Supplement

Increased apolipoprotein C3 drives cardiovascular risk in type 1 diabetes


Supplemental Methods

Liver targeted deletion of FoxO
Mice with hepatocyte-targeted deletion of FoxO1, FoxO3 and FoxO4 have been described previously (1). These mice were on a mixed C57BL/6J 129S1 background. Controls were littermates who did not express liver-specific α1-antitrypsin-Cre. Mice were fasted for 4 hours on the day of sacrifice during the dark cycle, and then blood and livers were collected for analysis. We also induced acute deletion of FoxOs in adult mice by injecting them with an adeno-associated virus (AAV8; 1 × 10^{11} viral genomes/mouse) expressing Cre recombinase under control of the Tbg (thyroxine hormone-binding globulin) promoter for liver specificity 4 weeks prior to the end of the experiment. Controls were injected with GFP-AAV8. Mice were fasted for 4 hours on the day of sacrifice. This method has also been described previously (1). Male mice were used for these experiments. The experiments were performed in accordance with the Columbia University Institutional Animal Care and Use Committee (protocol # AAAQ4433).

Chromatin immunoprecipitation (ChIP)
ChIP was carried out as previously described using the PIXUL platform (2) using antibodies to Pol II (clone 4H8, catalogue # Sc-47701, mouse monoclonal, Santa Cruz), H3K4m3 (catalogue # 39159 rabbit polyclonal, Active Motif, Carlsbad, CA) and H3K9m3 (catalogue # 39161 rabbit polyclonal, Active Motif). Data were normalized to none-immunoprecipitated DNA and expressed as % of input.

HDL isolation from human serum
Five test runs were completed to ascertain that the protocol for plasma worked for serum. Thus, HDL (density, 1.063-1.210 g/mL) was isolated by sequential ultracentrifugation from freshly thawed serum using the previously described method for plasma (3).

Digestion of isolated HDL from human serum
The protein concentration of HDL was determined using the Lowry assay (BioRad), with albumin as the standard. In a digestion plate (500 µL 96 well “V” bottom clear, AXYgen Scientific, Union City, CA), following the addition of freshly prepared methionine (5 mM final concentration) in 20% acetonitrile and 100 mM NH_{4}HCO_{3}, HDL protein (8 µg) was reduced with dithiothreitil and alkylated with iodoacetamide. After adding 0.4 µg of isotope-labeled [^{15}N]APOA1 as the internal standard, the HDL was incubated at 37°C
with 40:1 (w/w, HDL protein/enzyme) of sequencing grade modified trypsin (sequencing grade; Promega, Madison, WI) for 4 h. A second aliquot of trypsin (1:20 final, w/w, enzyme/HDL protein) was added, and the samples were incubated overnight at 37°C. Digestion was halted by acidifying the reaction mixture (pH 2-3) with trifluoroacetic acid, and the samples were dried and stored at −80°C until mass spectrometric analysis.

**HDL isolation from mouse plasma**

HDL (d=1.063–1.210 g/mL) was isolated from EDTA plasma by sequential density ultracentrifugation. Briefly, 335 μL of plasma was adjusted to density 1.21 g/ml by solid KBr and underlaid with 150 μL of KBr solution with a density 1.21 g/mL and spun at 511,260 x g at 5°C for 4.5 h in a T120.1 rotor (Beckman Coulter, CA). The top 125 μL was collected, adjusted to density of 1.063 g/mL with normal saline and HDL was separated from more buoyant lipoproteins with another 2 h spin at 511,260 x g in the T120.1 rotor. HDL was collected in 125 μL from the bottom of the tube and dialyzed against 20 mM potassium phosphate buffer with 100 μM DTPA, pH 7.4.

