Cyclodextrin protects podocytes in diabetic kidney disease

Short running title: Cholesterol and podocyte malfunction in DKD

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ABSTRACT

Diabetic kidney disease remains the most common cause of end-stage kidney disease despite multifactorial intervention. We demonstrated that increased cholesterol in association with down-regulation of ATP-binding cassette transporter ABCA1 occurs in normal human podocytes exposed to the sera of patients with type 1-diabetes and albuminuria (DKD+) when compared to diabetic patients with normoalbuminuria (DKD-) and similar duration of diabetes and lipid profile. Glomerular down-regulation of ABCA1 was confirmed in biopsies from patients with early DKD (n=70) when compared to normal living donors (n=32). Induction of cholesterol efflux with cyclodextrin (CD) but not inhibition of cholesterol synthesis with simvastatin prevented podocyte injury observed in vitro after exposure to patient sera. Subcutaneous administration of CD to diabetic BTBR ob/ob mice was safe and reduced albuminuria, mesangial expansion, kidney weight and cortical cholesterol content. This was followed by an improvement of fasting insulin, blood glucose, body weight, glucose tolerance in vivo and improved glucose stimulated insulin release in human islets in vitro. Our data suggest that impaired reverse cholesterol transport characterizes clinical and experimental DKD and negatively influences podocyte function. Treatment with CD is safe and effective in preserving podocyte function in vitro and in vivo and may improve the metabolic control of diabetes.
INTRODUCTION

Diabetic kidney disease (DKD) is responsible for nearly half of the incidence of end-stage kidney disease (ESKD) in the USA (1), yet our current understanding of the pathophysiological processes responsible for DKD has led to limited improvements in patient outcomes. Multifactorial intervention reduces the rate of progression of DKD, but does not prevent ESKD in type 1 (T1D) or type 2 diabetes (T2D) (2; 3). A key factor for this translation gap is the current lack of adequate mechanistic insight into DKD in humans.

The kidney glomerulus is a highly specialized structure that ensures the selective ultrafiltration of plasma so that essential proteins are retained in the blood (4). Podocytes are glomerular epithelial cells that contribute to the glomerular filtration barrier through a tight regulation of actin cytoskeleton remodeling (4). Currently, the diagnosis of DKD relies on the detection of microalbuminuria (5). However, a growing body of evidence suggests that key histological lesions precede the development of albuminuria (6; 7); among them, decreased podocyte number (podocytopenia) has been described as an independent predictor of DKD progression (8-12).

Although we have previously shown that podocyte insulin resistance and susceptibility to apoptosis is already present at the time of onset of microalbuminuria in experimental models of DKD, the cause of podocyte injury in early DKD remains unknown (13).

We used a previously established cell-based assay in which differentiated human podocytes are exposed to 4% patient sera for 24 hours (14) to identify new pathways and targets in DKD. Podocytes exposed to the sera of patients with DKD showed increased cholesterol accumulation in association with down-regulation of ATP-binding cassette transporter 1 (ABCA1) expression that was independent of circulating cholesterol.

ABCA1 is a major regulator of cellular cholesterol homeostasis by mediating efflux to lipid-poor apolipoprotein acceptors in the bloodstream (15). ABCA1 genetic variants are strongly associated
with the risk of coronary artery disease (16). Furthermore, the capacity of patient sera to induce ABCA1 mediated cholesterol efflux in macrophages is impaired in patients with T2D and incipient or overt nephropathy (17). Excessive cholesterol accumulation has been described in glomeruli of rodent models of T1D and T2D (18-20) and may contribute to DKD development and progression. Finally, inactivating mutations of ABCA1 result in Tangier disease, which causes premature atherosclerosis and proteinuria (21).

While interventions that increase ABCA1 expression (such as liver X receptor agonists) may be beneficial in DKD, they have a relatively high incidence of adverse events (22) as well as intrinsic lipogenic effects (23). We used β-cyclodextrins, cyclic oligosaccharides consisting of 7 β(1-4)-glucopyranose rings, to remove cholesterol from differentiated human podocytes in vitro and from diabetic animals in vivo. The exact mechanism by which cyclodextrins remove cholesterol from cells are not completely understood but the formation of cholesterol/cyclodextrin inclusion complexes at the membrane surface plays an important role in this process (24).

