Diabetic Complications Consortium

Application Title: APOC-III mediated dyslipidemia in diabetic kidney disease and atherosclerosis

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1. Project Accomplishments:

This project is aimed at investigating the excessive risk of atherosclerotic cardiovascular disease (CVD) in people with diabetes that also have underlying kidney disease. APOC3 is a key determinant of plasma triglycerides and is involved in mediating diabetic dyslipidemia (increased triglyceride-rich lipoproteins – TRLs), which is a known risk factor for CVD. Here we demonstrate that apolipoprotein C3 (APOC3) is elevated in the setting of DKD and that blocking APOC3 reduces DKD and atherosclerosis in mouse models of type 2 diabetes.

2. Specific Aims:

Specific aim: Does blocking diabetes-induced APOC3 prevent diabetic dyslipidemia, kidney disease and atherosclerosis in a novel mouse model of combined diabetic kidney disease and atherosclerosis?

Results:

APOC3 antisense oligonucleotide (ASO) treatment reduces diabetic dyslipidemia

Male and female BTBR (black and tan, brachyury) wildtype (WT) or BTBR mice homozygous for the leptin-deficiency mutation (*Lepob*; OB) mice were used for this study. Human-like dyslipidemia was induced in ½ the cohort of mice using an ASO against the low-density lipoprotein (LDL) receptor (LDLR) (Fig 1, groups 5-8; green) and the other ½ were treated with a control ASO (Fig 1, groups 1-4; white). To test the role of APOC3 ½ of all the mice were injected with APOC3 ASO (Fig. 1, groups 2, 4, 6 and 8), or control ASO. At the initiation of dyslipidemia with the ASO injections, the animals were fed a semi-purified, high fat, cholesterol-containing diet (40 % of calories from fat, 1.25% added cholesterol; TD 00244) for 14 weeks. Blood glucose, plasma cholesterol, and triglycerides (TGs) were measured at baseline, 2, 4, 8, and 14 weeks of the study. At baseline, no significant differences were observed between the treatment groups (data not shown). Plasma cholesterol and TG were elevated at 2 weeks with LDLR ASO treatment. They stayed elevated throughout the study in mice that were treated with LDLR ASO, and those levels were suppressed in mice treated with APOC3 ASO with the effect of the APOC3 ASO being more effective in mice also treated with LDLR ASO. The effect of APOC3 ASO on plasma TGs was more pronounced compared to the effect of APOC3 on plasma cholesterol (Fig. 1B-C, data not shown). In line with the known role for APOC3 in TG-metabolism and consistent with our data, the principal reduction observed was in very-low-density lipoproteins (VLDL) (Fig. 1D). Consistent with APOC3 being a key determinant for diabetic dyslipidemia, plasma APOC3 levels were elevated in leptin-deficient mice compared to WT mice. APOC3 levels were further elevated with LDLR ASO-mediated dyslipidemia. Importantly, plasma APOC3 levels

were significantly suppressed by APOC3 ASO treatment (Fig. 1E). In addition to effects on lipid levels, treatment with APOC3 ASO also suppressed blood glucose in leptin-deficient diabetic mice (Fig. 1F). The improvement in glycemia was associated with improved tolerance to an intraperitoneal glucose challenge (data not shown).

