contrast to the animals described above, the pig (Gottingen Mini-pig), dog (obese beagle), Golden Syrian hamster on chow or HFD (high fat diet), ZDF (Zucker diabetic fatty)
heterozygous (ZDF/+) rat, ZDF rat on HFD, C57BL/6 and db/db mouse models all carried the majority of plasma cholesterol on HDL particles and presented athero-protective profiles (Fig. 1). The pig model had comparable HDL-c to healthy humans, with plasma TC, TG, and LDL-c in the low end of normal range (Table 1). The obese beagle model had a borderline high TC level (196± mg/dL) but the highest HDL-c level (163±3 mg/dL) across all animal species, presenting the most athero-protective lipid profile among all models studied here, since high HDL-c level is associated with reduced risk for CHD(26). The hamster on chow diet, C57BL/6 and db/db mouse models had HDL-c accounting for about 70% of total cholesterol (Table 1 and Fig. 1). HFD induced an increase in TG levels in both hamster and ZDF rat, but to different extents: with relatively high TG level on chow diet, HFD-fed hamster had a ~2.4-fold increase in TG (534±43 mg/dL), while ZDF rat on HFD had a more than 17-fold increase of TG (1482±210 mg/dL).

Acute-phase reactant C-reactive protein (CRP) has been identified as a marker of inflammation; however there are debates about whether CRP is a predictor of cardiovascular events(27, 28). The range of CRP in normal humans is 0.1-4.8 μg/ml(29). NHPs, dog, and rabbit on chow all fell at the low end of this range, while mouse models were at the high end. Marmoset, pig, rabbit on cholesterol diet, and hamster on both chow and HFD had very low CRP levels. Extremely high CRP levels were observed in ZDF rat models (Table 1).

Fatty acid composition analysis for three major biosynthetic plasma lipid fractions across models

Major plasma lipid fractions and the diversity of lipid molecular species in humans have been summarized previously(30). Our lipidomic study covered most of the major plasma lipid fractions including CE, TG, DAG, FFA, PC, PE, LPC, and FC. Among these fractions, CE, TG,
and PC are the three most abundant, accounting for more than 75% of total plasma lipids(30). Fatty acids identified in plasma lipids can also be divided into three major categories: the non-essential fatty acids which include saturated fatty acids and mono-unsaturated fatty acids, the linolenic acid (omega-3) pathway which includes linolenic acid and its derivatives, and the linoleic acid (omega-6) pathway which included linoleic acid and its derivatives. The source of non-essential fatty acids can be either from de novo lipogenesis (DNL) or from diet, while fatty acids within the omega-3 or omega-6 pathways can only originate from two essential fatty acids obtained from diet (Fig. 2A).

Fatty acids within the CE, TG, and PC fractions were combined according to their synthetic pathways, and compared both quantitatively and qualitatively across animal species (Fig. 2B, 2C and 2D). In dyslipidemic humans, the mean absolute and relative amounts of fatty acids in plasma CE were: 987±50 nmol/g from the non-essential fatty acids (about 34% of total fatty acids), 58±5 nmol/g from the omega-3 pathway (about 2% of total), and 1866±63 nmol/g from the omega-6 pathway (about 64% of total) (Fig. 2B). In plasma TG of dyslipidemic humans, there was 2780±197 nmol/g from the non-essential fatty acids (about 73% of total), 48±9 nmol/g from omega-3 (about 3% of total), and 903±60 nmol/g from omega-6 (about 24% of total) (Fig. 2C). In plasma PC of dyslipidemic humans, there were 2009±80 nmol/g (about 54% of total), 190±28 nmol/g (about 5% of total), and 1486±52 nmol/g (about 40% of total) from the non-essential fatty acids, omega-3, and omega-6 pathways, respectively (Fig. 2D). Thus in plasma CE, TG, and PC of dyslipidemic humans, over 95% of fatty acids were from the non-essential fatty acids or omega-6 pathways. While the omega-6 pathway was the main source of fatty acids in plasma CE, the non-essential fatty acids provided the majority of fatty acids in TG and PC. Across all the animal species, the contribution of fatty acids from the omega-3
pathway was consistently small, with the highest percentage being 8% in CE (db/db mice), 7% in TG (ApoE<sup>−/−</sup> mice) and 9% in PC (rhesus with metabolic syndrome) (Fig 2B, 2C, and 2D).