We hypothesized that 2-Hydroxypropyl-β-cyclodextrin (CD), which was recently approved by the Food and Drug Administration (FDA) for the cure of Niemann-Pick disorder (25; 26), would be an effective way to sequester cholesterol and to protect podocytes from cholesterol dependent damage in DKD in vivo and in vitro.

**RESEARCH DESIGN AND METHODS**

*Patient sera and kidney biopsies.* Serum samples were obtained from ten healthy controls and twenty patients with T1D from the Finnish Diabetic Nephropathy Study (FinnDiane). T1D was defined as onset of diabetes before 40 years of age and permanent insulin treatment initiated within 1 year of diagnosis. Urinary albumin excretion rate (AER) was defined as normal AER (<30mg/24h), microalbuminuria (≥30<300mg/24h), and macroalbuminuria (≥300mg/24h).
Fasting glucose values were measured using a Hemocue device (Hemocue, Finland). Serum lipids were determined with a Konelab analyser (Thermo Scientific, Finland). Other biochemical analyses were performed in an accredited hospital laboratory (HUSLAB, Helsinki). For glomerular mRNA expression profiles, kidney biopsy specimens were procured from 70 Southwestern American Indians after obtaining informed consent. Human renal biopsies from pre-transplant, healthy living donors (n=32), membranous nephropathy (n=21) and focal segmental glomerulosclerosis (n=18) patients were obtained from the European Renal cDNA Bank.

**Illumina array platform analysis and Affymetrix GeneChip analysis.** For Illumina array platform analysis, four pools of sera were prepared per patient group, C, DKD-, DKD+, by combining sera from 2-3 patients. Podocytes were treated with 4% of the pooled sera (4 C, 4 DKD-, 4 DKD+ pools) for 24 h. More detailed protocol information and results are available at http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46900.

For glomerular mRNA expression profiles of *ABCA1, HMG-CoA, LDLR, SREBP1* and *SREBP2*, kidney biopsy specimens were procured from 70 Southwestern American Indians enrolled in a randomized, placebo-controlled, clinical trial to evaluate the renoprotective efficacy of losartan in T2D (ClinicalTrials.gov, #NCT00340678) after obtaining informed consent. Human renal biopsies from pre-transplant, healthy living donors (n=32), membranous nephropathy (n=21) and focal segmental glomerulosclerosis (n=18) patients were obtained from the European Renal cDNA Bank following the guidelines of local ethic committees. Biopsy tissue specimens were microdissected as described (27-29). Glomerular gene expression profiling was performed using Human Genome U133A and U133plus2 Affymetrix GeneChip arrays (27; 29). Raw image files were normalized and annotated. Log2 transformed datasets were batch-corrected using Combat (30) from Genepattern. Differential regulation analysis between controls and disease groups was
performed using Significance Analysis of Microarray (SAM) as implemented in the Mev software suite (31; 32). Genes passing an FDR correction (q-value) for multiple testing below 5% were considered significantly regulated.

**Human podocyte culture.** Human podocytes were cultured and differentiated in RPMI culture medium containing 10% FBS and 1% Penicillin/Streptomycin as previously described (33). Briefly, immortalized normal human podocytes were propagated at 33°C and then thermoshifted for differentiation for 14 days at 37°C. Terminally differentiated podocytes were serum and insulin starved in 0.1% FBS (Insulin experiments) or 0.2% FBS for all other experiments. When patient sera were utilized, starved cells were exposed to 4% patient sera in FBS-free culture medium for 24 hours. For insulin treatment experiments, 100 nmol insulin was added to the culture medium for 15 minutes after exposure to patient sera. For cyclodextrin or statin treatment experiments serum starved human podocytes were pretreated for 1 hour with 5 mM Methyl-beta-Cyclodextrin (CD, Sigma) or simvastatin (1 µM, Sigma).

**Immunofluorescence staining.** Cells cultured in chamber slides were fixed with 4% PFA for 30 minutes at 37°C and permeabilized with 0.1% Triton X-100, followed by exposure to mouse-anti-phosphorylated caveolin (pY14, BD Biosciences), anti-active-RhoA (New East Biosciences), or anti-vimentin (Sigma) antibodies. Fluorescence detection was performed using Alexa Fluor secondary antibodies (Invitrogen). For cholesterol determination, filipin (Sigma) staining was performed as described (34). F-actin was visualized by Rhodamine Phalloidin (Invitrogen). Two hundred consecutive cells per condition were studied. Slides were prepared with DAPI enriched mounting media (Vectashield) and analyzed by confocal microscopy.

