Bempedoic acid lowers low-density lipoprotein cholesterol and attenuates atherosclerosis in low density lipoprotein receptor-deficient (LDLR\textsuperscript{+/−} and LDLR\textsuperscript{−/−}) Yucatan miniature pigs.

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**Objective**—Bempedoic acid (ETC-1002) is a novel drug that targets hepatic adenosine triphosphate-citrate lyase to reduce cholesterol biosynthesis. In Phase 2 studies, bempedoic acid lowers elevated LDL-cholesterol (C) in hypercholesterolemic patients. In the present study, we tested the ability of bempedoic acid to decrease plasma-C and LDL-C, and attenuate atherosclerosis in a large animal model of familial hypercholesterolemia.

**Approach and Results**—Gene targeting has been used to generate Yucatan miniature pigs heterozygous (LDLR+/−) or homozygous (LDLR−/−) for LDL-receptor (R) deficiency (ExeGen). LDLR+/− and LDLR−/− pigs were fed a high-fat, cholesterol-containing diet (34% kcal fat; 0.2% cholesterol) and orally administered placebo or bempedoic acid for 160 days. In LDLR+/− pigs, compared to placebo, bempedoic acid decreased plasma-C and LDL-C up to 40% and 61%, respectively. In LDLR−/− pigs, in which plasma-C and LDL-C were 5-fold higher than in LDLR+/− pigs, bempedoic acid decreased plasma-C and LDL-C up to 27% and 29%, respectively. Plasma levels of triglycerides and HDL-C, fasting glucose and insulin, and liver lipids were unaffected by treatment in either genotype. In the aorta of LDLR+/− pigs, bempedoic acid robustly attenuated en face raised lesion area (~58%) and left anterior descending coronary artery (LAD) cross-sectional lesion area (~40%). In LDLR−/− pigs, in which lesions were substantially more advanced, bempedoic acid decreased aortic lesion area (~47%) and LAD lesion area (~48%).

**Conclusions**—In a large animal model of LDLR-deficiency and atherosclerosis, longer-term treatment with bempedoic acid reduces LDL-C and attenuates the development of aortic and coronary atherosclerosis in both LDLR+/− and LDLR−/− miniature pigs.
Non-standard abbreviations and acronyms

ACLY  ATP-citrate lyase

AMPK  AMP-activated protein kinase

BemA  bempedoic acid

HMG-CoA  3-hydroxy-3-methyl-glutaryl-coenzyme A

Srebf  sterol regulatory element binding factor

LAD  left anterior descending coronary artery
**Introduction**

Elevated plasma levels of LDL cholesterol (LDL-C), non-HDL cholesterol (non-HDL-C), or apolipoprotein B (ApoB) are independent risk factors for cardiovascular disease (CVD).\(^1,2\) Inhibitors of hydroxyl methyl glutaryl coenzyme A (HMG-CoA) reductase, or statins, have successfully prevented CVD events through reductions in LDL-C, consistent with the important role of LDL-C lowering in risk reduction.\(^3\) Despite the effectiveness of statins, new treatment strategies for further risk reduction are required. Some patients, such as those with familial hypercholesterolemia, fail to achieve LDL-C targets with statins alone, and others are either statin-intolerant or tolerate only sub-optimal low doses.\(^4-6\)

Bempedoic acid (BemA, ESP-55016, ETC-1002) was originally identified from a series of long hydrocarbon chain diacids for its ability to inhibit fatty acid and cholesterol synthesis *in vitro* and *in vivo*.\(^7,8\) In rodent models, BemA prevented diet-induced elevations in plasma cholesterol and triglycerides, hepatic steatosis, insulin resistance and obesity.\(^9-11\) Recent studies have shown that BemA-CoA is a direct and potent competitive inhibitor of ATP-citrate lyase (ACLY), consistent with increased hepatic citrate concentrations and decreased concentrations of acetyl-CoA, malonyl-CoA, and HMG-CoA in murine liver.\(^9\) Additionally, the CoA-derivative of BemA was required for activity; a reaction catalyzed by the liver-specific enzyme very long-chain acyl-CoA synthetase-1.\(^11\) Early studies reported that BemA also targeted AMP-activated protein kinase (AMPK), a kinase that regulates whole body energy metabolism and inhibits fatty acid and cholesterol synthesis.\(^9\) Subsequent studies revealed that AMPK activation by BemA is rodent-specific, and likely does not occur in humans.\(^11\) Furthermore, in *Apoe*\(^{-/-}\) mice deficient in hepatic AMPK, BemA treatment decreased plasma LDL-C and improved other parameters of lipid metabolism to the same extent as in *Apoe*\(^{-/-}\) mice with functional hepatic AMPK,\(^11\) implying that inhibition of ACLY is the primary mechanism for lipid regulation by BemA.

Studies have linked inhibition of cholesterol synthesis by BemA to LDL-receptor (LDLR) upregulation in primary human hepatocytes and livers of *Apoe*\(^{-/-}\) mice,\(^11\) indicating LDLR-mediated lipoprotein clearance as a mechanism for plasma LDL-C reductions. In short-term phase 2 human studies (12 weeks), BemA treatment reduced plasma LDL-C 21-43% in patients with hypercholesterolemia (reviewed in\(^12\)). Furthermore, BemA significantly reduced LDL-C when given in combination with ezetimibe or statins, and in statin-intolerant hypercholesterolemic patients. Parameters of triglyceride or carbohydrate metabolism were unaffected in these human studies. Longer-term BemA efficacy, safety, and CVD outcome trials are ongoing.

The ability of BemA to attenuate atherosclerosis was recently evaluated in high-fat fed *Apoe*\(^{-/-}\) and *Ldlr*\(^{-/-}\) mice treated for 12 weeks. In *Apoe*\(^{-/-}\) mice, BemA reduced plasma cholesterol (-18%) and modestly attenuated aortic sinus lesions (-21%).\(^11\) In *Ldlr*\(^{-/-}\) mice, BemA-treatment attenuated diet-induced hypercholesterolemia (-41%), hypertriglyceridemia (-52%), hyperglycemia, hyperinsulinemia, hepatic steatosis, and adiposity. BemA decreased aortic sinus atherosclerosis by 44%, with beneficial changes in plaque morphology.\(^10\) Prevention of atherosclerosis in mice by BemA is promising; however, atheroprotection in mouse models using other therapeutic interventions have not necessarily translated to humans.\(^13\) The pig represents a large animal model of human-like lipoprotein metabolism and responds to statins and other cholesterol-lowering medications.\(^14,15\) Recently, pig models of familial hypercholesterolemia have been created in which deletion of the *LDLR* or overexpression of the human gain-of-function PCSK9 D374Y mutation in Yucatan miniature pigs results in hypercholesterolemia and development of human-like atherosclerotic lesions in the aorta and coronary arteries.\(^16,17\) The objective of the present study was to examine the impact of longer-term BemA treatment on LDL-C and atherosclerosis in LDLR-deficient Yucatan miniature pigs. The efficacy of BemA in *Ldlr*\(^{-/-}\) mice\(^10\) prompted us to include homozygous as well as
heterozygous LDLR-deficient pigs. Pigs readily developed diet-induced hypercholesterolemia and atherosclerosis without high levels of dietary cholesterol or cholate. Treatment of both LDLR+/− and LDLR−/− pigs for 160 days with BemA significantly decreased LDL-C and attenuated the development of aortic and coronary atherosclerosis.

