




Reversal of hypertriglyceridemia in diabetic BTBR *ob/ob* mice does not prevent nephropathy

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Received: 10 September 2020 / Revised: 5 March 2021 / Accepted: 8 March 2021 / Published online: 28 April 2021
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Abstract

The etiology of diabetic nephropathy in type 2 diabetes is multifactorial. Sustained hyperglycemia is a major contributor, but additional contributions come from the hypertension, obesity, and hyperlipidemia that are also commonly present in patients with type 2 diabetes and nephropathy. The leptin deficient BTBR *ob/ob* mouse is a model of type 2 diabetic nephropathy in which hyperglycemia, obesity, and hyperlipidemia, but not hypertension, are present. We have shown that reversal of the constellation of these metabolic abnormalities with leptin replacement can reverse the morphologic and functional manifestations of diabetic nephropathy. Here we tested the hypothesis that reversal specifically of the hypertriglyceridemia, using an antisense oligonucleotide directed against ApoC-III, an apolipoprotein that regulates the interactions of VLDL (very low density lipoproteins) with the LDL receptor, is sufficient to ameliorate the nephropathy of Type 2 diabetes. Antisense treatment resulted in reduction of circulating ApoC-III protein levels and resulted in substantial lowering of triglycerides to near-normal levels in diabetic mice versus controls. Antisense treatment did not ameliorate proteinuria or pathologic manifestations of diabetic nephropathy, including podocyte loss. These studies indicate that pathologic manifestations of diabetic nephropathy are unlikely to be reduced by lipid-lowering therapeutics alone, but does not preclude a role for such interventions to be used in conjunction with other therapeutics commonly employed in the treatment of diabetes and its complications.

Introduction

Diabetes is the most common cause of end stage renal disease (ESRD) in Western countries and is an increasing cause of ESRD in less-developed countries [1]. It is estimated that ~40% of type 2 diabetic (T2D) patients will develop diabetic nephropathy (DN) [1–3]. In several populations that have been studied, DN is more common in the context of T2D than type 1 diabetes (T1D) [4–6]. The

higher risk occurs at every stratum of hemoglobin A1c, consistent with the concept that factors other than hyperglycemia contribute to the difference in incidence between those with T1D and T2D.

Dyslipidemia is a more common feature of T2D than T1D [6–11]. This is in part because dyslipidemia is uncommon in T1D patients with good glycemic control, whereas dyslipidemia persists in T2D patients, even when they control their hyperglycemia, in large part because it is associated with insulin resistance and obesity. A strong relationship between dyslipidemia and obesity also occurs in nondiabetic individuals. The most common dyslipidemia in T2D is hypertriglyceridemia, which is due to excessive VLDL production and is often exacerbated by a reduction in lipoprotein lipase (LpL)-mediated triglyceride removal from circulating triglyceride-rich lipoproteins; chylomicrons and VLDL [12].

Whereas the link between hyperlipidemia and atherosclerosis is extremely well established and has been a major part of atherosclerosis research for many years, the connection between lipids and nephropathy is far less

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established [12–14]. Nonetheless, there is evidence suggesting a causal link between dyslipidemia and DN. Patients with DN have elevations in apoB containing lipoproteins (VLDL and LDL) and an increased level of ApoC-III [11, 15]. Conversely, DN itself exacerbates these same lipid abnormalities [16].

The aim of this study was to test a highly targeted and effective therapy for hypertriglyceridemia in an established mouse model of DN. We have established that the leptin deficient *lep^{ob/ob}* BTBR (BTBR*ob/ob*) mouse model of T2D is a good model with both sustained hyperglycemia and hypertriglyceridemia that also exhibits many of the same pathological manifestations as human DN [17]. We have also demonstrated the reversibility of DN in this model with reversal of obesity and correction of hyperglycemia by administration of leptin [18]. Thus, we used this animal model to investigate the specific effect of triglyceride lowering on DN. We used antisense oligonucleotides directed against ApoC-III to reduce triglyceride levels in the BTBR*ob/ob* mice.