**Apoptosis analysis.** Apoptosis was assessed using the Caspase-3/CPP32 Colorimetric Assay kit (Biovision) according to the manufacturers description. Caspase 3 activity was normalized to cell number and expressed as fold change to controls.
**Determination of cholesterol content and cholesterol efflux assay.** Esterified cholesterol was determined as difference between total and free cholesterol using an enzymatic assay and normalized to cell protein content (35). The cellular content of lipid droplets was determined using Oil red O (ORO). Cells were fixed and permeabilized as described above, washed in PBS and in 60% isopropanol, followed by incubation in ORO (0.5% ORO in isopropanol, 1:3 diluted) for 15 min at room temperature and counterstaining with hematoxylin for 1 min. The fraction of ORO positive cells over two hundred consecutive cells was calculated by bright field microscopy. To measure cholesterol efflux from differentiated human podocytes a previously described method (36) was used with some modifications. Differentiated human podocytes were labeled with 2 µCi/ml 3H-cholesterol in medium with 2% FBS for 16 hours, the cells were washed with PBS, then incubated in RPMI containing 0.5 % patient sera for 6 hours. Four independent experiments were performed and each sample was run in duplicate or triplicate. Cholesterol efflux to medium containing serum was expressed as % of total cell cholesterol as previously described (36; 37).

**Western blotting and luminex.** Cell lysate collection and Western blotting was performed as previously described (14). The following primary antibodies were used: rabbit-polyclonal-anti-MyD88 (Cell Signaling), rabbit-polyclonal-anti-phosphorylated (Y473) or anti-total-AKT (Cell Signaling), mouse-monoclonal-anti-rhoA (Santa Cruz), or mouse-monoclonal-anti-Gapdh (6C5, Calbiochem) antibody. Secondary Anti-mouse-IgG-HRP or and Anti-rabbit-IgG-HRP Conjugate (Promega) were used. Image acquisition was performed using the chemiluminescent imager SRX-101A (Konica Minolta medical imaging, USA) and band densitometry was analyzed using ImageJ software (NIH). Alternatively, phosphorylated/total AKT was quantified using luminex technology as previously reported (38).
Quantitative Real Time PCR (QRT-PCR). Podocyte RNA was extracted using the RNAeasy Mini Kit (Qiagen). Reverse transcription was performed using the high-capacity cDNA reverse transcriptase kit (Applied Biosystems) according to the manufacturer’s protocols. QRT-PCR was performed in the StepOnePlus real-time PCR system (Applied Biosystems) using the PerfeCTa® SYBR® Green FastMix (Quanta Biosciences). Relative gene expression was determined as $2^{\Delta\text{Ct}}$, with $\Delta\text{Ct}$ being the difference between the cycle threshold (Ct) value of the target gene and Gapdh. For semi-quantitative expression analysis, PCR was performed and analyzed by gel electrophoresis. Amplification product intensities were determined using ImageJ software (NIH), values were normalized and expressed as fold changes in gene expression over GAPDH. The following primers were used: hABCA1-F, AACAGTTTGTTGGCCCTTTTG; hABCA1-R, AGTTCCAGGCTGGGTACTT; hLDL-R-F, TCACTCCATCTCAAGCATCG; hLDL-R-R, GGTTGGTCCTTCACACCAGT; hHMG-CoA-R-F, GGCATTTGACAGCAGCATG; hHMG-CoA-R-R, GCTGGAATGACAGCTTCACA; hGAPDH-F, GTCAGTGGTGGACCTGACCT; hGAPDH-R, Hs_ABCA1-SG QuantiTect-Primer-Assay (Qiagen); mAbca1-F, GGACATGCACAAGGTCCTGA; mAbca1-R, CAGAAAATCCTGGAGCTAAA.