Materials and Methods

Animals and diets
Male and female Yucatan miniature pigs with targeted disruption of the LDL receptor (LDLR) gene were obtained from Exemplar Genetics (Sioux City, Iowa).16 Twelve heterozygotes (LDLR+/−) and 12 homozygotes (LDLR−/−) were used in this study. In one experiment, liver samples from 2 wildtype pigs (LDLR+/+) were used. Pigs with initial body weights of 24-28 kg were housed individually in metabolic cages and were initially fed a pig chow diet (Prolab Mini-Pig Diet 5P94, PMI Nutrition, Brentwood MO) for 2 weeks. The chow contained 10.5% of kcal from fat, 22.1% kcal from protein and 67.3% kcal from carbohydrate. All pigs were subsequently transferred to a chow-based, fat and cholesterol containing diet (600 g/d) for 174 days. The high fat, high cholesterol (HFHC) diet provided 16%, 50% and 34% of calories from protein, carbohydrate and fat, respectively and 1.2 g/d of cholesterol (0.2% of diet, 0.4 mg/kcal). The HFHC diet mimics a standard western-type human diet. The fat ratio of 1:1:1 (Polyunsaturated:Monounsaturated:Saturated) was achieved by mixing lard, butter and safflower oil.15 Body weights were measured on days 0, 28, 49, 91 and 160 of the lipid and lipoprotein study. Blood samples were obtained through surgically implanted jugular vein catheters in conscious, unrestrained animals18 during the first 4 weeks. Subsequent samples were obtained by cranial vena cava venipuncture following anesthetization of pigs with a single intramuscular injection of tiletamine, zolazepam, zylazline and ketamine (1.6 mg/kg of each). Pigs were fasted for 20-22 hours prior to blood collection. The protocol was approved by the Animal Care Committee of the University of Western Ontario (Protocol #2013-071).

Administration of drugs and study protocol
Bempedoic acid (ETC-1002) was obtained from Esperion Therapeutics (Plymouth, MI) in powder form. Based on initial pharmacokinetic data in pigs and the assumption that bioavailability would be similar to that found in humans, doses of 120 mg/d and 240 mg/d were used. In humans, this dose range results in optimal LDL-C responses in Phase 2 studies (reviewed in12). Oral doses of compound were placed in gelatin capsules and fed by hand, just prior to daily feeding. A pharmacokinetic study was performed after 14 days of the HFHC diet. LDLR+/− (n=8) and LDLR−/− (n=8) pigs were administered a single dose of bempedoic acid (120 mg; or 240 mg). On day 15 of the HFHC diet (study day 0), 12 LDLR+/− pigs were administered either bempedoic acid (n=4 at 120 mg/d; n=4 at 240 mg/d) or placebo (n=4) and 12 LDLR−/− pigs were administered either bempedoic acid (n=4 at 120 mg/d; n=4 at 240 mg/d) or placebo (n=4) for 160 days. Fasting blood samples were obtained weekly from day 0 to day 28 and monthly from day 56 to day 160. Animals were sacrificed on day 160. The protocol is summarized in Figure 1A.

A total of 15 barrows (castrated male); 7 LDLR+/− and 8 LDLR−/− and a total of 9 gilts (female – not bred); 5 LDLR+/− and 4 LDLR−/− were used in the study. Pigs were randomized to drug treatment groups by age and starting body weight. Littermates, males and females were distributed equally among placebo and drug treatment groups. There were no obvious differences between males and females for any of the study parameters examined and thus data was not sub-grouped by gender.

Adverse events
Two pigs died during the study. One pig, an LDLR\(^{+/ -}\) animal at the 240 mg/d dose, presented with significant hind-limb paralysis on day 80 and had to be euthanized. Autopsy revealed a large ventricular thrombus and bacterial endocarditis due to the prolonged catheter use. This was deemed not to be drug related. The second, an LDLR\(^{-/-}\) placebo pig died suddenly on day 120 due to a complication of blood sampling from the cranial vena cava. The data from these two animals were not used for long-term lipid analyses (after day 77 and after day 120, respectively), liver analyses or atherosclerosis studies.

**Pharmacokinetic study**

A pharmacokinetic study was performed on day 14 in HFHC-fed pigs. On the day of study, fasted pigs (22 h) were administered a dose of bempedoic acid orally by hand, along with a small amount of diet. The drug was ingested within 30 sec and the normal daily feed was then provided. Blood samples were obtained through the indwelling catheter at time 0, 0.5, 1, 2, 4, 6, 12, and 24 h, into tubes containing EDTA.Na\(_2\). Blood samples were kept on ice, immediately centrifuged (1500 rpm, 30 min. at 4\(^\circ\)C) to obtain plasma and samples were stored at -80\(^\circ\)C until analysis. Plasma concentrations of bempedoic acid (ETC-1002) and its main metabolite, ESP15228, and their respective deuterated (D4) standards (provided by Esperion) were measured by liquid chromatography-tandem mass spectrometry as described previously.10

**Analytical assays**

Plasma and lipoprotein lipid analyses have been described previously.14, 15, 18 Lipoprotein separations were performed by ultracentrifugation of fresh plasma and samples were subsequently stored at -80\(^\circ\)C for lipid analysis. Plasma and lipoprotein lipids were measured on a Cobas Mira S Autoanalyzer (Roche Diagnostics, Montreal, QC, Canada) using enzymatic reagents as described previously.10 Precision testing was provided by OneWorld Accuracy (Burnaby, BC, Canada). Lipoprotein cholesterol distributions were evaluated in fresh plasma samples (50 μL) from 3 placebo, 3 bempedoic acid 120 mg/d and 3 bempedoic acid 240 mg/d pigs of each genotype by fast protein liquid chromatography (FPLC) and fractions analyzed for total cholesterol as described previously.10 Plasma lathosterol concentrations were assayed by liquid chromatography-tandem mass spectrometry as described previously.19 Plasma glucose was measured enzymatically using a Cobas Mira S Autoanalyzer with enzymatic reagents from Roche Diagnostics. Plasma insulin concentrations were measured using a pig-specific ELISA (Alpco, Salem, NH, # 80-INSPO-E01). Plasma levels of uric acid, total bilirubin, creatinine, creatine kinase (CK) and alanine transaminase (ALT) were performed using standard assays by the London Laboratory Services Group, Clinical Laboratories, Department of Pathology and Laboratory Medicine, London Health Sciences Centre, London, ON.

**Animal sacrifice and tissue analyses**

Approximately 22 hours after the last dose of drug on day 160, pigs were sacrificed. Pigs were pre-anesthetized with an intramuscular injection of tiletamine, zolazepam, zylazine and ketamine (1.6 mg/kg of each). Sedated pigs were transported to the large animal surgical suite and intubated. Isoflurane was administered at a maintenance dose of 1.5% for the duration of tissue harvesting. Blood was drawn from the cranial vena cava and put into tubes containing EDTA.Na\(_2\) on ice. Plasma was isolated for lipid and lipoprotein analyses as described above. Liver was excised and samples were flash frozen in liquid nitrogen and stored at -80\(^\circ\)C. The heart and aorta (to the aortic bifurcation) were dissected from the surrounding tissue, rinsed in 1X phosphate buffered saline and immersion fixed in 4% paraformaldehyde for histological examination. The paraformaldehyde was replaced at 24 h and 72 h post-sacrifice.

In liver, the concentrations of triglyceride (TG), total cholesterol (TC) and free cholesterol (FC) were measured using enzyme-based assays (Triglyceride with glycerol blank: Trig/GB
Roche/Hitachi # 11877771 216; Cholesterol: WAKO #439-17501; and Free cholesterol WAKO #435-35801) in samples from lipid extracts, containing radioactive recovery standards, that were re-solubilized with triton, as previously described. 10 Cholesteryl ester (CE) was calculated by difference.