The animal species most similar to dyslipidemic humans in terms of the fatty acid composition of plasma CE were: all NHP models, pig, dog, hamster, ZDF rat, C57BL/6, db/db, LDL<sup>−/−</sup>, and CETP<sup>+/−</sup>/LDL<sup>−/−</sup> mice. Both the absolute amounts and the relative distribution of fatty acids in plasma CE in these animal species were similar to dyslipidemic humans, with omega-6 being the largest component (Fig. 2B). ZDF rat, C57BL/6, and db/db mice had an even greater percentage of fatty acids from the omega-6 pathway (over 75%). A high-fat diet in hamster or ZDF rat did not impact either the absolute amount or the percentage of fatty acids from the omega-6 pathway. In LDL<sup>−/−</sup> and CETP<sup>+/−</sup>/LDL<sup>−/−</sup> mice, however, a cholesterol containing diet not only increased the absolute amount of fatty acids in CE (with a much more significant impact on LDL<sup>−/−</sup> mice), it also shifted the composition of fatty acids from the omega-6 to the non-essential fatty acids (Fig. 2B). Rabbit and ApoE<sup>−/−</sup> mice were the animal species least comparable to dyslipidemic humans since the non-essential fatty acids count for the majority of fatty acids under a chow diet condition. A cholesterol diet not only dramatically increased the total amount of CE fatty acids, it also pushed the percentage of the non-essential fatty acids even higher (Fig. 2B).

The non-essential fatty acids were the major source for plasma TG for all animal species. However, a significant impact of diet was seen in rabbit, hamster, ZDF rat, ApoE<sup>−/−</sup>, LDL<sup>−/−</sup>, and CETP<sup>+/−</sup>/LDL<sup>−/−</sup> mice. A high-fat diet in hamster and ZDF rat increased both the absolute amount of the non-essential fatty acids (with a greater increase in ZDF rat) and the percentage of the non-essential fatty acids in plasma TG. A cholesterol containing diet had little impact on total TG in rabbit, ApoE<sup>−/−</sup>, and CETP<sup>+/−</sup>/LDL<sup>−/−</sup> mice, although the composition of fatty acids
shifted toward the non-essential fatty acids in \(ApoE^{-/-}\) and \(CETP^{+/-}/LDLr^{-/-}\) mice (Fig. 2C). A cholesterol containing diet in \(LDLr^{-/-}\) mice increased both the absolute amount and relative contribution of the non-essential fatty acids (Fig. 2C).

Compared to plasma CE and TG, the total amount of fatty acids in PC varied less and the fatty acid composition remained much more stable across animal species and dietary conditions. About 50-60% of the total came from the non-essential fatty acids and 30-40% from the omega-6 pathway for all the animal species. A HFD or cholesterol diet increased total fatty acids, but did not substantially change the fatty acid composition of PC in rabbit, hamster, ZDF rat, \(ApoE^{-/-}\), \(LDLr^{-/-}\), and \(CETP^{+/-}/LDLr^{-/-}\) mice, although slight increases in the percentage of the non-essential fatty acids were seen in \(ApoE^{-/-}\), \(LDLr^{-/-}\) and \(CETP^{+/-}/LDLr^{-/-}\) mice (Fig. 2D).

Amounts of eight major circulating lipid fractions in various animal models compared to dyslipidemic humans

To get a more comprehensive understanding of the similarities and differences of different animal models compared to dyslipidemic humans, the amounts of eight major circulating lipid fractions (CE, TG, DAG, FFA, PC, PE, LPC and FC) in all the animal models were measured and compared with dyslipidemic human. For any given lipid fraction, first the mean for that lipid fraction was calculated in each animal species, then the difference was compared with the mean of the same lipid fraction in dyslipidemic human. The differences were weighted based on the proportion of that lipid fraction over total lipid in dyslipidemic human so that abundant lipid fractions (such as CE, TG and PC) contributed more than less abundant lipid fractions in the comparison. The animal models were then sorted by distance (Fig. 3) (see Methods for a more detailed description). Most NHP models were relatively close to dyslipidemic humans,
with the rhesus metabolic syndrome model having the greatest similarity. Although dietary manipulation is most commonly used in generating dyslipidemic or atherosclerotic models, in most non-NHP models, such as LDLr<sup>-/-</sup> mice, ApoE<sup>-/-</sup> mice, ZDF rat, rabbit, and hamster, the similarity between these animal species and dyslipidemic humans with respect to the distribution of the eight major lipid fractions actually decreased with a cholesterol diet or HFD, with most significant changes seen in LDLr<sup>-/-</sup> mice, hamster, and ZDF rat (Fig. 3). CETP<sup>+/+</sup>/LDLr<sup>-/-</sup> mice on cholesterol-diet is the only model that showed closer similarity to dyslipidemic human than the mice on chow diet. It also showed the closest similarity to dyslipidemic human among all non-NHP models (Fig. 3). The mean values of each lipid fraction in each animal model are also shown in Supplemental Table 3.