Methods

BTBR T+ Ipr3tf/J and C57BL/6J *Lep^{ob/+}* mice were obtained from the Jackson Laboratory. A congenic strain was derived by crossing these two strains and back-crossing into the BTBR background, creating the inbred BTBR *ob/ob* leptin deficient strain [19]. The mice have been maintained in the Attie Lab for >20 years and were rederived at JAX to make them available to the research community (Jax #004824 BTBR.Cg-*Lep^{ob}/WiscJ*). The genomic region on chromosome 6 surrounding the *Lep* gene has been reduced to ~24 Mb through marker-assisted selection [20]. Mice aged 2.6–4.9 wks were injected intraperitoneally with 2nd generation antisense oligonucleotides (ASOs; Ionis, Inc.) that targeted *Apoc3* at a dose of 12.5 mg/kg/wk on a weekly basis for 8 wks. The dose was determined by previously reported dose responses in multiple experimental systems. The control ASO was Ionis # 141923-91 [21]. The number of mice in each cohort was: female controls ASO = 5, females treated with ApoC3 ASO = 7, male controls = 12, and males treated with ApoC3 = 11.

Blood samples were collected via retro-orbital bleeding after fasting for 4 h, starting at 8 a.m. and ending at noon. Plasma triglyceride levels were measured using a commercially available kit (TR22421, Thermo Fisher Scientific). Urine samples were obtained by placing the mouse in an empty cage and collecting urine as it was voided. The collected urine was frozen for subsequent analysis of creatinine and albumin. Creatinine and albumin were measured using well-established protocols recommended by the Diabetic Complications Consortium (www.diacomp.org),

including standard protocols using the Albuwell M kit (Exocell, Inc, Philadelphia PA, US) and the Urinary Creatinine companion kit (Exocell).

Histological analysis was performed by staining routinely processed formalin fixed, paraffin embedded kidney tissue with Jones Silver methanamine reagent, and the resulting stain assessed by computerized morphometry using ImageProPlus to measure the percentage of positively stained mesangial matrix as compared to the total glomerular area, as previously described [17, 18]. Podocytes were counted using the Venkatareddy et al. method [22], as previously utilized by our laboratory [23]. Briefly, formalin fixed tissue sections were stained with antibodies specific for Wilm's tumor antigen (WT-1) (Abcam, Cambridge, MA, ab89901). Glomerular tuft area, and podocyte nuclear size were measured using Image J in 50 consecutive glomeruli, moving in a serpentine pattern through the cortex. The number of WT-1 positive podocytes were counted in the same 50 glomeruli and total glomerular podocyte number was calculated using the Venkatareddy et al. formula [22].

Results

We used antisense oligonucleotides (ASOs) kindly provided to us by Ionis, Inc. to suppress the expression of *Apoc3*. Ionis' ASOs against *Apoc3* were previously evaluated in several mouse and rat models, including C57BL6/J mice on high-fat diet, LDL receptor-knockout mice, *ob/ob* mice, LDL receptor-knockout mice expressing CETP, rats on a fructose diet, and the ZDF rat [21]. The ASOs resulted in ~70% decrease in circulating ApoC-III protein levels (Fig. 1).