**Tissue mRNA determination**

RNA was isolated from aliquots of liver using Trizol reagent (Life Technologies, Mississauga, Ontario, Canada). Gene-specific mRNA quantitation for *APOB, HMGCR, LDLR*, sterol response element binding factor (*SREBF2, SREBF1c*), proprotein convertase subtilisin/kexin type 9 (*PCSK9*), cytochrome P450 7A (*CYP7A*), microsomal triglyceride transfer protein (*MTTP*), ATP binding cassette A1 (*ABCA1*), Niemann-Pick C1-like 1 (*NPC1L1*), solute carrier 27A (*SLC27A*) and glyceraldehyde-3 phosphate dehydrogenase (*GAPDH*) was performed by quantitative real-time PCR (qRT-PCR) on an ABI ViiA 7 Sequence Detection System (Applied Biosystems) as described previously. 10 Expression levels for each gene were normalized to *GAPDH* expression levels. For genes with known pig (*S. scrofa*) sequences, (*APOB, HMGCR, SREBF2, CYP7A, MTTP, ABCA1, SLC27A and GAPDH*), primer and probe sets were designed using Primer Express 2.0 software (Applied Biosystems). 15 For *LDLR* and genes with unknown pig sequences, (*SREBF1c, NPC1L1 and PCSK9*), PCR primers were designed against the mRNA sequence of the human, mouse and rat homologues, if known, using PrimerQuest software (Integrated DNA technologies) as described previously. 15 Using these primers, a pig PCR product was sequenced and used to design pig-specific primer probe sets using Primer Express 2.0 as described. 15 All primers were obtained from Sigma-Genosys, and the fluorogenic probes 5’-labeled with 6-carboxyfluorescein (FAM) and 3’-labeled with a minor groove binder (MGB) were obtained from Applied Biosystems. Primers and probes for qRT-PCR analyses are listed in Table I (online-only Supplement).

**Immunoblotting**

The preparation of total tissue lysates from snap-frozen liver, separation of proteins by SDS-PAGE (7.5% polyacrylamide gels) and transfer to PVDF membranes were performed as previously described. 10 Membranes were probed using antibodies against LDLR (2 ng/mL) (BioVision, Inc., Milpitas, CA, USA) and alpha tubulin (0.1 ng/mL) (Abcam, Inc., Cambridge, UK) overnight at 4ºC. Secondary antibodies (LI-COR, Lincoln, NE, USA) were raised against the IgG of the host species of the primary antibodies, tagged with an infrared fluorophore signaling at either 680 or 800nm. Imaging and quantitation of immunoblots were performed using the LI-COR Odyssey Fc (LI-COR Biosciences) and LI-COR Image Studio Software 5.0.

**Histology**

The aortae, stored in 4% paraformaldehyde, were rinsed in 1X phosphate buffered saline. Each aorta was opened longitudinally, immersed in 70% ethanol (10 sec) and immersed in Sudan IV stain (Sigma-Aldrich; 5 g/L in ethanol:acetone:water, 1:1:1, 15 min) with agitation. Samples were de-stained (15 min) with agitation in 80% ethanol. Aortae were splayed and pinned flat on a foam board. Images were obtained with a Canon Rebel digital camera and digital images downloaded to a computer. Total stained area (all Sudan IV-stained lesion area including raised lesions) in the full length aorta and raised lesion area in the abdominal aorta (from the aortic hiatus to the aortic bifurcation) were identified visually, traced manually and the areas calculated using Axiovision software. 20 The percent of total aortic surface area with stained lesions (or raised lesions) were calculated.

A cross section of the abdominal aorta (1 cm), perpendicular to the direction of blood flow, was obtained from the *en face* preparation, just proximal to the aortic bifurcation, and embedded in
paraffin. Sections (5 µm) were prepared from the distal edge of each segment. A section was stained with hematoxylin and eosin, and an adjacent section was stained with Movat's Pentachrome. Cross-sections were examined by light microscopy to visualize lesion details. A digital image of each section was downloaded to quantify intimal area (including lesions) using Axiovision software.

The left coronary artery and the left anterior descending coronary artery (LAD) were dissected from the paraformaldehyde-fixed heart. Cross sections of the LAD (1 cm) were obtained 1 cm and 2 cm distal to the left circumflex bifurcation, and paraffin-embedded. Sections (5 µm) were prepared from the proximal edge of each segment. A section was stained with hematoxylin and eosin, and adjacent sections were stained with Movat's Pentachrome. LAD sections were also immunostained for CD68 as previously described. Sections from the spleen of a pig from a local abattoir were used as a positive control. Peroxidase activity was blocked by incubating sections (5 min) with Peroxidase block (Dako Envision+ System-HRP (DAB), K4006, Agilent, Santa Clara, CA) followed by blocking for non-specific protein binding using Protein Block Serum-Free (X0909, Agilent). Primary antibody against pig CD68 (BioRad, MCA2317GA, clone BA4D5 (1mg/mL)) was diluted 1:1000 and applied overnight at 4°C. Sections were incubated with secondary antibody (Dako Envision+ System-HRP (DAB), K4006, Agilent) for 30 minutes at room temperature followed by DAB+ (Dako Envision+ System-HRP (DAB), K4006, Agilent) for 7 minutes. Sections were counterstained with hematoxylin (Sigma-Aldrich).

All LAD sections were examined by light microscopy to visualize lesion details. A digital image of each section was used to quantify intimal area (including lesions) and medial area, using Axiovision software. Each aortic and LAD Pentachrome-stained section was scored according to the classification of Virmani et al. Classification of sections were: no lesion, intimal thickening, intimal xanthoma, pathological intimal thickening and fibrous cap atheroma. The largest, most advanced plaque type in each section was recorded for between-group comparisons.

### Statistical analyses

For the comparison of three or more groups, data was initially tested for normal distribution using the Shapiro-Wilk test and equal variance by F-test. If the data was normally distributed, a One-Way ANOVA was performed, followed by a Tukey’s post-hoc test. P<0.05 was considered significant. For data evaluated over time, body weight and % change LDL-cholesterol, a Repeated Measures ANOVA, followed by a Tukey’s post-hoc test was performed. If the data was not normally distributed, the non-parametric Kruskal-Wallis One-Way ANOVA on Ranks, was performed, followed by a Tukey’s post-hoc test. P<0.05 was considered significant.

For the comparison of two groups, data was initially tested for normal distribution using the Shapiro-Wilk Test and equal variance by F-test. If the data was normally distributed, a Student’s t-test was performed. P<0.05 was considered significant. For data not normally distributed, the non-parametric Mann-Whitney Rank Sum test was used. P<0.05 was considered significant. All statistical analyses were performed using SigmaPlot 14.0 or GraphPad Prism 7.

### Results

**Body weight, clinical chemistry and pharmacokinetics**

$LDLR^{+/−}$ pigs (n=12) and $LDLR^{-/−}$ pigs (n=12) were fed a HFHC diet for 14 days. Pigs of each genotype were then subdivided into groups receiving placebo (n=4), BemA at 120 mg/d (n=4) or BemA at 240 mg/d (n=4) in addition to the HFHC diet for an additional 160 days (Figure 1A).

Most pigs gained weight similarly (~85 g/d) over 160 days (Figure 1B,C). $LDLR^{-/−}$ pigs treated with BemA at 120 mg/d gained weight more slowly (64 g/d), such that at 160 days they
weighed 15% less than the other five groups. The reason for this difference is unapparent, as caloric intake was the same and all pigs were otherwise healthy. Plasma levels of urea, creatinine, total bilirubin, ALT, and CK were measured at days 0, 28 and 160 (Table II and III in the online-only supplement). All measurements were normal and there were no differences among placebo, BemA at 120 mg/d or BemA at 240 mg/d in either genotype or with time.

Plasma pharmacokinetic analyses were performed following a single dose of BemA (120 mg or 240 mg) in drug-naive pigs fed the HFHC diet for 2 weeks (Figure 1 D-F). Parameters for C_{max}, T_{max}, AUC_{0-24} and t_{1/2} did not differ between genotypes. The combined genotype data for T_{max} and t_{1/2} were similar at 120 mg and 240 mg doses. In both genotypes, C_{max} was ~2-fold higher at 240 mg compared to 120 mg and the AUC_{0-24} was ~2-fold higher at 240 mg compared to 120 mg (Figure 1G). At C_{max}, plasma concentrations of the main active metabolite of BemA, ESP15228, were less than 3% of the parent compound and pharmacokinetic parameters did not differ between doses or genotypes (data not shown).

Plasma lipids and lipoproteins

BemA significantly reduced plasma cholesterol in both rodents and humans so we first assessed plasma lipids in treated pigs. Total plasma-C in LDLR^{+/−} pigs fed the HFHC diet with placebo initially increased from 3.95 to 4.75 mmol/L and subsequently remained stable (Figure IA, in the online-only supplement). BemA at 120 and 240 mg/d reduced total plasma-C by 17% and 26%, respectively, from baseline to 160 days, such that at 160 days total plasma-C was 32% and 40% lower than placebo (Figure 2A).