**Impact of simvastatin treatment on plasma lipid profiles**

To further understand the utility of different animal models for identification of new targets in drug discovery, the response of plasma lipid profiles in various animal models to simvastatin treatment was determined. Percent changes in plasma lipids (TC, VLDL-c, LDL-c, HDL-c, and TG) upon simvastatin treatment and their statistical significance are shown in Table 2. Consistent with the literature, statin treatment caused a significant decrease in plasma TC and LDL-c level in the dyslipidemic humans studied here (16% and 24% decrease respectively upon 40mg/day simvastatin treatment for 2 weeks)(31). Statin treatment also has been shown to decrease plasma VLDL-c and TG and increase HDL-c in a subset of patients(31, 32). Here simvastatin treatment significantly decreased plasma TG (-18%) but the effect on HDL-c was minimal (+1%).
Similar to dyslipidemic humans, a greater than 15% decrease in TC and 20% decrease in LDL-c were seen in all the NHP models, with dyslipidemic AGM being the most responsive model to simvastatin treatment (Table 2). Decreases of VLDL-c were seen in most NHP models except rhesus with metabolic syndrome. Among the non-primates, dog was the only species that showed responses of plasma TC, VLDL-c, and LDL-c (-34%, -43%, and -80% respectively). Significant decreases in plasma TC and VLDL-c were seen in rabbit on cholesterol, hamster on HFD, and ZDF rat on HFD; however, no decrease was seen in LDL-c. In ZDF rat on HFD, statin treatment increased LDL-c more than 2-fold (+112% increase). All mouse models had no change or increases in plasma TC, VLDL-c, and LDL-c upon simvastatin treatment.

Changes in plasma TG levels varied among NHP models, with AGM and cynomolgus having the most significant decrease similar to dyslipidemic humans, and no decreases in plasma TG in dyslipidemic AGM, rhesus with metabolic syndrome, and marmoset. In non-primate species, dog, hamster on HFD, ZDF rat on HFD, and db/db mice showed significant decreases in plasma TG upon simvastatin treatment. Similar to our observation in dyslipidemic human, no significant increase in HDL-c was seen in any animal species, including NHP models (Table 2).

Changes in the fatty acid composition of plasma lipids (CE, TG and PC) upon statin treatment

To obtain more detailed insight into the similarities and differences of various animal models to dyslipidemic humans in statin responsiveness, statin-induced changes in SFAs, MUFAs, omega-3 pathway derived fatty acids, and omega-6 pathway derived fatty acids in plasma CE, TG, and PC upon simvastatin treatment were calculated (Fig. 4). In dyslipidemic humans, significant decreases were seen in CE and TG fatty acids across all four categories (except
MUFAs in CE), along with a consistent, but not statistically significant, decrease in PC fatty acids. AGM, cynomolgus, and hamster on HFD were the animal models closest to dyslipidemic humans in overall responsiveness on CE, TG, and PC (Fig. 4). Three diseased rhesus models (rhesus with metabolic syndrome, diabetic rhesus, and rhesus with DIO) showed responses in plasma CE and PC to simvastatin treatment that were similar to dyslipidemic humans; however, changes in plasma TG were not significant, due to the large biological variation. Marmosets did not show any statistically significant changes across CE, TG, and PC in any fatty acid categories, despite significantly decreased plasma TC upon simvastatin treatment (Fig. 4 and Table 2).

Hamster on HFD, dog and rabbit were the only non-primate species that showed decreased fatty acids in all four categories of CE upon simvastatin treatment. CETP+/−/LDLr−/− mice on cholesterol diet showed decreased omega-3 and omega-6 pathway related fatty acids, but increased SFAs. ZDF rat on HFD and all the other mouse models either had no change or had increases in the majority of fatty acid categories in plasma CE (Fig. 4). For plasma TG, ZDF rat on HFD, db/db, and ApoE−/− mice all showed similar response in fatty acid composition compared to dyslipidemic humans. Three atherogenic-diet animal models, rabbit on cholesterol diet, ApoE−/− mice on cholesterol diet, and CETP+/−/LDLr−/− mice on cholesterol diet, showed increases in each component of TG upon simvastatin treatment. A trend toward a small decrease, either significant or non-significant, was seen in most of the animal models, except dog, rabbit on cholesterol diet, and ZDF rat on HFD.

To statistically compare the responsiveness to statin treatment between each animal model to dyslipidemic human and obtain an overall ranking of the similarity, we calculated the distance between the set of 12 log ratios in Fig. 4 for dyslipidemic humans and each of the animal
models. Three distance measures were used, each reflecting a different aspect of the difference between sets of log ratios. Euclidean method measures the "ordinary" distance which reflects the magnitude of statin responsiveness in each animal model. Variance-based Euclidean method also considers the variation of each fatty acid within the same category, so categories with high variability were down-weighted. Uncentered correlation-based (1-Cosine distance) method assesses only the pattern of statin responsiveness across fatty acid categories (Fig. 5; see Methods for more detailed description). Rhesus DIO, cynomolgus, AGM, hamster on HFD, and db/db mouse models were closest to dyslipidemic humans in their responses to simvastatin treatment, followed by rhesus with metabolic syndrome, dog, marmoset, and diabetic rhesus. Those models showed close distances to dyslipidemic human in all three measures. \( LDLR^{-/-} \) mice on cholesterol diet, \( CETP^{+/-}/LDLR^{-/-} \) mice on chow or cholesterol diet, and \( ApoE^{-/-} \) mice on chow or cholesterol diet had large cosine-based distances from dyslipidemic humans, indicating that the patterns of their response in fatty acid composition of plasma CE, TG, and PC were very different from dyslipidemic humans (Fig. 5). In ZDF rat on HFD the magnitudes of the simvastatin effects were very different from dyslipidemic humans, shown by the large values of the Euclidean and variance-weighted distances. Simvastatin effects in rabbit on cholesterol diet were unlike dyslipidemic humans by all three distance measures (Fig. 5).