ASO-treated BTBR *ob/ob* mice showed no difference in body weight (Fig. 2A) or fasting plasma glucose (Fig. 2B) compared to control mice. In contrast, treatment with the *Apoc3* ASOs caused a significant drop in circulating triglyceride levels after 1 month of treatment (8 weeks of age)

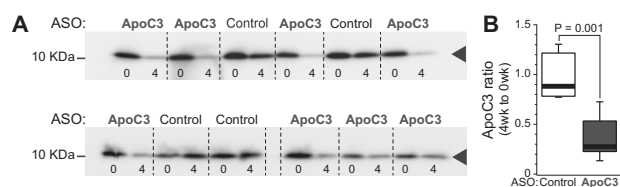


Fig. 1 Antisense oligonucleotide (ASO) knockdown of ApoCIII. **A** Western blot analysis shows the decrease in circulating ApoCIII protein levels in both male and female BTBR *ob/ob* mice with ASO treatment, compared to control ASO prior to the start of treatment (0) and after 4 weeks of treatment (4). **B** The ratio of circulating ApoCIII protein levels prior to treatment and after 4 weeks of treatment (4 to 0 wk) shows an approximate 70% decrease with ApoCIII ASO treatment compared to control ASO treatment.

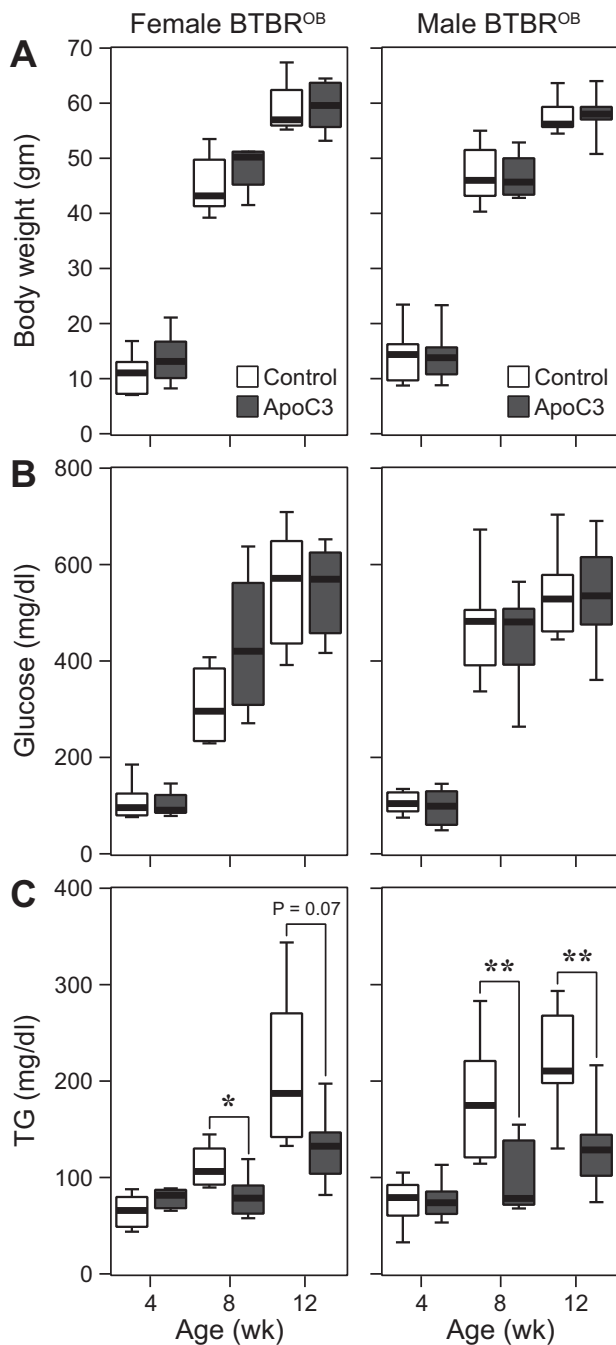


Fig. 2 Metabolic analysis of ApoCIII ASO treatment. **A** Treatment with ApoCIII ASO had no effect on body weight in either male or female BTBR *ob/ob* mice compared to control ASO at week 0, after 4 weeks of treatment or after an additional 4 weeks post treatment (week 8). **B** Plasma glucose was also unchanged with ApoCIII ASO treatment, shown by levels at 0, 4, and 8 weeks. **C** Circulating triglyceride levels were significantly decreased in both male and female BTBR *ob/ob* mice with 4 weeks of ApoCIII ASO treatment, and this decrease persisted for another 4 weeks post treatment, shown by levels at 8 weeks.

that was sustained for an additional month of the study (Fig. 2C). There was a range of tryglyceride levels in the various study cohorts consistent with what commonly has

been observed in obese mice compared to lean mice. Thus, the *Apoc3* ASOs dissociated dyslipidemia from hyperglycemia in BTBR-*ob/ob* mice, enabling us to specifically evaluate the effect of lowered TGs on DN.