LDL-C concentrations increased in LDLR^{+/−} pigs fed the HFHC diet with placebo over the first 28 days, and subsequently remained stable (Figure IB, in the online-only supplement). BemA at 120 and 240 mg/d reduced LDL-C 32% and 42%, respectively, from baseline to 160 days, such that at 160 days LDL-C was 51% and 61% lower than placebo (Figure 2C).

In LDLR^{−/−} pigs fed the HFHC diet plus placebo, total plasma-C initially increased from 19.9 to 21.0 mmol/L and increased more slowly through 160 days (Figure IIA, in the online only supplement). BemA at 120 and 240 mg/d reduced total plasma-C by 12% and 15%, respectively, from baseline to 160 days, such that at 160 days total plasma-C was 23% and 27% lower than placebo (Figure 2B).

LDL-C concentrations increased in LDLR^{−/−} pigs fed the HFHC diet plus placebo from 18.1 to 18.6 mmol/L through 160 days (Figure IIB, in the online only supplement). BemA at 120 and 240 mg/d reduced LDL-C by 16% and 18%, respectively from baseline to 160 days, such that at day 160 LDL-C was 27% and 29% lower than placebo (Figure 2D).

Size exclusion chromatography of plasma confirmed that BemA treatment (120 mg/d and 240 mg/d) predominantly reduced LDL-C in both LDLR^{+/−} and LDLR^{−/−} pigs (Figures 2E,F, Figure IIIA,B in the online-only supplement). In LDLR^{+/−} pigs, BemA at 120 mg/d slightly increased HDLC-C concentrations but HDLC-C was unaffected at 240 mg/d. In LDLR^{−/−} pigs, HDLC-C was unaffected by BemA at either dose. HDLC profiles reflect the lack of effect of BemA on plasma HDLC-C (Figure IIIC,D, in the online only supplement). Peak elution volumes of cholesterol within each lipoprotein fraction were unaffected by treatment in either genotype, suggesting BemA did not alter lipoprotein sizes.

Plasma lathosterol levels, a marker of cholesterol synthesis, were higher in LDLR^{−/−} pigs (Figure 2G), reflecting increased cholesterol synthesis in homozygotes, similar to observations in human familial hypercholesterolemia. At both 28 and 160 days, BemA treatment decreased lathosterol concentrations in both LDLR^{+/−} (-50 to -51%) and LDLR^{−/−} (-43 to -55%) pigs. Both doses showed similar reductions that persisted when lathosterol was expressed as a ratio to total cholesterol (Figure 2H). Lower lathosterol levels in
BemA-treated pigs are consistent with BemA-induced inhibition of hepatic cholesterol synthesis. Together with the reductions of LDL-C in both genotypes, these results indicate that BemA was active in liver following its conversion to the CoA-derivative by liver-specific very long-chain acyl-CoA synthetase-1. mRNA expression of the pig enzyme (SLC27A) was confirmed to be liver-specific as shown in Figure IV in the online only supplement.

In LDLR+/- and LDLR-/- pigs, plasma triglyceride concentrations were low at day 160 (0.27 mmol/L and 0.29 mmol/L, respectively) (Figure 3A,B). The HFHC diet and BemA had no significant effect on plasma triglycerides (Figure 3A,B). Similarly, VLDL-triglycerides were low in LDLR+/- (0.17 mmol/L) and LDLR-/- (0.23 mmol/L) pigs, and unaffected by diet or BemA treatment (Figure 3A,B).

**Plasma Glucose and Insulin**

In contrast to studies in rodents, there were no consistent differences in fasting plasma glucose or insulin among genotypes or treatment groups (Figure 3C-F). In LDLR+/- pigs at 160 days, plasma glucose was ~20% (not significant, N.S.) lower in pigs treated with BemA compared to placebo (Figure 3C). Conversely, in LDLR+/- pigs, plasma glucose increased 10-18% (N.S.) with BemA treatment compared to placebo (Figure 3E). There was no difference in fasting plasma insulin concentrations among genotypes or treatment groups (Figure 3D,F).

**Liver lipids**

BemA reduced hepatic lipids in rodents; therefore, we measured hepatic cholesterol and triglyceride concentrations. Compared to LDLR+/- placebo pigs fed the HFHC diet, liver total cholesterol, free cholesterol, and cholesteryl ester in LDLR-/- placebo pigs fed the HFHC diet were 1.8-fold, 1.6-fold, and 2.9-fold higher, respectively (Figure 3G,H). In either genotype, BemA had no significant effect on liver total cholesterol, free cholesterol or cholesteryl ester at either dose. BemA at 240 mg/d modestly reduced cholesteryl ester (~10%, N.S.) in both genotypes. Liver triglycerides were 1.2-fold higher in LDLR-/- placebo pigs compared to LDLR+/- placebo pigs, but were unaffected by BemA treatment.

**Liver gene and protein expression**

To understand the molecular mechanisms underlying the BemA-mediated LDL-C reduction, select hepatic gene expression and protein levels were examined (Figure 4). As predicted, liver LDLR mRNA and LDLR protein were not detectable in LDLR-/- pigs (Figure 4A-C). In LDLR+/- pigs, administration of BemA at both 120 and 240 mg/d modestly decreased LDLR mRNA (N.S.) compared to placebo, but had no effect on LDLR protein. We anticipated that BemA treatment would increase LDLR mRNA in LDLR+/- pigs because BemA is an inhibitor of hepatic cholesterol synthesis. We speculate that with long-term BemA treatment, hepatic cholesterol homeostasis may have reached a new steady state, such that LDLR mRNA returned to baseline levels. In support of this concept, in a small number of LDLR+/- pigs consuming the same diet and treated with 240 mg/d of BemA for 6 weeks, hepatic LDLR mRNA increased 1.5-fold compared to placebo (Figure 4D).

Liver mRNA for SREBF2 and other SREBF2 target genes, HMGCR, PCSK9, and NPC1L1 were generally unaffected by genotype or BemA treatment (Figures 4E-H). SREBF2, HMGCR and NPC1L1 trended higher in LDLR-/- pigs compared to LDLR+/- pigs, consistent with increased cholesterol synthesis in homozygotes, however, these differences were not significant. In LDLR+/- pigs, BemA at 120 mg/d increased PCSK9 mRNA 2-fold compared to placebo, although no effect was observed at the 240 mg/d dose (Figure 4G). The explanation for this observation is unknown. APOB, SREBF1c, CYP7A1, MTTP, and ABCA1 expressions were unaffected by genotype or BemA treatment (Figure 4I).
Aortic Atherosclerosis

The significant reduction in LDL-C with BemA treatment suggests that BemA may affect atherosclerosis development in LDLR deficient pigs. In these pigs, aortic atherosclerosis developed in a characteristic pattern, with raised lesions primarily in the abdominal aorta and non-raised lesions in the ascending aorta and aortic arch (Figure 5, Figure V, VI in the online-only supplement). En face analyses of Sudan IV-stained aortic area relative to total aortic area and raised lesion area in the abdominal aorta relative to total abdominal aortic area were performed. Stained lesion and raised lesion areas in LDLR+/− placebo pigs were 6-fold and 12-fold greater, respectively, compared to LDLR+/− placebo pigs (Figure 5).

In aortae from LDLR+/− pigs, BemA at 120 mg/d and 240 mg/d reduced total and raised lesions compared to placebo-treated pigs (Figure 5A, Figure V, in the online-only supplement). Quantitation revealed that BemA decreased total lesion area 65% at 120 mg/d and 48% (N.S.) at 240 mg/d, compared to placebo (Figure 5B). BemA treatment decreased raised lesions in the abdominal aorta 66% (N.S.) at 120 mg/d and 54% (N.S.) at 240 mg/d, compared to placebo (Figure 5C). As there was no significant dose-dependent effect with respect to LDL-C or lesion reductions, lesion data from both doses were combined. In LDLR+/− pigs, BemA treatment decreased total lesion area 58% and raised lesion area 58%, compared to placebo (Figure 5B,C).