BTBR ob/ob mice treatment and analysis. BTBR ob/ob mice were purchased from Jackson Laboratories. Mice were injected subcutaneously with 4,000 mg/kg CD or saline as reported previously (39), three times per week for 5 months. Urine was collected, and body weight and glycemia (OneTouch) were determined weekly. Six mice per group were analyzed. All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC). After isotonic saline perfusion, the right kidney was removed for cholesterol content determination and mRNA extraction. One left kidney pole was embedded in OCT, a second pole fixed in 4% PFA and paraffin-embedded for histological analysis. Blood samples were analyzed for CBC, lipid panel, AST, ALT, Alkaline Phosphatase, GGT, and BUN in the Comparative Laboratory Core
Facility, University of Miami. Serum creatinine was determined by tandem mass spectrometry at the UAB-UCSD O'Brien Core Center, University of Alabama at Birmingham, using the methods previously described (40). The urine albumin content was measured by ELISA (Bethyl Laboratories). Urinary creatinine was assessed by an assay based on the Jaffe method (Stanbio). Values are expressed as µg albumin/mg creatinine. Fasting plasma insulin was determined by ELISA (Mercodia, SW). Intraperitoneal glucose tolerance tests (IPGTT) were performed 4 months after treatment; after 5-hr fasting, blood glucose was recorded at baseline and up to 180 minutes after a glucose bolus (1.5 g/kg). For insulin sensitivity, glycemia was monitored at baseline and up to 150 minutes after intraperitoneal injection of 4 mU/g of short acting insulin. Human islets from four different isolations were pretreated with 0.5 mM CD for one hour and perfused as described before to determine insulin release in response to glucose and KCl (38).

**Histology, assessment of mesangial expansion and glomerular surface area.** Periodic acid- Schiff’s (PAS) staining of 4 µm thick tissue sections was performed. Twenty glomeruli per section were analyzed for mesangial expansion by semiquantitative analysis (scale 0-4) performed by two blinded independent investigators. The glomerular surface was delineated in each encountered glomerulus and the mean surface area calculated as described (41).

**Statistical analysis.** Data are shown as mean and standard deviations. Four to 8 independent experiments were performed for *in vitro* studies. Six mice per group were used for *in vivo* experiments. Statistical analysis was performed with one-way ANOVA. When one-way ANOVA showed statistical significance, results were compared using *t*-test after Tukey’s correction for multiple comparisons. Results were considered statistically significant at *p*<0.05.

**RESULTS**

*Clinical laboratory measurements and patient population*
We studied 30 male subjects divided into three groups based on clinical characteristics at the time of collection of the sera samples. The study subjects included: 1) 10 patients with T1D, normoalbuminuria and normal creatinine, defined as patients without diabetic kidney disease (DKD-), 2) 10 patients with T1D, albuminuria and altered creatinine, defined as patients with diabetic kidney disease (DKD+), 3) 10 healthy controls (C). The three groups were not significantly different for age, total cholesterol, HDL-, LDL-cholesterol and triglycerides. All diabetic patients were taking agents to block the renin-angiotensin-aldosterone system. Lipid lowering agents were utilized in 2/10 controls, 3/10 DKD- and 4/10 DKD+. Duration of diabetes, fasting plasma glucose and HbA1C were not significantly different among DKD+ and DKD-patients (Table 1). The mean estimated glomerular filtration rate (eGFR) was 101 ml/min/1.73m² in DKD-, 97 ml/min/1.73m² in C and 43 ml/min/1.73m² in the DKD+ group. Sera collected 6 ± 1.2 years prior from five of the patients with DKD (mean eGFR declined from 109 to 75 ml/min/1.73m²) was utilized in selected experiments.

Podocytes exposed to DKD+ sera have a characteristic cRNA signature

RNA was extracted from differentiated podocytes cultured in the presence of patient sera as we previously reported (14). Gene heatmap analysis (Agilent Technologies) revealed strong regulation of several GSEA KEGG pathways with dysregulation of genes involved in actin cytoskeleton regulation (RAC), insulin signaling (ISP), cytokines and cytokine receptor interaction (CCRI) primarily through TLR4, TNFα and IL1β and apoptosis (APOP) (Figure 1a). Microarray data are available at http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46900. We validated these findings at the protein level demonstrating by western blotting that in DKD+ treated podocytes, MyD88 expression was increased (Figure 1b), the ability of insulin to phosphorylate AKT was impaired (Figure 1c) and the amount of cleaved caspase 3 was increased (Figure 1d).
Normal human podocytes exposed to sera of patients with DKD exhibit cell blebbing