The mice treated with the control and *Apoc3* ASOs did not differ in their urinary albumin/creatinine ratio (ACR), a sensitive measure of kidney function (Fig. 3A). The corresponding functional studies showed no significant differences in urinary albumin excretion between mice treated with the antisense reagent and untreated control mice of comparable age (Fig. 3A). Using each mouse as its own control, measurement of each diabetic mouse treated with antisense at the start, midpoint, and endpoint of the treatment demonstrated no sustained beneficial effect on protein excretion at the end of the 8 week course of therapy (data not shown). Renal function, as measured by ACR demonstrated no statistical difference between treated and untreated cohorts (Fig. 3A). There was considerable variability in the measured ACR, in particular in the 4 week group. The BTBR*ob/ob* mice have previously been shown to have variability in ACR measurements [17, 18]. There was no correlation between the ACR and podocyte number, podocyte density, or the amount of mesangial matrix in all of the cohorts of mice.

Histologic examination of kidney tissue revealed no qualitative differences between male and female mice, nor were there differences in histology in treated or untreated mice (Fig. 3B). All experimental mouse cohorts had comparable glomerular mesangial volumes (Fig. 3) and podocyte number (female controls: 28.7 ± 3.2 ; female ApoC3: 30.8 ± 2.9 ; male controls: 29.7 ± 1.8 ; male ApoC3: 34.1 ± 3.1 podocytes/glomerulus) and density (female controls: 68.2 ± 5.5 ; female ApoC3: 74.7 ± 5.0 ; male controls: 78.7 ± 9.1 ; male ApoC3: 79.0 ± 7.7 podocytes/ $10^6 \mu\text{m}^3$ (Fig. 4), and were without features of glomerular mesangiolysis, segmental sclerosis, or significant inflammatory cell infiltration. The qualitative assessment of similar volumes of mesangial matrix in all cohorts was confirmed in the morphometric analyses (Fig. 3C). The tubulointerstitial parenchyma was well preserved in all cohorts, and lacked features of interstitial fibrosis, tubular atrophy, inflammation, or acute tubular injury in both treated and untreated cohorts

Discussion

We previously established that *leptin*^{*ob/ob*} BTBR (BTBR*ob/ob*) mice are an excellent model of DN, exhibiting many of the same pathological manifestations of human DN [17]. BTBR-*ob/ob* mice show mesangial expansion and podocyte loss, important features of the human disease. Beginning as early as 6 weeks of age, these mice demonstrate progressive