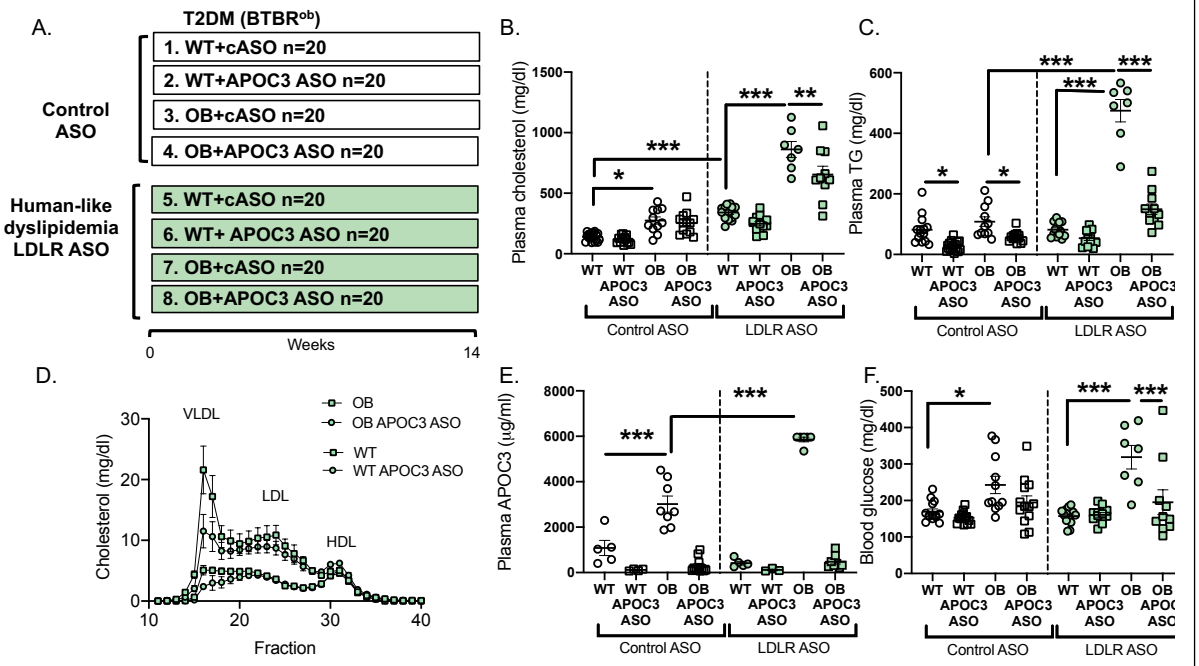

Figure 1. **A**. Study set up. Male and female BTBR WT and leptin-deficient mice (OB) mice were injected with either a control antisense (ASO) or and ASO against LDLR to induce human-like dyslipidemia. The N's are the number of mice we attempted to achieve. Within each group ½ of the mice were given a control ASO (cASO) or an APOC3 ASO. Upon initiation of the ASO treatment the mice were also fed an atherogenic diet for 14 weeks. **B**. Plasma cholesterol at week 14. **C**. Plasma TG at week 14. **D**. Cholesterol lipoprotein profile from LDLR ASO injected mice. Two lower lines are the WT mice. **E**. Plasma APOC3 at the end of the study. **F**. Blood glucose at the end of the study. N=7-15, 1-way ANOVA.

APOC3 ASO significantly reduces diabetes-accelerated atherosclerosis in dyslipidemic leptin-deficient mice

At the end of the study, we analyzed atherosclerosis at three separate sites, namely the aorta, the aortic sinus, and the brachiocephalic artery. The aorta was open longitudinally and stained with the lipophilic stain Sudan IV to identify atherosclerotic lesions. Consistent with previous reports, neither WT nor leptin-deficient mice without human-like dyslipidemia developed significant atherosclerosis either in the aorta or the BCA (Fig. 2A-C). WT mice treated with the LDLR ASO had some atherosclerosis; however, atherosclerosis was primarily present in leptin-deficient LDLR ASO treated mice (Fig. 2A-C). In line with our data from mice with type 1 diabetes, APOC3 ASO treatment significantly reduced atherosclerosis in the mice with type 2 diabetes in the aorta, and the BCA (data from the aortic sinus is pending).

Furthermore, APOC3 ASO treatment completely prevented APOC3 accumulation in the artery wall (Fig. 2D). Also, fewer macrophages accumulated in the BCA when APOC3 was reduced. The reduced macrophage accumulation was not due to changes in white

blood cell concentrations or composition, as APOC3 ASO did not suppress total white blood cells, monocytes or neutrophils in WT or leptin-deficient mice (data not shown). APOC3 ASO treatment did, however, reduce a marker of lipid accumulation in monocytes, which was selectively elevated in LDLR ASO treated leptin-deficient mice.