In summary, considering basal levels and composition of plasma lipids and their response to simvastatin treatment, the NHP models, including two AGM models, cynomolgus, and three rhesus models, were the species most similar to dyslipidemic humans. They were thus the best models among those tested for both studying disease mechanism and validation of potential drug targets for dyslipidemia. The exception among the NHPs was the marmoset. Marmosets were the only new world monkeys in our study, and they are much smaller in size than the
other NHPs. In addition, in our study marmosets exhibit extremely elevated cortisol levels (data not shown), which may account for the high variability seen across numerous endpoints (33). The dog displayed significant similarities to dyslipidemic humans despite being an HDL-dominated animal species, especially in response to statin treatment, supporting the use of the dog model in studies of many aspects of dyslipidemia, in particular LDL lowering. Data from the hamster on HFD and db/db mouse models suggested that they can be used as models for study of hypertriglyceridemia. Most of the commonly used dyslipidemic or atherosclerotic animal models, such as $ApoE^{-/-}$ mice on chow or cholesterol diet, $LDLr^{-/-}$ mice on cholesterol diet, rabbit on cholesterol diet, and ZDF rat on HFD, however, fell out of the range of dyslipidemic humans and demonstrated the least similarity of all models, raising concern about relating experimental data from those models to dyslipidemic humans.
Discussion

Lipid metabolism is a dynamic process that involves multiple pathways with both intracellular, and extracellular regulation and modulation. While the cross-talk and interactions among these pathways are essential to maintain lipid homeostasis, disregulation of each pathway can contribute substantially to the development of dyslipidemia. Management of dyslipidemia (with plasma LDL-c over 130 mg/dL and TC over 200 mg/dL) with first-line medications such as statins has been shown to decrease risk for CHD by epidemiological studies. Hypertriglyceridemia (with plasma TG over 200 mg/dL) is also linked to CHD risk, and management of non-HDL-c levels in those patients has been proposed as secondary prevention(34). Considering those risk factors as well as responsiveness of lipid profiles to treatment with statins, we compared plasma lipid profiles under basal and simvastatin-treated conditions for various animal models to dyslipidemic humans. Our goal was to identify the optimal model(s) for validating new targets for treatment of dyslipidemia in humans.

Suitable preclinical models for dyslipidemia with elevated plasma LDL-c

At baseline, the dyslipidemic human subjects studied here had borderline high plasma TC and LDL-c, and a balanced LDL-c to HDL-c ratio, which are representative of the majority of dyslipidemic patients with elevated CVD risk. Looking across the 24 non-diseased or diseased animal models in this study, the NHP models, especially the diseased NHP models such as dyslipidemic African Green monkey and three diseased rhesus models, were the most similar to humans with respect to basal plasma TC, ratio of LDL-c to HDL-c, and lipoprotein traces. Also similar to humans, in NHP models the majority of fatty acids within plasma CE were linoleic acid and its derivatives (the omega-6 pathway), and the vast majority of fatty acids in
TG were SFAs and MUFAs. Statin treatment lowered plasma TC and LDL-c levels in NHP models to a similar extent as in dyslipidemic humans, as well as decreasing fatty acids from all three categories (the non-essential fatty acids from DNL or diet, fatty acids from omega-3 pathway, and fatty acids from omega-6 pathway). By these parameters, NHP models are the best preclinical models for dyslipidemia for both mechanistic studies and target validation. This is not surprising since, phylogenetically, NHPs are the closest mammals to human. They also have similar omnivorous dietary preferences, as well as similar activity and substrate specificity for key plasma lipid enzymes, such as CETP and LCAT (lethicin-cholesterol acyltransferase)(13, 35). While CETP directly determines cholesterol content distribution within plasma lipoproteins by transferring CE from HDL to LDL, LCAT and ACATs (acyl-CoA: cholesterol acyltransferase) are responsible for the conversion of FC to CE. LCAT takes fatty acids from the sn-2 position of PC (which contains mostly poly-unsaturated fatty acids) and converts FC to CE while PC is converted to LPC in plasma during HDL biogenesis(36). ACATs add free fatty acids (MUFAs from the DNL pathway) from either exogenous dietary sources or endogenous lipogenesis to FC(37). While activation of LCAT increases athero-protective HDL-c levels, ACAT-derived CE is the predominant atherogenic lipid in blood(38).