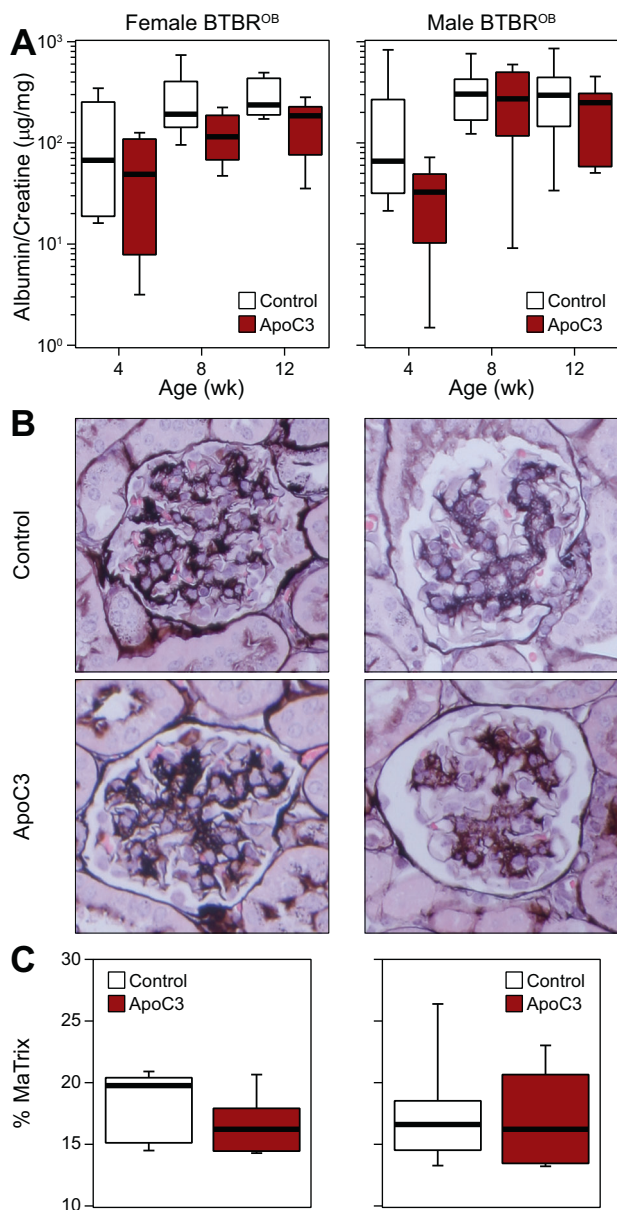


Fig. 3 Functional and morphologic analysis of study mice. **A** Analysis of urine samples to determine albumin/creatinine ratios (ACRs) show no significant differences between males and females and between treated and untreated mice. **B** Representative glomeruli from male and female mice treated with either ApoCIII ASO or control ASO (silver methenamine stain). There was no histologic difference seen within glomeruli or the tubulointerstitium between the groups. **C** Computerized morphometric analysis of randomized glomeruli demonstrates no differences in the accumulation of mesangial matrix between the study groups.

proteinuria, glomerular hypertrophy, and glomerular lesions that resemble those of human DN, including, increased basement membrane thickness, increased mesangial matrix, diffuse mesangial sclerosis, mesangiolysis, and loss of podocytes. Focal mild interstitial fibrosis is also a feature in mice with sustained diabetes and aged 18 weeks or longer [17, 24]. In addition, it has been shown that podocyte loss