**Digestion of isolated HDL from mouse plasma**

HDL (10 μg protein) was solubilized with 0.5% sodium deoxycholate (SDC) (Sigma-Aldrich, USA) in 200 mM NH₄HCO₃, spiked with 0.05 μg of [¹⁵N]APOA1 as internal standard (4), reduced with dithiothreitol, alkylated with iodoacetamide, and digested with two additions of trypsin (1:20, w/w HDL protein; sequencing grade, Promega) for 4 hours. After precipitation of SDC with formic acid (1% final concentration), samples were frozen and stored at −20°C until analysis (less than a week). For the liquid chromatography mass spectrometry analysis, an equivalent of 200 ng of the HDL protein was injected (5).

**Proteomics analysis of mouse HDL**

The abundance of selected HDL-associated proteins was quantified by mass spectrometry using data independent analysis. Tryptic digests of HDL were desalted on a C18 trapping column (Reprosil-Pur 120 C18-AQ, 5 μm, 0.1 x 40 mm, Dr. Maisch HPLC GmbH, Germany) (trapping flow rate 4 μL/min), separated on a capillary analytical column (Reposil-Pur 120 C18-AQ, 5 μm, 250x0.075 mm, Dr. Maisch HPLC GmbH) with a multi-step linear gradient 1-5% solvent B in 2 min, 5-25% in 50 min, 25-35% in 10 min followed by ramp to 80% B and re-equilibration (A - 0.1% formic acid in water, B - acetonitrile, 0.1% formic acid, flow rate of 0.4 μL/min) using a nanoAquity UPLC (Waters, MA), and analyzed in a Thermo Fusion Lumos (Thermo Fisher, San Jose, CA) tribrid mass spectrometer with electrospray ionization using data-independent analysis.

Data-independent analysis was performed as follows: MS1 scan (395-1005 Th) at resolution 120,000, maximum injection time 50 ms, was followed by 60 MS/MS scans on 10 Th mass windows across 400-1000 Th range (resolution 15,000 with loop time 3 sec and maximum injection time 22 ms, higher-energy collisional dissociation activation at normalized collision energy 30%). Data was further processed using Skyline (6) to extract fragment ion chromatograms of top 5 fragment ions for the peptides derived from proteins of interest with 10 ppm accuracy windows. Chromatograms were integrated and peak areas were exported for further analysis.
Determination of mouse HDL particle concentrations and HDL functional assays

HDL particle concentrations were quantified by calibrated ion mobility analysis, as described previously (7). HDL peak areas were converted into aqueous particle concentrations, using glucose oxidase calibration curves. Serum HDL cholesterol efflux capacity was assessed with J774 macrophages labeled with [³H]cholesterol and stimulated with a cAMP analogue. Efflux by the ABCA1 pathway was measured using BHK cells containing mifepristone-inducible human ABCA1. The cells were radiolabeled with [³H]cholesterol and 1 mg/mL fatty acid–free albumin (8). Cholesterol efflux was calculated as the percentage of radiolabel in the medium at the end of the incubation time divided by the total radioactivity of the medium and cells. ABCA1-specific cholesterol efflux was monitored as the difference in cholesterol efflux in cells with and without induction of ABCA1.