In aortae from LDLR−/− pigs, BemA at 120 mg/d and 240 mg/d reduced total and raised lesions compared to placebo (Figure 5D, Figure VI, in the online-only supplement). Lesion quantitation demonstrated that BemA decreased total lesion area 43% (N.S.) at 120 mg/d and 50% at 240 mg/d, compared to placebo (Figure 5E). BemA treatment decreased raised lesion area in the abdominal aorta 40% (N.S.) at 120 mg/d and 60% (N.S.) at 240 mg/d, compared to placebo (Figure 5F). Again, as there was no significant dose-dependent difference with respect to LDL-C or lesion reductions, lesion data from both doses were combined. BemA treatment decreased total lesion area 47% and raised lesion area 50% (Figure 5E,F).

To further characterize the aortic atherosclerotic plaques, cross-sections of abdominal aorta, obtained at a standard distance proximal to the aortic bifurcation, were stained with Movat’s Pentachrome (Figure 5A,D). In LDLR−/− pigs, aortic plaque morphology, typed according to the human classification of Virmani22, was primarily intimal thickening, consisting of mainly smooth muscle cells, extracellular matrix, and intimal xanthomas, with foam cell infiltration in the intimal layer (Figure 5A). In LDLR+/− pigs, in addition to intimal thickening and xanthomas, a large number of lesions showed pathological intimal thickening (Figure 5D). These lesions had readily apparent extracellular lipid, no areas of necrosis, but a smooth-muscle cell containing fibrous cap overlying areas of lipid (Figure 5D). Similar lesions have been observed in LDLR−/− pigs. Cross-sectional measurements revealed intimal plaque area was ~10-fold greater in LDLR−/− pigs compared to LDLR+/− pigs. BemA reduced mean intimal areas, including lesions, in both LDLR−/− and LDLR+/− pigs, although differences were not statistically significant (data not shown). Within each genotype, macroscopic analyses of the largest raised lesion in each abdominal aortic section did not reveal significant BemA-induced differences in plaque classification (data not shown).

Similar to humans, pigs develop coronary atherosclerosis. Atherosclerosis in the left anterior descending (LAD) coronary artery was assessed in sections obtained at standardized distances distal to the origin of the circumflex artery. Sections were stained with Movat’s Pentachrome to permit assessment of intimal and medial areas and plaque classification. Representative LAD cross-sections and higher magnification of representative lesions are shown in Figure 6A,D. Intimal area (3-fold), intimal/medial area ratio (3-fold), and percentage of advanced lesions (84% vs 0%) were substantially higher in LDLR+/− pigs compared to LDLR+/−.
pigs (Figure 6B,C,E,F). In LDLR<sup>+</sup>- pigs, LAD lesions were predominantly classified as intimal thickening and intimal xanthomas, whereas in LDLR<sup>-</sup>- pigs, pathological intimal thickening and fibrous cap atheroma were more abundant. Quantitatively, in LDLR<sup>+</sup>- pigs, BemA decreased LAD intimal area 44% (N.S.) at 120 mg/d and 48% (N.S.) at 240 mg/d (Figure 6B). Combining the data for both doses, BemA decreased intimal area 45%. Medial area was unaffected by treatment. Intimal area as a percentage of medial area decreased with BemA at 120 mg/d (-38%), BemA at 240 mg/d (-43%) and by combining the data of both doses (-40%). Morphological analyses of the largest lesion in each section demonstrated that BemA treatment decreased the percent of lesions classified as intimal xanthomas (38% vs 14%), increased the percent of lesions classified as intimal thickening, and increased the number of sections with no lesions (Figure 6C). The improvement in lesion class was significant for the combined dose data (Figure 6C). Histological analysis revealed that macrophage content of lesions in LDLR<sup>-</sup>- pigs were very low and unquantifiable (Figure VII, in the online-only supplement).

In LDLR<sup>-</sup>- pigs, BemA treatment reduced LAD intimal area, including plaques, 70% at 240 mg/d, but not at 120 mg/d (-12%) or when doses were combined (-42%, NS) (Figure 6E). Medial area was decreased 22% at 240 mg/d compared to placebo. The intimal/medial area ratio was reduced by BemA at 120 mg/d (-28%, N.S.), at 240 mg/d (-70%), and by combining the data of both doses (-48%) (Figure 6E). Morphological analyses of the largest lesion in each section showed BemA treatment reduced the percentage of lesions classified as either pathological intimal thickening or fibrous cap atheroma, and increased the percent classified as intimal xanthomas or intimal thickening (Figure 6F). In placebo-treated pigs, 84% of sections had at least one lesion classified as more advanced, whereas only 50% of lesions were classified as such in BemA-treated LDLR<sup>-</sup>- pigs. The improvement in lesion class was significant for the combined dose data (Figure 6F). Histological analysis revealed that macrophage content of lesions in LDLR<sup>-</sup>- pigs were low (~0.75% of total lesion area) and unaffected by BemA treatment (Figure VII, in the online-only supplement).

**Discussion**

The ability of the novel ACLY inhibitor BemA to reduce plasma LDL-C longer-term and attenuate atherosclerosis in a large-animal model that recapitulates human lipoprotein metabolism and disease pathophysiology had not been established. In the present study, BemA treatment of LDLR-deficient miniature pigs for 160 days decreased LDL-C up to 63% in LDLR<sup>+</sup>- animals and up to 29% in LDLR<sup>-</sup>- animals. BemA treatment did not affect plasma triglycerides, HDL-C, glucose and insulin. In both LDLR<sup>+</sup>- and LDLR<sup>-</sup>- pigs, BemA treatment attenuated atherosclerosis development up to 58% in the aorta and up to 45% in the LAD coronary artery. The drug was well tolerated.

In LDLR<sup>-</sup>- pigs fed the HFHC diet, BemA significantly decreased LDL-C as early as 28 days of treatment, and was sustained up to 160 days. Doubling the dose did not significantly improve LDL-C lowering, an effect also reported in hypercholesterolemic patients treated with similar doses of BemA. An analogous observation was reported with statin treatment of hypercholesterolemic patients, where doubling the statin dose improves the percent LDL-C reduction by only 6-8%. The mechanism underlying BemA-induced LDL-C reduction is not readily apparent. We anticipated that BemA-mediated cholesterol synthesis inhibition would decrease hepatic cholesterol concentrations and upregulate hepatic LDLR expression in heterozygotes through the SREBP2 pathway. Although BemA decreased cholesterol synthesis, as evidenced by lower plasma lathosterol levels, treatment did not affect hepatic cholesterol content or SREBF2 expression, and LDLR mRNA and LDLR protein expressions were unchanged. The expression of other hepatic SREBP2 target genes, including HMGCR, PCSK9 and NPC1L1 were also unaffected. One potential explanation is that at 160 days of treatment, hepatic cholesterol metabolism has reached a new steady state, such that hepatic
cholesterol and LDLR expression have returned to pretreatment levels. In support of this concept, BemA treatment of \(LDLR^{+/−}\) pigs consuming the same diet for 6 weeks increased hepatic LDLR mRNA 1.5-fold compared to placebo. Another possibility is that BemA inhibited hepatic production of VLDL and/or LDL, a mechanism for LDL reduction described previously in pigs treated with statins.\(^{14, 15}\) ApoB kinetic studies are required to test these possibilities.