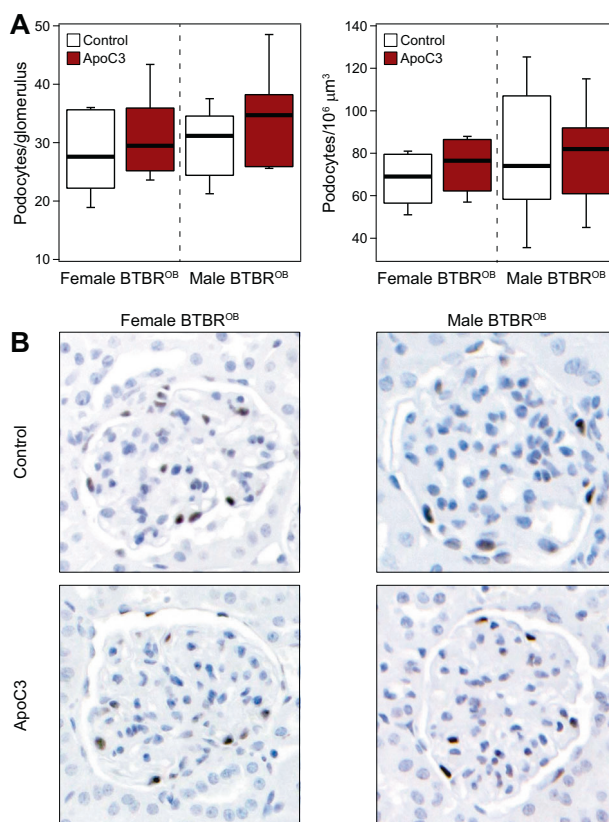


Fig. 4 Quantification of glomerular podocytes. **A** Podocyte numbers and density show no significant differences between males and females and between treated and untreated mice. **B** Representative glomeruli from male and female mice treated with either ApoCIII or control ASO (WT-1 immunohistochemistry).

and mesangial expansion is reversible upon restoration of normoglycemia resulting from leptin administration [18]. The development of some features of advanced DN in a relatively short time frame (18 weeks) compared to other available mouse models of DN, and the reversibility of these lesions of DN with restoration of a normal metabolic milieu, made this mouse an especially attractive model in which to test the hypothesis that triglyceride lowering can ameliorate the pathologic and functional manifestations of DN.

Although severity and duration of hyperglycemia predicts development and progression of diabetic microvascular complications in both type 1 and type 2 diabetes, correction of hyperglycemia does not guarantee reversal of these complications, indicating the effects of a multi-factorial injury processes [2]. We hypothesized that the dyslipidemia that is common in diabetes, especially type 2 diabetes, could play an essential role in the development and persistence of DN.

This study was designed to test whether a highly targeted and effective therapy for hypertriglyceridemia would ameliorate nephropathy in an established mouse model of DN. In a prior study, we tested the effect of a PPAR α agonist on

DN in BTBR-*ob/ob* mice [25]. In that previous study hyperglycemia and hypertriglyceridemia were reduced, but DN persisted. PPAR α potentially induces genes involved in fatty acid oxidation. Intermediates in fatty acid oxidation can generate reactive oxygen species and it has been hypothesized that excessive fatty acid flux into mitochondria generate intermediates that cause metabolic dysfunction [26]. We hypothesized that lipid oxidation epitopes are implicated in DN. This would predict that inducing β -oxidation throughout all tissues may be detrimental. Instead, we proposed that a better strategy is to promote the sequestration of lipids, primarily in adipose tissue. Indeed, it has been argued that lipotoxicity in tissues like liver, heart, β cells, and kidney is caused by inadequate adipose lipid sequestration, as occurs in lipodystrophy [27, 28].

An analogous situation informing our studies is that of atherosclerosis. Atherosclerosis is an inflammatory disease [29]. There is strong evidence to support a pathway by which lipid peroxidation modifies proteins to produce epitopes that activate both the innate and adaptive immune responses [30]. Most of the lipids that are subject to peroxidation are fatty acids esterified to triglycerides, phospholipids, and cholesterol on the lipoprotein particles. Eight classes of macrophage scavenger receptors that bind to epitopes derived from lipid peroxidation have been described [29]. Several of these receptors were first discovered for their ability to bind to modified lipoproteins. But they also bind microbial pathogens. They can present antigens to elicit the adaptive immune response and thus link the innate and adaptive immune responses [30].

The pioneering work of Witztum and co-workers has shown that lipid-derived epitopes comprise a major stimulus for the innate and acquired immune responses and inflammation, leading to atherosclerosis [31, 32]. Witztum developed a monoclonal antibody, “E06”, that recognizes these epitopes. Remarkably, natural antibodies against the oxidation epitopes have identical sequences in the antigen binding variable region to that of E06 [33]. Most importantly, administration of a recombinant single-chain E06 antibody protects LDL receptor-knockout mice against atherosclerosis [34]. Lipid peroxidation products like those recognized by the E06 antibody can be detected in kidneys from diabetic subjects [35]. The premise of our study was that there is overlap between the inflammatory processes that underlie atherosclerosis and DN.