Supplemental references

Supplemental figure 1. Hepatic FoxO1, 3 and 4 deficiency does not reduce Apoc3 mRNA levels in fasted mice. Liver-specific deletion of FoxO1, FoxO3 and FoxO4 was generated by inducing recombination of the floxed alleles using hepatocyte-targeted α1-antitrypsin Cre in Foxo1<sup>fl/fl</sup>, Foxo3<sup>fl/fl</sup>, Foxo4<sup>fl/Y</sup> mice. Mice were fasted for 4 hours at the beginning of the dark cycle. A. Hepatic Foxo1, Foxo3 and Foxo4 mRNA. B. Hepatic Apoc3 mRNA (n=4-5). ***p<0.001, two-tailed unpaired t-test.
Supplemental figure 2. **Neither diabetes nor insulin have dramatic transcriptional effects on Apoc3.** Female Ldlr\(^{-/-}\);Gp\(^{Tg}\) mice were rendered diabetic (D) using LCMV. Saline was used as control in non-diabetic (ND) mice. The mice were maintained for 10 days while on a low-fat, semipurified diet. A subset of diabetic mice received an acute injection of insulin 30 minutes prior to euthanasia (0.2-0.3 U/ mouse; acute study). A. Blood glucose. B. Liver insulin signaling (n=3). C-D. Chromatin immunoprecipitation (ChIP) in liver samples using antibodies to Pol II (transcriptional activation), H3K4me3 (histone modification associated with active chromatin) and H3K9me (histone modification associated with inactive chromatin). C. ChIP in acute insulin study. D. ChIP in intense insulin study. E. Blood glucose from onset of diabetes in intense insulin study. F. Plasma APOC3 levels from the onset of diabetes. Note that time point 8 days contains the same data as in figure 2I. N=4, *p<0.05, **p<0.01, ***p<0.001, one-way ANOVA (A) followed by Tukey's multiple comparison test.
Supplemental figure 3. **Lack of effects of Apoc3 ASO treatment on intestinal Apoc3 mRNA, liver triglycerides and liver toxicity.** Diabetes (D) was induced in female Ldlr<sup>-/-</sup>;Gp<sup>Tg</sup> mice using LCMV. Saline was used as control in non-diabetic (ND) mice. The mice were maintained for 12 weeks and treated with control antisense (cASO) or antisense to Apoc3 (Apoc3 ASO). **A.** Intestine Apoc3 mRNA at the end of the 12-week study (n=4). **B.** Liver triglycerides were measured after a modified Bligh and Dyer lipid extraction, and normalized to liver wet weight or liver protein. The data were similar regardless of normalization strategy (data not shown) (n=8). **C.** Plasma levels of liver enzyme alanine aminotransferase as a marker of potential hepatotoxicity (ALT; n=4). No significant differences were detected (one-way ANOVA).
Supplemental figure 4. **Diabetes results in increased plasma levels of APOC3 and APOE, which are normalized by Apoc3 ASO treatment.** Diabetes (D) was induced in female Ldlr⁻/⁻;Gptg mice using LCMV. Saline was used as control in non-diabetic (ND) mice. The mice were maintained for 12 weeks and treated with control antisense (cASO) or antisense to Apoc3 (Apoc3 ASO). Relative levels of plasma APOC3 (A) and APOE (B) were measured by targeted mass spectrometry at the indicated time-points. The data from the 12-week time-point are shown in figures 3-4. N=4-8, *p<0.05, **p<0.01, ***p<0.001, one-way ANOVA followed by Tukey’s multiple comparison tests.
Supplemental figure 5. Neither altered HDL cholesterol efflux capacity nor HDL particle concentration explains diabetes-accelerated atherosclerosis or the effect of Apoc3 ASO treatment on atherosclerosis. Female Ldlr⁻/⁻;GpTg mice were rendered diabetic (D) using LCMV. Saline was used as control in non-diabetic (ND) littermates. The mice were maintained for 12 weeks. At the onset of diabetes, animals were switched to a low-fat, semi-purified diet. Animals were treated twice/week with 25 mg/kg (intraperitoneal injections) of antisense to Apoc3 (Apoc3 ASO) or a control antisense (cASO) starting 2 days after onset of diabetes. Doses were adjusted every 2 weeks based on body weight. A. The same amount of PEG-precipitated APOB-depleted serum (serum HDL) was added to cholesterol-loaded J774 macrophages, and cholesterol efflux was measured. B. The same amount of serum HDL was added to cholesterol-loaded BHK cell overexpressing inducible ABCA1, and ABCA1-specific cholesterol efflux was measured (A-B; n=17-19). C. Total HDL particle concentration was quantified using calibrated ion mobility analysis (n=12-14). D. HDL was isolated by sequential density centrifugation (d= 1.063–1.21 mg/mL), tryptically digested and analyzed by tandem mass spectrometry. HDL-associated APOC3 (n=9-10). *p<0.05, **p<0.01, ***p<0.001, one-way ANOVA followed by Tukey's multiple comparison tests.
Supplemental figure 6. Diabetes and Apoc3 ASO do not alter atherosclerosis by changing levels of circulating white blood cells or systemic inflammation. During week 12 of the study, mice were sedated and bled for analysis of leukocyte numbers. A. Total white blood cell (WBC) counts using a Hemavet automatic cell counter. B-D. Flow cytometric analysis of whole blood cell populations normalized to total WBC. B. Monocytes. C. Ly6C\(^{hi}\) monocytes. D. Neutrophils. E. Platelets determined by Hemavet (A-E; n=7-14) F. Plasma SAA levels were measured by ELISA at the end of the study (N=6-13; 3 statistical outliers were removed from F; 2 in the ND cASO group and 1 in D cASO group). At the end of the study resident macrophages were isolated by peritoneal lavage. G. Macrophage *Il1b* mRNA was measured by real-time PCR. H. Macrophage *Tnfa* mRNA (G-H; n=10-14). *p<0.05, **p<0.01, ***p<0.001, one-way ANOVA followed by Tukey’s multiple comparison tests. ND, non-diabetic mice; D, diabetic mice.
Supplemental figure 7. **Validation of APOC3, APOE and APOB antibodies for immunohistochemistry.** Female Ldlr<sup>−/−</sup>;Gp<sup>Tg</sup> mice were rendered diabetic (D) using LCMV. Brachiocephalic arteries were stained with a rabbit polyclonal anti-APOC3 antibody or control rabbit antiserum, both at a final 1:1000 dilution. A rabbit monoclonal antibody against APOE or a rabbit monoclonal antibody was used at a final concentration of 0.4 µg/ml. No staining was observed in sections from APOE-deficient mice (data not shown). A biotinylated goat-anti APOB antibody or biotinylated goat IgG was used at 4 µg/ml. Representative adjacent cross-sections are shown. Scale bar is 100 µm.
Supplemental figure 8. **Diabetes results in more advanced lesions characterized by increased necrotic cores and increased APOC3.**