The effect of BemA in \(LDLR^{−/−}\) pigs was unanticipated because of the complete LDLR deficiency. However, this group was included based on the LDL-C lowering response in BemA-treated \(Ldlr^{−/−}\) mice.\(^{10}\) In \(LDLR^{−/−}\) pigs, LDL-C was up to 29% lower with BemA treatment compared to placebo. Although the percent lowering of LDL-C was less than that observed in \(LDLR^{−/−}\) pigs, the absolute LDL-C reduction in treated \(LDLR^{−/−}\) pigs was much larger (~5.4 vs ~1.6 mmol/L). We observed no dose response in \(LDLR^{−/−}\) pigs, suggesting that the mechanism for LDL-C lowering in homozygotes may have been maximized at 120 mg/d. Although cholesterol synthesis was higher in \(LDLR^{−/−}\) pigs, BemA-treatment decreased endogenous cholesterol synthesis to a similar extent as in \(LDLR^{+/−}\) pigs, which is consistent with the similar pharmacokinetic profiles of BemA in both genotypes. As was observed in heterozygotes, BemA treatment of \(LDLR^{−/−}\) pigs did not affect hepatic cholesterol concentrations, and gene expression analyses did not provide further mechanistic insights. One obvious possibility is that BemA decreased hepatic secretion of VLDL and/or LDL apoB. Alternatively, BemA may upregulate non-LDLR-mediated LDL clearance. ApoB kinetic studies would provide initial insights into either of these hypotheses.

BemA treatment had little effect on plasma triglycerides, HDL-C, glucose and insulin in either genotype, although differences may have been masked by the small sample size. However, our results for these parameters mirror the effect of BemA in clinical studies of patients with hypercholesterolemia (reviewed in\(^{12}\)). In contrast, studies in BemA-treated rodents consistently demonstrated reduced fatty acid synthesis, liver triglycerides, liver cholesterol, hepatic VLDL secretion, plasma triglycerides, and adiposity, as well as improved glucose tolerance.\(^{8-10, 27}\) Reasons for these differences are not entirely clear. In the present study, liver triglyceride content in LDLR-deficient pigs is <10% of that in high fat diet-fed mice.\(^{10}\) It is possible that compared to rodents, rates of hepatic de novo fatty acid synthesis are low in pigs and in humans studied to date, such that blocking fatty acid synthesis would have little effect on triglyceride metabolism. Furthermore, BemA had little effect on hepatic cholesterol content in LDLR-deficient pigs, whereas in high fat diet-fed mice BemA decreased hepatic cholesterol ~50%. This may be related to the lower liver cholesterol content in LDLR-deficient pigs (<40%) compared to high fat diet-fed mice.\(^{10}\) It is possible that BemA inhibition of hepatic cholesterol synthesis in pigs is sufficient to influence the hepatic cholesterol regulatory pool, but not cholesterol mass within the whole liver.

The development of LDLR-deficient Yucatan miniature pigs, a model of human familial hypercholesterolemia, has provided a versatile model for the study of atherosclerosis.\(^{16}\) In the present study, we examined the impact of BemA on atherosclerosis in both \(LDLR^{+/−}\) and \(LDLR^{−/−}\) minipigs fed a high-fat diet containing 0.2% cholesterol. We anticipated that 0.2% cholesterol would be sufficient to induce atherosclerosis in both genotypes, and reasoned that a very high, non-physiological level of dietary cholesterol (1 to 2%) might mask or overwhelm the efficacy of cholesterol-lowering drugs, including BemA. Indeed, aortic atherosclerosis, as well as LAD coronary artery atherosclerosis developed in both \(LDLR^{+/−}\) and \(LDLR^{−/−}\) pigs. As anticipated, atherosclerosis in \(LDLR^{−/−}\) pigs was more advanced than in \(LDLR^{+/−}\) pigs. The extent of lesion development and class of lesions in the aorta and coronary arteries were similar to those previously reported in LDLR-deficient pigs and in D374Y PCSK9 transgenic pigs,\(^{16, 17}\) even though these studies used diets with higher dietary cholesterol (1-2%).
In both \textit{LDLR}^{+/−} and \textit{LDLR}^{−/−} pigs, BemA treatment markedly decreased total and raised lesion area in the aorta. In the LAD, BemA reduced intimal/medial area and shifted the classification of lesions to less advanced lesions. This robust improvement in atherosclerosis is most likely due to the large, sustained reductions in LDL-C in both genotypes, compared to placebo. However, we could not exclude a direct effect of BemA on cells within the arterial wall.\textsuperscript{9-10, 27} Lesion macrophage content was low, as previously reported in atherosclerotic pigs.\textsuperscript{21} In \textit{LDLR}^{+/−} pig coronary lesions, macrophage content was too low to be quantitated and in \textit{LDLR}^{−/−} pigs, the low macrophage content was unaffected by BemA. Attenuation of atherosclerosis in both genotypes did not appear to be dose-responsive, consistent with the observation that LDL-C reductions by BemA at the two doses were not statistically different. It is possible that with a larger sample size, a dose-response relationship would emerge. The effect of BemA on atherosclerosis in \textit{LDLR}^{−/−} pigs was not anticipated. However, the significant BemA-induced reduction in LDL-C, translated into a significant attenuation of atherosclerosis. Comparatively, previous reports demonstrated that atorvastatin (3 mg/kg/d) treatment of \textit{LDLR}^{+/−} pigs decreased aortic and coronary artery lesion area,\textsuperscript{28} and pitivastatin (40 mg/d) treatment of \textit{LDLR}^{−/−} pigs attenuated coronary lesion size and severity.\textsuperscript{29}

There are several limitations to the present study. Although the number of pigs studied was similar to previous reports,\textsuperscript{16, 17, 30} the sample size may have been insufficient to achieve statistical significance for some parameters at each dose tested. However, combining the atherosclerosis data for both doses, justified by the lack of dose-specific differences in plasma LDL-C, allowed for statistically significant treatment effects to emerge. ApoB kinetic studies are required to provide insight into the mechanism underlying plasma LDL-C reductions, especially in \textit{LDLR}^{−/−} pigs. Although BemA attenuated early lesion development in \textit{LDLR}^{+/−} pigs, a longer diet and treatment time would be required to assess BemA-mediated effects on more advanced lesions in this genotype. Differences in experimental outcomes between males and females were not apparent; however, the numbers of animals studied were insufficient to assess gender effects. Finally, it will be important to utilize an intervention protocol to examine the impact of BemA on lesion regression.

In conclusion, treatment of both \textit{LDLR}^{+/−} and \textit{LDLR}^{−/−} pigs with BemA significantly decreased plasma LDL-C and attenuated aortic and coronary atherosclerosis. These studies have several important implications. First, they underscore the value of LDLR-deficient Yucatan minipigs in preclinical evaluation of therapeutics that impact lipoprotein metabolism and atherosclerosis. Second, the efficacy of BemA in \textit{LDLR}^{+/−} pigs implies that attenuated secretion of hepatic apoB100-containing lipoproteins contributes to BemA-induced LDL-C reductions, and that BemA use could expand beyond the anticipated target patient population and serve as adjunct therapy for patients with homozygous familial hypercholesterolemia. Third, they fully support the pursuit of LDL-C lowering as a BemA therapeutic indication, as well as ongoing BemA CVD outcome trials in patients.

\textbf{Acknowledgements}

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\textbf{Disclosures}

Roger S. Newton is an employee of Esperion Therapeutics Inc.
References


**Highlights**

- Bempedoic acid is a novel inhibitor of the enzyme ATP-citrate lyase.
- In LDLR<sup>+/−</sup> and LDLR<sup>−/−</sup> pigs fed a cholesterol-containing diet, bempedoic acid significantly reduces plasma and LDL-cholesterol.
- Longer-term treatment with bempedoic acid attenuated the rate of atherosclerotic lesion growth in both the aorta and LAD coronary artery in LDLR<sup>+/−</sup> and LDLR<sup>−/−</sup> pigs.
Figures Legends

Figure 1. Body weight, plasma biochemical parameters and pharmacokinetics in LDLR-deficient pigs treated with bempedoic acid. LDLR+/– and LDLR–/– pigs were fed a high-fat, 0.2% cholesterol diet (HFHC) supplemented with bempedoic acid (BemA 0, 120 or 240 mg/d) for 160 days. A, Experimental timeline for all studies performed. B, C, Body weight (kg) over 160 days in both genotypes. D–F, Plasma BemA concentration profiles over 24 hr following a dose of D, 120 mg, E, 240 mg and F, combining the dose data. G, Values for the maximal concentration (Cmax), time of maximal concentration (Tmax), area under the concentration/time curve over 24 hr (AUC0-24) and half life (t1/2) for each dose. Data is presented as mean ± SEM. * P<0.05 vs Placebo and 240 mg BemA, Repeated Measures Two-Way ANOVA with post-hoc Tukey’s test. ** P<0.05, Student’s t-test. N.S. indicates no significant differences; ANOVA.