In humans, much of the fatty acid substrate for hepatic triglyceride production is derived from adipose tissue, although hepatic de novo lipogenesis also contributes to triglyceride (TG) production [36]. Adipose tissue is the most insulin-sensitive tissue in the body. Insulin exerts a potent inhibitory effect on the hydrolysis of adipose triglycerides. Thus, insulin resistance and relative insulin deficiency, which is commonly associated with T2D, leads

to excess adipose tissue lipolysis and increases flux of free fatty acids to the liver. These fatty acids are re-esterified to form triglycerides, which are stored as triglyceride droplets. When in excess, this leads to hepatic steatosis. The triglycerides are also packaged into very low density lipoprotein (VLDL) particles, which are secreted into the circulation. Thus, hepatic steatosis and hypertriglyceridemia are common features of insulin resistance.

VLDL triglycerides are hydrolyzed by LpL, which is bound to the luminal surface of the capillary endothelium, primarily in adipose tissue and heart. Apolipoproteins mediate the interaction of VLDL particles with the vascular wall and the action of LpL. It was previously thought that the *primary* mechanism of action of Apo-CIII is to inhibit LpL, but recent evidence indicates that its *primary* mechanism of action is by blocking VLDL interactions with the LDL receptor and the LDL receptor related protein-1 [37]. We cannot completely exclude the possibility that Apo-CIII is able to inhibit LpL.

The relationship between elevated LDL and coronary heart disease (CHD) has been known for a very long time and is supported by a vast array of genetic and intervention studies, as well as mechanistic studies in animal models [12–14]. The relationship between hypertriglyceridemia and atherosclerosis has been more complicated and controversial. People with hypertriglyceridemia tend to have low HDL levels. Accordingly, it was widely believed that HDL was the “good cholesterol” and low HDL, not high triglycerides, was the culprit in promoting atherosclerosis. However, recent Mendelian randomization studies with mutations in endothelial lipase, which only affect HDL cholesterol, show no effect on CHD [38]. On the other hand, rare loss-of-function mutations in ApoC-III lead to a 39% reduction in serum triglyceride and a 40% reduction in CHD [39]. Finally, 185 common variants affecting triglyceride levels were analyzed and the clear conclusion is that their effect on triglycerides, not HDL, predicts the risk of CHD [40].

Like humans with type 2 diabetes, BTBR-*ob/ob* mice have hypertriglyceridemia and hyperglycemia. Given the above rationale, we hypothesized that elevated lipids, combined with alleles specific to BTBR mice, lead to development of DN. Using ASO-mediated repression of *Apoc3* expression, a now well established means to lower serum triglycerides [41], we succeeded in reducing circulating ApoC-III protein levels, bringing TG levels to near-normal. Despite this reduction in serum TG, we did not observe any discernible functional or structural improvement in DN, as evaluated through the urine ACR or histological analysis. Although this is a negative finding, when considered in conjunction with our earlier studies on the effect of a PPAR α agonist in this same mouse model it provides important evidence that the pathologic

manifestations of DN are unlikely to be reduced by lipid-lowering therapeutics alone. Nonetheless, there may still be benefit from such agents when used in conjunction with other therapeutics commonly employed in the treatment of diabetes and its complications, such as selective endothelin receptor antagonists, SGLT2 inhibitors, and inhibitors of the renin–angiotensin–aldosterone system.

Author contributions AA and MK performed study concept and design. AA, KS, MK, KM, SS, KH, and CA performed development of methodology and writing, review, and revision of the paper; KM, SS, KH, and KM provided acquisition of data and statistical analysis; MG and RL provided technical and material support. All authors read and approved of the final paper.

Funding This study was supported by a grant from NIDDK 5R01DK101573-07. Financial support for this work provided by the NIDDK Diabetic Complications Consortium (RRID:SCR_001415, www.diacomp.org), grants DK076169, and DK115255.

Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

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