The fact that the majority of fatty acids in plasma CE in human and NHPs are from the omega-6 pathway suggest that LCAT could be the major enzyme for CE biosynthesis in these species. The Gottingen mini-pig and obese beagle models in this study showed omega-6 pathway-enriched fatty acid composition in plasma CE, and non-essential fatty acids enriched fatty acid composition in plasma TG, similar to humans. However their lipoprotein profiles were distinctly different. In pigs and dogs, CETP activity either is very low or not detectable (39, 40). It has been shown that CETP expression can be upregulated by an atherogenic diet in humans.
and rabbits, whether the same might be true in pigs or dogs, or to what extent the increase contribute to the susceptibility to CVDs, remains to be studied(41-43). Previous studies showed that under an atherogenic diet a different pig strain, the Yucatan pig, can develop atherosclerotic plaque which is similar to human athero-lesions(44). Statin treatment was not examined in the pig model in our study, but has very little effect on plasma TC and LDL-c levels in Yutacan mini-pigs (45). Whether all mini-pigs or a specific mini-pig model are suitable for the study of dyslipidemia in humans requires further validation. Although the basal plasma lipid profile is not similar to dyslipidemic human, the dog model showed significant TC and LDL-c lowering upon statin treatment in our study, which was also seen in a previous report(46). Looking at fatty acid composition of CE in the dog model, statin treatment decreased fatty acids synthesized from all three categories (non-essential fatty acids, omega-3, and omega-6) with similar magnitude as in dyslipidemic humans (Fig. 4). Dog also demonstrated close similarity to dyslipidemic human in all three distance analyses looking at simvastatin effects on CE, PC and TG (Fig. 5). These data suggested that the dog is another useful model to examine the effect of LDL-c lowering agents for the treatment of hypercholesterolemia.

Rabbits on normal diet had very low plasma TC and LDL-c levels (~30 mg/dL and 8 mg/dL respectively), which is due to the very low amount of cholesterol in the normal rabbit diet and the high metabolic clearance rate(47). Upon cholesterol diet (0.5% cholesterol) challenge, there was a 27-fold increase in TC and 100-fold LDL-c increase, due to a lowered metabolic clearance rate(47). This was one of the most dramatic changes among all the models we analyzed. Unlike dyslipidemic humans, in cholesterol-fed rabbits over 75% of the fatty acids were SFAs and MUFAs, suggesting that CE biosynthesis might be via ACATs instead of
LCAT. Another major difference of rabbits from humans is the absence of hepatic lipase (HL), an enzyme that has both triglyceride lipase and phospholipid lipase activity(48). Under a cholesterol-fed condition, absence of HL in rabbit causes massive cholesterol accumulation in chylomicron remnant and β-VLDL lipoprotein particles(49).

Dramatic changes in plasma TC and LDL-c were also seen in cholesterol-fed ApoE<sup>-/-</sup>, LDLr<sup>-/-</sup>, and CETP<sup>+/−</sup>/LDLr<sup>-/-</sup> mice. Unlike the same models on chow-diet, which have the majority of fatty acids in plasma CE from the omega-6 pathway, the majority of fatty acids in CE in these cholesterol-fed models were from the non-essential fatty acids, consistent with the previous observation that expression of ACATs increases on a cholesterol diet(50).

Statin treatment did not decrease plasma LDL-c levels in any of the rabbit or rodent models. Although hamster on chow and on HFD had similar fatty acid compositions in plasma CE and TG as human, statin had no effect on LDL-c levels. The data suggest that these models are not suitable for target validation or drug discovery for dyslipidemia with elevated LDL-c.

Representative preclinical models for dyslipidemic populations with other risk factors such as elevated non-HDL cholesterol and TG

Recently, non-HDL cholesterol (including LDL-c and remnant lipoproteins such as VLDL-c and IDL-c) has been proposed to be a better estimate of total atherogenic burden than LDL-c, especially in patients with elevated plasma triglycerides between 200 and 500 mg/dL(26, 34). Elevated plasma TG levels are also often associated with low HDL-c levels. Although highly variable due to dietary status and often associated with other complications such as type 2 diabetes and metabolic syndrome, elevated TG has been considered another independent risk factor for CHD by a number of studies(34). Among the animal models in our study that had
similar omega-6 pathway-enriched fatty acid compositions as humans in plasma CE, many showed elevated VLDL-c and/or TG levels, including several NHPs models (dyslipidemic African Green, rhesus with metabolic syndrome, diabetic rhesus, and DIO rhesus), dog, hamster on HFD, and db/db mouse (Table 1)(51, 52). Statin treatment lowered VLDL-c and/or TG levels in all these models (Table 2), suggesting that they are also useful as models for studying plasma remnant lipoprotein and TG metabolism and secondary risk prevention for dyslipidemic humans. Marmosets being the exception showed no decrease in TG upon statin treatment and are a suboptimal model due to their sensitivity to stress (data not shown). ZDF rats on HFD also had elevated TG and demonstrated VLDL-c and TG-lowering upon statin treatment. However their plasma baseline TG level was abnormally high (1482±210 mg/dL), and the majority of cholesterol resided in less atherogenic larger VLDL and chylomicron particles. Thus the lowering of non-HDL levels is less reliable as an indicator of lowered CHD risk(26).