Podocytes exposed to the sera of patients with DKD experienced pronounced actin cytoskeleton remodeling with localized decoupling of the cytoskeleton from the plasma membrane (blebbing), which was evident in both the phallloidin staining (F-actin) and the brightfield images (Figure 2a) and which was very different from what we have reported in focal and segmental glomerulosclerosis (14). Quantitative analysis of cell blebbing (percentage of cells with blebs out of a total of 200 consecutive cells analyzed) revealed this phenotype in 80% of cells exposed to DKD+ sera, but in only 20% of cells exposed to DKD- sera and 5% in the controls (Figure 2b, p<0.001). Cell blebbing was accompanied by the redistribution of active RhoA at the site of cell blebbing (Figure 2c) and by an increase in total RhoA (Figure 2d). Cell blebbing was not a consequence of reduced GFR in the DKD+ group, as historical sera collected from five of the patients with T1D and normal GFR that ultimately developed DKD caused the same degree of cell blebbing in cultured podocytes as the sera from the same patients collected on average 6 ± 1.2 years later, when the patients had established DKD with macroalbuminuria and low GFR (Figure 2e).

Impaired reverse cholesterol transport in podocytes exposed to DKD+ sera and in glomeruli from patients with early diabetes

As inflammation, insulin resistance, apoptosis and cytoskeleton remodeling are linked by the intracellular accumulation of lipids in the pathogenesis of non-alcoholic steatohepatitis (NASH syndrome) (42), and accumulation of cholesterol has been described in the cortex of the kidneys of mice with DKD (20), we explored if podocytes cultured in the presence of sera of patients with DKD accumulate intracellular cholesterol. We were able to demonstrate an increased number of ORO (Figure 3a and 3c) and filipin positive cells (Figure 3b) in DKD- and DKD+ treated cells, more so in the DKD+ treated cells. Quantitative analysis of total (Figure 3d), free
(Figure 3e) and esterified cholesterol (Figure 3f) revealed significantly increased esterified cholesterol in DKD+ treated cells when compared to cells treated with sera from control subjects. This increase was likely due to impaired cholesterol efflux, as LDL-receptor and HMG-CoA reductase expression were unchanged (Figure 3g and 3h), while $ABCA1$ mRNA expression was down-regulated in DKD+ treated podocytes (Figure 3i). We then studied the mRNA expression of lipid related genes in glomeruli from additional 70 patients with T2D and early DKD when compared to 32 normal living donors (Supplementary Table 1), and demonstrated significant down-regulation of $ABCA1$ in DKD (Figure 3j). Interestingly, down-regulation of $ABCA1$ mRNA expression was a feature of DKD only, as $ABCA1$ was not regulated in other proteinuric glomerular diseases (Figure 3j) such as membranous nephropathy (MN, n=21) and focal segmental glomerulosclerosis (FSGS, n=18). In order to assess if downregulation of $ABCA1$ expression is associated with physiologically decreased cholesterol efflux in DKD sera treated human podocytes, we performed cholesterol efflux assays using $^3$H-cholesterol. When cholesterol labeled podocytes were incubated with 0.5% serum from patients for 6 hours, both DKD- (p<0.01) and DKD+ (p<0.001) serum were significantly less effective at promoting cholesterol efflux compared to C serum (Figure 3k). These results indicate that downregulation of $ABCA1$ expression in DKD+ sera treated podocytes is associated with impaired cholesterol efflux, which may partially account for the cellular cholesterol observed differences.

Cyclodextrin protects podocytes in vitro

As the exposure of podocytes in culture to DKD+ sera caused accumulation of total cholesterol in association with decreased $ABCA1$ expression, a transporter responsible for cholesterol efflux, we went on to test the hypothesis that cyclodextrin would protect podocytes from the actin cytoskeleton remodeling and cell blebbing observed after exposure to the sera from patients with DKD. We were able to demonstrate that CD prevented cell blebbing and the localization of
phosphorylated caveolin to focal adhesion sites (Figure 4a). Quantitative cholesterol analysis showed that CD prevented the accumulation of total and esterified cholesterol in DKD+ treated podocytes (Figure 4b and 4c). Furthermore, prevention of intracellular cholesterol accumulation with CD also prevented DKD+ induced apoptosis (Figure 4d), insulin resistance (Figure 4e) and MyD88 expression (Figure 4f). Blockade of HMG-CoA reductase with simvastatin in podocytes did not protect from DKD+ induced actin cytoskeleton remodeling (Supplementary Figure S1).