**A. Study set-up.** Female *Ldlr<sup>-/-</sup>*; *Gp<sup>Tg</sup>* mice were fed a high-fat diet with 1.25% cholesterol for 12 weeks, switched to chow for 2 weeks then injected with LCMV to induce diabetes (D) or saline (non-diabetic; ND). Once diabetic, the mice were maintained for 4 weeks on a low-fat diet.

**B. Blood glucose.**

**C. Plasma cholesterol.**

**D. Sinus lesion Movat’s pentachrom and APOC3**

**E. Sinus lesion size**

**F. Sinus necrotic core**

**G. Sinus necrotic core**

**H. Sinus APOC3 area**

Supplemental figure 8. Diabetes results in more advanced lesions characterized by increased necrotic cores and increased APOC3. A. Study set up. Female *Ldlr<sup>-/-</sup>*; *Gp<sup>Tg</sup>* mice were fed a high-fat diet with 1.25% cholesterol for 12 weeks, switched to chow for 2 weeks then injected with LCMV to induce diabetes (D) or saline (non-diabetic; ND). Once diabetic, the mice were maintained for 4 weeks on a low-fat diet. B. Blood glucose at the end of the study. C. Plasma cholesterol. D. Example of aortic sinus lesions stained with Movat’s pentachrome (top) and APOC3 immunohistochemistry. E. Quantification of aortic sinus lesion size beginning at the appearance of all three aortic valve leaflets. F. Quantification of aortic lesion necrotic cores (diabetes significant effect, p=0.035). G. Quantification of aortic lesion necrotic cores at the + 90 µm expressed as % of lesion area. H. Quantification of APOC3 immunohistochemistry at + 95 µm. N=7-10. *p<0.05, **p<0.01, ***p<0.001, t-test or 2-W ANOVA (E and F). Scale bar 100 µm. Arrow indicates necrotic core.
Uncropped p-Akt and p-GSK3β for figure S2B

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Uncropped total Akt for figure S2B

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Uncropped total GSK3β for figure S2B

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