Other than statin, which in several clinical trials have confirmed the benefit in patients with atherogenic dyslipidemia(26), fibrates also demonstrated beneficial effects. For example, fenofibric acid alone or in combination with statins has been shown to improve plasma TG, HDL-c, and LDL-c in dyslipidemic humans(53). In other species, fibrates were shown to be effective in lowering plasma TG and cholesterol levels in pig and dog, as well as in species for which statins are not effective and poorly tolerated, such as hamster, rat, and mouse models(54-56). Other agents for treatment of atherogenic dyslipidemia include nicotinic acid (niacin) alone or in combination with statins, CETP inhibitors, and long-chain omega-3 polyunsaturated fatty acids(57, 58). In future studies it might be useful to evaluate the effects one or more of these agents alone or in combination with statins, to provide more information
for better assessment of the suitability of different models for target validation in human dyslipidemia.

In the study of atherosclerosis and plaque progression, the most widely used preclinical models are rabbit, ApoE−/− mice, and LDLr−/− mice on cholesterol diet, due to their ability to quickly form plaques. Although not studied here, it has been shown that the histology of lesion development in mouse models is similar to human; however, the major limitation of mouse models is that the most common complications of plaque rupture and superimposed thrombosis (and subsequent acute myocardial infarction and ischemic stroke) rarely occur in mouse models (59). Recently, NHPs, pigs, dogs, and hamsters on atherogenic diets have been used as models of human atherosclerosis (60-62). The hamster model is questioned since no consistent lesion development has been observed in several hamster strains (63). Larger animals, such as dog, pig, and NHPs are more attractive due to the ability to apply interventional procedures and imaging on the larger vessels, making them more suitable as models for clinical practice (59).

Future analysis of lipid composition in plaques before and after statin treatment will be helpful to further understand the applicability of those animal models to dyslipidemic humans.

Given the role of phospholipids as precursors of eicosanoid synthesis, their fatty acid composition is of clear interest to model selection for cardiovascular disease research. Fig. 2D shows the relative abundance and fatty acid composition of plasma PCs. There is a marked degree of similarity in the latter between the animal species. The ratio of saturated to unsaturated fatty acid in this particular lipid fraction is carefully balanced by de novo synthesis and strongly influenced by remodeling via hydrolysis and reacylation (the Lands cycle). The result is a relatively well controlled composition in which a saturated fatty acid occupies the sn1 position and an unsaturated fatty acid occupies the sn2 position (64, 65). The acyl chain
specificities for some of the enzymes involved in remodeling of phospholipids have been investigated in several animal species, including rat and pig, and in many cases were found to have similar preferences for n6 and n3 fatty acids (66). Given this, the similarity in PC composition between animal species reported here is not surprising. Further complexities involving dietary PCs and their bacterial breakdown products, and correlation with cardiovascular disease risk in humans and in mice, suggest that more detailed studies of dietary PC and the plasma sequelae in models of interest are warranted.

Inflammation is involved in all phases of atherosclerosis. As a sensitive marker for inflammation, CRP has been proposed as a predictor of cardiovascular risk. The recently completed JUPITER trial found that rosuvastatin reduces vascular events in low-risk older subjects with normal LDL-c levels but elevated high-sensitivity C-reactive protein (hs-CRP), supporting the inclusion of hs-CRP levels among cardiovascular risk factors (67). However, due to methodology limitations on measuring CRP levels in non-human, non-rodent species, the level of CRP failed to improve our understanding of applicability of animal species. The evaluation of CRP as well as other inflammatory markers, and the effect of statins, will be discussed in detail in subsequent manuscripts focusing on specific animal models.

Taken together, our findings indicated that among the animal models we considered, NHPs are in general the best for both mechanistic studies and for target validation of primary and secondary pharmacological treatments for dyslipidemia in humans. The dog model can also be considered for developing pharmacological agents to lower LDL-c. For validation of targets related to secondary CVD risks such as high non-HDL cholesterol and high TG, dog, hamster on HFD, and db/db mice can also be considered. Further profiling of the lipid composition of lipoprotein fractions, plaque, and activated macrophages, as well as responsiveness to
additional pharmacological agents targeting dyslipidemia, such as fibrates, niacin, CETP inhibitors, and fish oil, will provide more insights for understanding the applicability of various animal models. Clearly, choice of an animal model requires careful consideration of the specific target or pathway of interest, since each model can have common characteristics with humans and be uniquely useful for addressing questions in dyslipidemia research. The information gathered as more mechanisms and animal models are evaluated out will allow the circular translation from the bench to the clinic and back.
References:


Figure Legends

**Fig. 1** Representative FPLC traces of plasma lipoprotein cholesterol levels of dyslipidemic human and various animal models. Three peaks representing VLDL, LDL, and HDL are labeled in each panel. Except the four panels in the bottom, The Y-axis in each panel was adjusted to the same level for easier comparison.

**Fig. 2** Fatty acid composition in plasma CEs, TGs, and PCs across animal species. A) Schematic showing fatty acids within three categories: non-essential fatty acids (blue), omega-3 (purple) pathway fatty acids, and omega-6 (yellow) pathway fatty acids. Highlighted fatty acids were measured in this study. B) Measured fatty acids were summed for each pathway. Left panel shows the absolute amounts (nmol) of fatty acids in each pathway and right panel shows the relative amounts (%) for plasma CEs, C) TGs, and D) PCs.