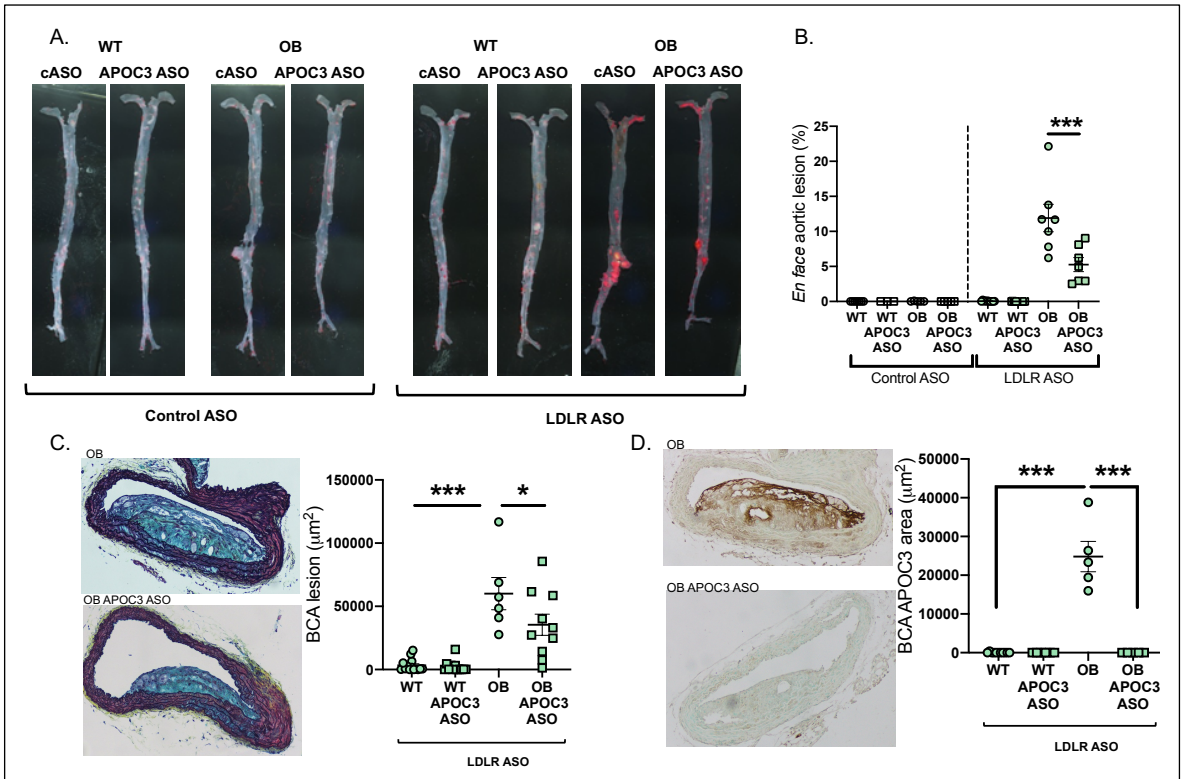

Figure 2. Male and female BTBR WT and leptin-deficient mice (OB) mice were injected with either a control antisense (ASO) or and ASO against LDLR to induce human-like dyslipidemia . Within each group ½ of the mice were given a control ASO (cASO) or an APOC3 ASO. Upon initiation of the ASO treatment the mice were also fed an atherogenic diet for 14 weeks. **A**. Representative aortic atherosclerosis analyzed *en face* after Sudan IV-staining (red stain). **B**. Quantification of aortic *en face* lesions. **C**. Representative lesions in the brachiocephalic artery (BCA) stained with Movat's pentachrome stain in LDLR ASO treated leptin-deficient mice with and without APOC3 ASO (left) and quantification to the right. **D**. Representative lesions in the brachiocephalic artery (BCA) stained with an APOC3 antibody in LDLR ASO treated leptin-deficient mice with and without APOC3 ASO (left) and quantification to the right. N=7-15 (B), N=6-11 (C-D), 1-way ANOVA.

Human-like dyslipidemia results in glomerular lipid accumulation which is suppressed by APOC3 ASO treatment

At the end of the study, in addition to atherosclerosis, kidney pathology was also evaluated. Parts of kidneys were embedded freshly in OCT, cryosectioned and stained with oil red-o to visualize neutral lipid accumulation. Almost no lipid accumulation was observed in WT or leptin-deficient mice that did not display human-like dyslipidemia (did not get LDLR ASO). Adding human-like dyslipidemia to leptin-deficient mice resulted in almost every single glomerulus having neutral lipid accumulation (Fig 3A-B). The glomerular lipid accumulation was suppressed by APOC3 ASO treatment. Furthermore,

the droplets that were accumulating under APOC3 ASO conditions were smaller and fewer (data not shown).

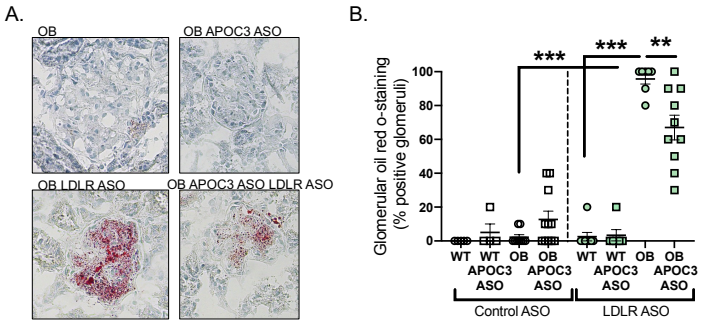

Figure 3. Male and female BTBR WT and leptin-deficient mice (OB) were injected with either a control antisense (ASO) or and ASO against LDLR to induce human-like dyslipidemia . Within each group ½ of the mice were given a control ASO (cASO) or an APOC3 ASO. Upon initiation of the ASO treatment the mice were also fed an atherogenic diet for 14 weeks. A. Representative glomerulus stained with Oil Red O. **B**. Quantification of number of Oil red O-positive glomeruli. N=6-11, 1-way ANOVA.