Figure 2. Bempedoic acid decreases plasma LDL-C and lathosterol concentrations in LDLR+/– and LDLR–/– pigs. LDLR+/– and LDLR–/– pigs were fed a high-fat, 0.2% cholesterol diet (HFHC) supplemented with bempedoic acid (BemA 0, 120 or 240 mg/d) for 160 days. A–D, Percent change in plasma cholesterol (A, B) and LDL-cholesterol (C, D) concentrations from baseline in LDLR+/– (A, C) and LDLR–/– (B, D) pigs (n=3-4/group). E, F, Plasma was subjected to FPLC at day 160 (n=3/group). Cholesterol was measured in eluted fractions and the mean concentration in each fraction is shown. LDL-C area under the curve calculations are shown in Figure IV, online-only supplement. G, Plasma lathosterol concentrations and H, ratio of plasma lathosterol:plasma cholesterol concentrations at days 28 and 160 (n=3-4/group). Data is presented as mean ± SEM. * P<0.05 vs placebo, Repeated Measures Two-Way ANOVA with post-hoc Tukey’s test. Different lower-case letters indicate significant differences; ANOVA with post-hoc Tukey’s test (P<0.05). ** P<0.05 vs placebo; Student’s t-test. *** P<0.05 vs placebo; Mann-Whitney Rank Sum Test. N.S. indicates no significant differences; ANOVA.

Figure 3. Bempedoic acid does not affect plasma triglyceride, glucose, insulin or liver lipid concentrations. LDLR+/– and LDLR–/– pigs were fed a high-fat, 0.2% cholesterol diet (HFHC) supplemented with bempedoic acid (BemA 0, 120 or 240 mg/d) for 160 days. A, B, Plasma total and VLDL triglyceride concentrations at day 160 in LDLR+/– and LDLR–/– pigs (n=2-4/group). Fasting plasma glucose concentrations at day 160 in C, LDLR+/– and E, LDLR–/– pigs (n=3-4/group). Fasting plasma insulin concentrations at day 160 in D, LDLR+/– and F, LDLR–/– pigs (n=3-4/group). G, H, Hepatic total cholesterol, free cholesterol (FC), cholesteryl ester (CE) and triglyceride (TG) content (n=3-4/group). Pl; placebo, 120; 120 mg/d BemA, 240; 240 mg/d BemA. n=4/group, except LDLR+/– 120 mg/d and LDLR–/– placebo, where n=3/group. Data are presented as mean ± SEM. N.S. indicates no significant differences; ANOVA. n.s. indicates no significant differences, Kruskal-Wallis One-Way ANOVA on Ranks.

Figure 4. Bempedoic acid does not affect hepatic expression of LDLR mRNA or protein. LDLR+/– and LDLR–/– pigs were fed a high-fat, 0.2% cholesterol diet (HFHC) supplemented with bempedoic acid (BemA 0, 120 or 240 mg/d) for 160 days. A, Liver LDLR mRNA expressed relative to GAPDH. B, C, Representative immunoblots (n=2/group, all from the same blot) of liver LDLR, normalized to α-tubulin, with quantitation. D, Liver LDLR mRNA from another group of pigs fed the same diet and treated with placebo or 240 mg/d of BemA for 6 weeks. For LDLR+/–, n=1; LDLR+/– and LDLR–/–, n=2/dose. Mean data is presented. E–I, mRNA expression of genes involved in hepatic lipid metabolism, expressed relative to GAPDH. For A–C and E–I n=4/group, except LDLR–/– 120 mg/d and LDLR–/– placebo, where n=3. Data are presented as mean ± SEM. N.S. indicates no significant differences; ANOVA. n.s. indicates no significant differences, Kruskal-Wallis One-Way ANOVA on Ranks.
Figure 5. Bempedoic acid attenuates aortic atherosclerosis in LDLR\(^{+/-}\) and LDLR\(^{-/-}\) pigs. LDLR\(^{+/-}\) and LDLR\(^{-/-}\) pigs were fed a high-fat, 0.2% cholesterol diet (HFHC) supplemented with bempedoic acid (BemA 0, 120 or 240 mg/d) for 160 days. A,D Representative Sudan IV-stained en face preparations of abdominal aortae from LDLR\(^{+/-}\) and LDLR\(^{-/-}\) pigs and representative cross-sections of abdominal aortae stained with Movat’s Pentachrome. Histological features are shown, lumen (L), internal elastic lamina (IEL), media (M) and neointima (NI). Total surface area covered by atherosclerotic lesions in aortae expressed as percent of total aortic area in B, LDLR\(^{+/-}\) and E, LDLR\(^{-/-}\) pigs. Surface area covered by raised lesions expressed as percent of total abdominal aortic area in C, LDLR\(^{+/-}\) and F, LDLR\(^{-/-}\) pigs. n=4/group, except LDLR\(^{+/-}\) 120 mg/d and LDLR\(^{-/-}\) placebo, where n=3. Data are presented as mean ± SEM. Different lower-case letters indicate significant differences, no letters indicate no significant differences assessed by ANOVA with post-hoc Tukey’s test (\(P<0.05\)). ** \(P<0.05\) vs placebo, Student’s t-test.

Figure 6. Quantification and classification of atherosclerosis in the left anterior descending coronary artery. LDLR\(^{+/-}\) and LDLR\(^{-/-}\) pigs were fed a high-fat, 0.2% cholesterol diet (HFHC) supplemented with bempedoic acid (BemA 0, 120 or 240 mg/d) for 160 days. A,D Representative cross-sections of the LAD, obtained 1 cm distal to the left circumflex artery bifurcation and stained with Movat’s Pentachrome. Enlarged images (boxes) for each dose of BemA are shown below with histological features identified: lumen (L), internal elastic lamina (IEL), external elastic lamina (EEL), adventitia (ADV) and neointima (NI). B,E LAD coronary artery morphometry presented as total intimal area, total medial area, and the intimal:medial ratio of each LAD cross-section. C,F Plaque classification and distribution of the largest lesion in each LAD section. Data, expressed mean ± SEM, is from 2 LAD sections/animal, obtained 1 and 2 cm distal to the left circumflex artery bifurcation. n=8 LAD sections/group, except LDLR\(^{+/-}\) 120 mg/d and LDLR\(^{-/-}\) placebo, where n=6 LAD sections. Different lower-case letters indicate significant differences; ANOVA with post-hoc Tukey’s test (\(P<0.05\)). Different upper-case letters indicate significant differences; Kruskal-Wallis One-Way ANOVA on Ranks, followed by a Tukey’s post-hoc test. ** \(P<0.05\) vs placebo, Student’s t-test. *** \(P<0.05\) vs placebo, Mann-Whitney Rank Sum Test. N.S. indicates no significant differences; ANOVA. n.s. indicates no significant differences, Kruskal-Wallis One-Way ANOVA on Ranks.
**Fig 1**

**A**

Day: -28 -14 0 28 160

Chow HFHC

Weekly blood samples

12 LDLR<sup>−/−</sup> pigs
12 LDLR<sup>−/−</sup> pigs

Pharmacokinetic study + Initiation of BemA
12 LDLR<sup>−/−</sup> pigs, 12 LDLR<sup>−/−</sup> pigs

4 ea of placebo, 120 mg BemA, 240 mg BemA

Monthly blood samples

Sacrifice with blood and tissue collection

**B**

LDLR<sup>−/−</sup>

△ Placebo

△ BemA 120

▾ BemA 240

Body weight (kg)

Study day

**C**

LDLR<sup>−/−</sup>

● Placebo

● BemA 120

● BemA 240

Body weight (kg)