**Fig. 3** Dendrogram comparison of baseline plasma lipid similarity based on 8 major circulating lipid fractions (CE, TG, DAG, PE, FFA, LPC, PC and FC) across species. The difference of any given lipid fraction between the means of each animal model and dyslipidemic human was calculated and weighted according to the proportion the same lipid fraction over total lipid in humans. The overall weighted distance of each animal model from dyslipidemic humans was calculated, and the models were sorted by distance (see Methods for a more detailed description).

**Fig. 4** Simvastatin effect on fatty acid composition in plasma CEs, TGs, and PCs in dyslipidemic humans and various animal models. Fatty acids were summed into four categories: SFA, MUFA, omega-3, and omega-6. The effect of simvastatin treatment on each category was estimated, and is shown as a log_{10} ratio with 95% confidence interval. Statistically significant effects (p < 0.05) are highlighted in red.
Fig. 5 Distance comparison from various animal models to dyslipidemic humans with respect to the responsiveness to simvastatin treatment on plasma CE, PC and TG. Three distance measures each represents a different aspect of the difference on the comparison are shown: Euclidean (purple solid circle), a distance calculation based on the log ratios from Fig. 4, which represents the similarity based on the magnitude of responsiveness; variance-weighted Euclidean (green solid triangle), which represents the also Euclidean comparison, but weighted the contribution of variability of the responses, so the fatty acid categories with high variability were down-weighted; uncentered-correlation-based distance (1-Cosine distance, red solid square), which represents the similarity based on pattern of statin responsiveness.
Table 1. Basal plasma lipid comparison across species

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<th>TG</th>
<th>TC</th>
<th>VLDL</th>
<th>LDL-c</th>
<th>HDL-c</th>
<th>CRP (ug/ml)</th>
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<td>71±3</td>
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<td>194±18</td>
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<td>308±16</td>
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<td>10±2</td>
<td>41±2</td>
<td>37±4</td>
<td>6±0.2^{C}</td>
</tr>
<tr>
<td>CETP^{-}/LDLr^{-} (Chol)</td>
<td>10</td>
<td>Yes</td>
<td>59±6</td>
<td>202±4</td>
<td>51±4</td>
<td>96±4</td>
<td>54±3</td>
<td>7±0.3^{C}</td>
</tr>
</tbody>
</table>

A: measured with human kit by RBM as described in Methods
B: measured with BioAnalytical method as described in Methods
C: Measured with rodent kit by RBM as described in Methods
Table 2. Comparison of changes on plasma lipids upon simvastatin treatment across the species

<table>
<thead>
<tr>
<th>Species</th>
<th>Statin responsiveness</th>
<th>TC</th>
<th>VLDL-c</th>
<th>LDL-c</th>
<th>HDL-c</th>
<th>TG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dyslipidemic human</td>
<td>Yes</td>
<td>-16%</td>
<td>**</td>
<td>-24%</td>
<td>**</td>
<td>1%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>NA^{A}</td>
<td></td>
<td></td>
<td>NS^{B}</td>
</tr>
<tr>
<td>Dyslipidemia African green</td>
<td>Yes</td>
<td>-67%</td>
<td>**</td>
<td>-57%</td>
<td>**</td>
<td>-69%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>-24%</td>
<td>**</td>
<td></td>
<td>1%</td>
</tr>
<tr>
<td>African Green</td>
<td>Yes</td>
<td>-36%</td>
<td>***</td>
<td>-36%</td>
<td>NS</td>
<td>-51%</td>
</tr>
<tr>
<td>Cynomolgus</td>
<td>Yes</td>
<td>-25%</td>
<td>**</td>
<td>-55%</td>
<td>NS</td>
<td>-22%</td>
</tr>
<tr>
<td>Rhesus, Met syn</td>
<td>Yes</td>
<td>-16%</td>
<td>NS</td>
<td>3%</td>
<td>NS</td>
<td>-22%</td>
</tr>
<tr>
<td>Rhesus, diabetic</td>
<td>Yes</td>
<td>-24%</td>
<td>**</td>
<td>-33%</td>
<td>NS</td>
<td>-23%</td>
</tr>
<tr>
<td>Rhesus DIO</td>
<td>Yes</td>
<td>-15%</td>
<td>NS</td>
<td>-24%</td>
<td>NS</td>
<td>-26%</td>
</tr>
<tr>
<td>Marmoset</td>
<td>Yes</td>
<td>-29%</td>
<td>*</td>
<td>-66%</td>
<td>NS</td>
<td>-25%</td>
</tr>
<tr>
<td>Dog</td>
<td>Yes</td>
<td>-34%</td>
<td>**</td>
<td>-43%</td>
<td>NS</td>
<td>-80%</td>
</tr>
<tr>
<td>Rabbit (Chol)</td>
<td>No</td>
<td>-46%</td>
<td>**</td>
<td>-76%</td>
<td>**</td>
<td>-7%</td>
</tr>
<tr>
<td>Hamster HFD</td>
<td>No</td>
<td>-8%</td>
<td>*</td>
<td>-33%</td>
<td>NS</td>
<td>-7%</td>
</tr>
<tr>
<td>ZDF rat HFD</td>
<td>No</td>
<td>-28%</td>
<td>**</td>
<td>-51%</td>
<td>**</td>
<td>112%</td>
</tr>
<tr>
<td>db/db</td>
<td>No</td>
<td>-4%</td>
<td>NS</td>
<td>-4%</td>
<td>NS</td>
<td>19%</td>
</tr>
<tr>
<td>ApoE^{+/-}</td>
<td>No</td>
<td>59%</td>
<td>**</td>
<td>89%</td>
<td>**</td>
<td>25%</td>
</tr>
<tr>
<td>LDLr^{+/-} (Chol)</td>
<td>No</td>
<td>14%</td>
<td>NS</td>
<td>19%</td>
<td>NS</td>
<td>7%</td>
</tr>
<tr>
<td>CETP^{+/-}/LDLr^{-/-}</td>
<td>No</td>
<td>13%</td>
<td>NS</td>
<td>25%</td>
<td>NS</td>
<td>53%</td>
</tr>
<tr>
<td>CETP^{+/-}/LDLr^{-/-}(Chol)</td>
<td>No</td>
<td>7%</td>
<td>NS</td>
<td>20%</td>
<td>NS</td>
<td>12%</td>
</tr>
</tbody>
</table>