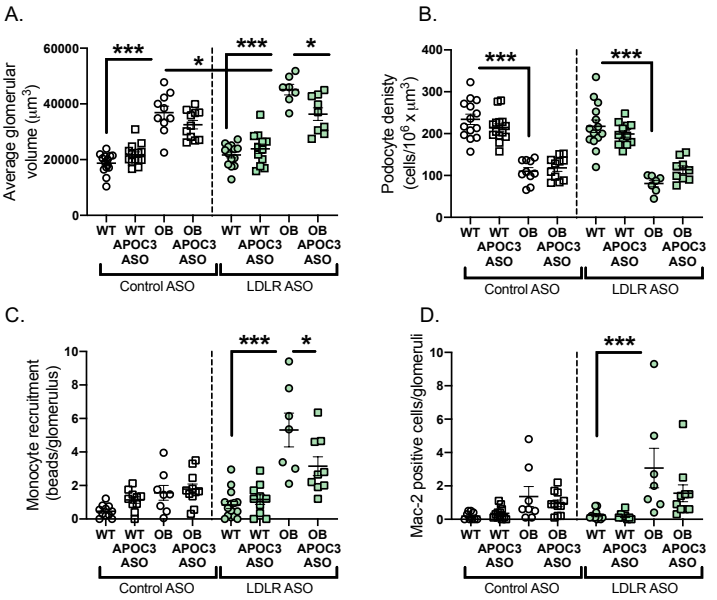

Figure 4. Male and female BTBR WT and leptin-deficient mice (OB) were injected with either a control antisense (ASO) or and ASO against LDLR to induce human-like dyslipidemia . Within each group ½ of the mice were given a control ASO (cASO) or an APOC3 ASO. Upon initiation of the ASO treatment the mice were also fed an atherogenic diet for 14 weeks. **A**. Quantification of glomerular volume. **B**. Podocyte was determined based on p57 staining and quantified. **C**. Yellow-green latex beads were injected 4 days prior to euthanasia. The beads are primarily taken up by monocytes (specifically Ly6C^{lo} monocytes). The number of beads per glomerulus was enumerated. **D**. Glomerular macrophage accumulation based on Mac-2 staining. N=7-14, 1-way ANOVA.

Diabetes results in glomerular hypertrophy which is further exacerbated by dyslipidemia.

We have previously demonstrated that the addition of human-like dyslipidemia (induced by targeted deletion of the LDL receptor) results in worsening diabetic kidney disease, especially augmenting the inflammatory response observed in diabetes. Consistent with this, we found that leptin-deficiency resulted in glomerular hypertrophy (Fig. 4A). The glomerular hypertrophy was augmented by dyslipidemia, which in turn was suppressed by APOC3 ASO treatment. Podocytes play an essential role in maintaining the glomerular barrier function. The number of podocytes was suppressed by leptin-deficiency (Fig. 4B). Although not statistically significant when comparing all 8 groups, induction of human-like dyslipidemia appeared to further reduce podocyte numbers, which in turn was rescued by APOC3 ASO treatment. To evaluate if diabetes and/or dyslipidemia stimulated monocyte recruitment to the glomerulus, monocytes were labeled with yellow-green latex beads 4 days before

euthanasia and followed into the kidneys. Importantly, similar labeling was achieved between the different cohorts of mice. In leptin-deficient mice treated with LDLR ASO, more beads accumulated in the glomerulus compared to in WT mice, which was suppressed by APOC3 ASO treatment (Fig. 4C). Consistently, Mac-2 staining, which stains macrophages, was also increased in leptin-deficient mice treated with LDLR ASO with an apparent suppression in APOC3 ASO treated mice (Fig. 4D).

In summary, the addition of human-like dyslipidemia results in accelerated atherosclerosis in a model of type 2 diabetes, which can be suppressed by APOC3 inhibition. Furthermore, the same dyslipidemia that accelerates atherosclerosis also promotes markers of kidney disease through a process associated with augmented myeloid cell infiltration. Again, this is abrogated by APOC3 silencing

3. Publications:

1. Kanter JE: FOXP1: A Gatekeeper of Endothelial Cell Inflammation. Circ Res. 2019 Aug 30;125(6):606-608. doi: 10.1161/CIRCRESAHA.119.315687. Epub 2019 Aug 29. PMID:31465266

2. Kanter JE, Hsu C-C, Bornfeldt KE: Monocytes and macrophages as protagonists of diabetic complications. Front Cardiovasc Med. 2020 Feb 14;7:10. doi: 10.3389/fcvm.2020.00010. eCollection 2020. Review. PMID: 2118048