Study day

**D**

LDLR<sup>−/−</sup>

○ LDLR<sup>−/−</sup>

BemA (µg/mL)

Hours

**E**

LDLR<sup>−/−</sup>

■ LDLR<sup>−/−</sup>

BemA (µg/mL)

Hours

**F**

LDLR<sup>−/−</sup>

▲ 120 mg

▲ 240 mg

BemA (µg/mL)

Hours

**G**

**n.s.**

▲ 120 mg

▲ 240 mg

C<sub>max</sub> (µg/mL)

T<sub>max</sub> (h)

AUC (µg/mL•h)

T<sub>1/2</sub> (h)

n.s.
Fig 2

A. % change from baseline LDLR 

B. % change from baseline LDLR 

C. % change from baseline LDLR 

D. % change from baseline LDLR 

E. Cholesterol (µg/mL of fraction) for LDL, HDL, and VLDL 

F. Cholesterol (µg/mL of fraction) for LDL, HDL, and VLDL 

G. Plasma lathosterol (µg/mL) for Placebo, BemA 120, BemA 240, and BemA combined doses 

H. Plasma lathosterol-cholesterol for Placebo, BemA 120, BemA 240, and BemA combined doses.
Fig 3

A. LDLR−/−

B. LDLR−/−

C. LDLR−/−

D. LDLR−/−

E. LDLR−/−

F. LDLR−/−

G. LDLR−/−

H. LDLR−/−
Fig 5

A  
**LDLR**

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### Major Resources Table

#### Animals

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<td>Abcam, Cambridge, MA, USA</td>
<td>ab7291</td>
<td>0.1 μg/mL</td>
<td>GR138941-7</td>
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<td>Goat anti-rabbit IgG (800 nm fluorophore)</td>
<td>Li-COR Biosciences, Lincoln, NE, USA</td>
<td>926-3221</td>
<td>0.06 μg/mL</td>
<td>C50331-05</td>
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<td>Goat anti-mouse IgG (680 nm fluorophore)</td>
<td>Li-COR Biosciences, Lincoln, NE, USA</td>
<td>926-68070</td>
<td>0.06 μg/mL</td>
<td>C50113-05</td>
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<td>Macrophages (CD68)</td>
<td>Bio-Rad Laboratories Inc., Hercules, CA, USA</td>
<td>MCA2317GA</td>
<td>1 μg/mL</td>
<td>1609</td>
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<td>Peroxidase labelled polymer, goat anti-mouse IgG</td>
<td>Agilent, Santa Clara, CA, USA</td>
<td>K4006</td>
<td>No Dilution</td>
<td>10126068</td>
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SUPPLEMENTAL MATERIAL

Bempedoic acid lowers LDL-cholesterol and attenuates atherosclerosis in LDL receptor-deficient (LDLR$^{+/+}$ and LDLR$^{-/-}$) Yucatan miniature pigs.

Burke AC, Telford DE, Sutherland BG, Edwards JY, Sawyez CG, Barrett PHR, Newton RS, Pickering JG and Huff MW

Supplemental Figures (7) and Tables (3)
Figure I

**A**

Total plasma cholesterol

**B**

LDL cholesterol

**Figure I.** Bempedoic acid decreases plasma and LDL-cholesterol concentrations in *LDLR*⁺⁻ pigs. *LDLR*⁺⁻ pigs were fed a high-fat, 0.2% cholesterol diet (HFHC) supplemented with bempedoic acid (BemA 0, 120 or 240 mg/d) for 160 days. **A,** Plasma total cholesterol concentrations in mmol/L from day 0 to day 160 in *LDLR*⁺⁻ pigs (n=4/group). **B,** Plasma LDL-cholesterol concentrations in mmol/L from day 0 to day 160 in *LDLR*⁺⁻ pigs (n=4/group). Data is presented as mean ± SEM. * P<0.05 vs baseline; ANOVA with post-hoc Tukey’s test; at day 160.
Figure II. Bempedoic acid decreases plasma and LDL-cholesterol concentrations in \(LDLR^{-/-}\) pigs. \(LDLR^{-/-}\) pigs were fed a high-fat, 0.2% cholesterol diet (HFHC) supplemented with bempedoic acid (BemA 0, 120 or 240 mg/d) for 160 days. A, Plasma total cholesterol concentrations in mmol/L from day 0 to day 160 in \(LDLR^{-/-}\) pigs (n=4/group). B, LDL-cholesterol concentrations in mmol/L from day 0 to day 160 in \(LDLR^{-/-}\) pigs (n=4/group). Data is presented as mean ± SEM. † \(P<0.05\) vs placebo, ANOVA with post-hoc Tukey’s test; at day 160.
Figure III. Bempedoic acid decreases plasma LDL-C and has no effect on HDL-C in LDLR\textsuperscript{+/−} and LDLR\textsuperscript{−/−} pigs. LDLR\textsuperscript{+/−} and LDLR\textsuperscript{−/−} pigs were fed a high-fat, 0.2% cholesterol diet (HFHC) supplemented with bempedoic acid (BemA 0, 120 or 240 mg/d) for 160 days. Plasma was subjected to FPLC at day 160 (n=3-4/group). Cholesterol was measured in eluted fractions and curves generated measuring the cholesterol concentration in each fraction. The area under the curves was calculated for both LDLR\textsuperscript{+/−} (A) and LDLR\textsuperscript{−/−} (B) pigs. Plasma HDL-cholesterol concentration in LDLR\textsuperscript{+/−} (C) and LDLR\textsuperscript{−/−} (D) pigs determined by precipitation of apoB-containing lipoproteins from plasma at day 160 (n=3-4/group). Data is presented as mean ± SEM. Different lower-case letters indicate significant differences; ANOVA with post-hoc Tukey’s test (P<0.05). N.S. indicates no significant differences; ANOVA.
Figure IV. The mRNA of porcine very long chain acyl-CoA synthetase-1 (SLC27A) is specifically expressed in the liver. SLC27A expression relative to GAPDH in liver, adipose, intestine and muscle in LDLR<sup>+/+</sup> (n=2), LDLR<sup>+/−</sup> (n=6) and LDLR<sup>−/−</sup> (n=6) pigs. Data are presented as mean ± SEM.
Figure V. Bempedoic acid attenuates aortic atherosclerosis in \textit{LDLR}^{+/-} pigs. \textit{LDLR}^{+/-} pigs were fed a high-fat, 0.2\% cholesterol diet (HFHC) supplemented with bempedoic acid (BemA 0, 120 or 240 mg/d) for 160 days. Aortae were dissected, cut longitudinally and stained with Sudan IV. Red area indicates atherosclerotic plaque. Shown is a representative aorta from each treatment group (Placebo, 120 mg/d BemA and 240 mg/d BemA).
Figure VI. Bempedoic acid attenuates aortic atherosclerosis in LDLR–/– pigs. LDLR–/– pigs were fed a high-fat, 0.2% cholesterol diet (HFHC) supplemented with bempedoic acid (BemA 0, 120 or 240 mg/d) for 160 days. Aortae were dissected, cut longitudinally and stained with Sudan IV. Red area indicates atherosclerotic plaque. Shown is a representative aorta from each treatment group (Placebo, 120 mg/d BemA and 240 mg/d BemA).
Figure VII

A

Too low to be quantitated

B

Figure VII. Left anterior descending coronary artery atherosclerosis macrophage content. LDLR°/° and LDLR°/° pigs were fed a high-fat, 0.2% cholesterol diet (HFHC) supplemented with bempedoic acid (BemA 0, 120 or 240 mg/d) for 160 days. 

A. Percent of lesions occupied by macrophages (n=4-6 sections/group). 

B. Representative cross-sections of the LAD, obtained 1 cm distal to the left circumflex artery bifurcation immunostained for CD68 and counterstained with hematoxylin. Histological features identified: lumen (L), media (M), and adventitia (ADV). Spleen sections were used as a positive control. Arrows indicate positive staining for CD68 (brown). Data are presented as the mean ± SEM. n.s. indicates no significant differences, Kruskal-Wallis One-Way ANOVA on Ranks.