*: p<0.05; ** p<0.01, *** p<0.001 by paired or unpaired t-test as indicated in Methods
A: not available
B: not significant
**Fig. 1** Representative FPLC traces of lipoprotein cholesterol levels of different animal models in comparison with dyslipidemic human.

<table>
<thead>
<tr>
<th>Model</th>
<th>Cholesterol Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dyslipemia human</td>
<td></td>
</tr>
<tr>
<td>African Green</td>
<td></td>
</tr>
<tr>
<td>Cynomolgus</td>
<td></td>
</tr>
<tr>
<td>Rhesus</td>
<td></td>
</tr>
<tr>
<td>Rhesus, Met Syn</td>
<td></td>
</tr>
<tr>
<td>Rhesus, diabetic</td>
<td></td>
</tr>
<tr>
<td>Rhesus DIO</td>
<td></td>
</tr>
<tr>
<td>Marmoset</td>
<td></td>
</tr>
<tr>
<td>Pig</td>
<td></td>
</tr>
<tr>
<td>Dog</td>
<td></td>
</tr>
<tr>
<td>Hamster</td>
<td></td>
</tr>
<tr>
<td>Hamster HFD</td>
<td></td>
</tr>
<tr>
<td>ZDF/+ rat</td>
<td></td>
</tr>
<tr>
<td>ZDF rat HFD</td>
<td></td>
</tr>
<tr>
<td>C57BL/6 mice</td>
<td></td>
</tr>
<tr>
<td>db/db mice</td>
<td></td>
</tr>
<tr>
<td>Rabbit</td>
<td></td>
</tr>
<tr>
<td>LDLr-/- mice</td>
<td></td>
</tr>
<tr>
<td>CETP+/-/LDLr-/- mice</td>
<td></td>
</tr>
<tr>
<td>CETP+/-/LDLr-/- mice (chol)</td>
<td></td>
</tr>
<tr>
<td>Rabbit (chol)</td>
<td></td>
</tr>
<tr>
<td>LDLr-/- mice (chol)</td>
<td></td>
</tr>
<tr>
<td>ApoE-/- mice</td>
<td></td>
</tr>
<tr>
<td>ApoE-/- mice (chol)</td>
<td></td>
</tr>
</tbody>
</table>

mAU vs. Time (Min)
Fig. 2

A). Biosynthetic pathways for circulating lipids

Non-essential fatty acids

Linolenic acid (omega 3) pathway

Linoleic acid (omega 6) pathway

B). FA Composition in CE (nmol)

C). FA Composition in TG (nmol)

D). FA Composition in PC (nmol)
Fig. 3 Dendrogram comparison of baseline plasma lipid similarity based on 8 major circulating lipid fractions across species.
Fig. 4

A graphic showing the simva effect (log ratio with 95% CI) for various conditions and species.

Species/Conditions:
- Dyslipidemic human
- African Green
- Cynomolagus
- Rhesus, Met Syn
- Rhesus, diabetic
- Rhesus DIO
- Marmoset
- Dog
- Rabbit (chol)
- Hamster HFD
- ZDF rat HFD
- C57BL/6 mice
- db/db mice
- ApoE KO mice
- ApoE KO (chol)
- Ldr KO mice (chol)
- CETPtg/ldr KO mice
- CETP tg/ldr KO mice (chol)

Effects with unadjusted p < 0.05 in red.
Fig. 5 Distance comparison of various animal models to dyslipidemic human with respect to the responsiveness to statin treatment on plasma CE, PC and TG.