Subcutaneous administration of CD protects BTBR ob/ob mice from the development of DKD

BTBR (black and tan, brachiuric) ob/ob (leptin deficient) mice have been described as a mouse model of progressive DKD (43). After establishing a dose and a mode of administration based on preliminary toxicology studies, we treated 4-week old BTBR mice with subcutaneous administration of three weekly injections of hydroxypropyl-β-cyclodextrin (CD, 4,000 mg/kg body weight) for five months. Although no changes in albumin excretion rates were observed up to 2 months after treatment initiation in homozygous mice, at 3 months a significant down-regulation of the albumin/creatinine ratios was observed in the morning spot urine samples (p<0.001) in CD treated when compared to untreated BTBR ob/ob mice. This decrease persisted until sacrifice (5 months after initiation of treatment, Figure 5a). At sacrifice, CD treated mice showed a reduction of kidney weight (Figure 5b). CD did not affect ABCA1 mRNA expression in the kidney cortex (Figure 5c) but resulted in a significant reduction of total cholesterol (Figure 5d). Blood urea nitrogen (BUN) and creatinine were not significantly affected by CD treatment (Figure 5e and 5f). However, CD treatment resulted in a reduction of mesangial expansion (Figure 5g and 5h) without affecting the glomerular surface area (Figure 5i). After four months of treatment, we also observed a reduction of body weight (Figure 6a and 6b), which was accompanied by a concomitant improvement of random glycemia (Figure 6c). Furthermore, sera collected at sacrifice demonstrated a significant improvement of fasting plasma insulin and
fasting plasma glucose (Figure 6d and 6e). IPGTT performed one week prior to sacrifice were improved in CD treated homozygous mice when compared to homozygous controls (Figure 6f). As improvement was observed despite a similar insulin sensitivity test (Figure 6g), we analyzed the effect of low dose CD on the function of four different preparations of human islet cells. A significant improvement in glucose stimulated insulin release was observed in CD treated human islets when compared to untreated human islets (Figure 6h). To determine whether the beneficial effect of CD on islet cell function was associated with the modulation of ABCA1 expression in pancreatic islets, we performed immunofluorescence staining using a rabbit polyclonal ABCA1 antibody (gift from Dr. A. Mendez) and determined ABCA1 expression as mean fluorescence intensity per pancreata analyzed (Figure 6i). As expected, pancreata from homozygous BTBR ob/ob mice were characterized by significantly decreased ABCA1 expression when compared to heterozygous littermates (p<0.001) and CD treatment significantly increased ABCA1 expression in pancreata of homozygous BTBR ob/ob (p<0.001) and heterozygous BTBR ob/+ mice (p<0.01). As hemolytic anemia and liver toxicity have been described while using other cyclodextrin derivatives in rodents and humans (26), and because we administered high dose CD for a period of 5 months, we studied hemoglobin, aspartate aminotransferase (AST), alanine transaminase (ALT) and gamma-glutamyltransferase (GGT) at sacrifice. No abnormalities due to chronic CD administration were observed indicating that the chronic use of CD is not accompanied by adverse side effects (Table 2).

**DISCUSSION**

As diabetes is a multifactorial disorder, it has been difficult to prevent or cure diabetic complications by targeting specific risk factors such as hyperglycemia and hypertension. In fact, even in the setting of multifactorial intervention, a subset of patients still develops DKD in both T1D and T2D (2; 3). Evidence points to a strong crosstalk between different target organs in
diabetes (44), which raises the possibility that multiple circulating factors other than glucose may directly affect podocyte function in DKD. In order to identify the key pathways that are activated in podocytes irrespectively of the nature of circulating factors, we utilized a cell-based bioassay where human podocytes exposed to sera of healthy individuals were compared to human podocytes exposed to sera of patients with long lasting diabetes without diabetic kidney disease (DKD-) and to sera of patients with long lasting diabetes and nephropathy (DKD+). We have successfully utilized this bioassay to identify predictors for the development of proteinuria in another glomerular disorder caused by circulating permeability factors, i.e. focal and segmental glomerulosclerosis (14).

Our results demonstrate that DKD+ sera treated podocytes have a characteristic microarray signature, which allows distinguishing them from DKD- or control serum treated podocytes. In addition, we identified four key pathways differentially regulated in DKD+ when compared to DKD-: inflammation, insulin resistance, apoptosis and actin cytoskeleton remodeling (Figure 1 and 2). Differently from what we described for FSGS, rearrangements of the actin cytoskeleton observed in DKD+ sera treated podocytes were typical of cell blebbing, as the cell protrusions were vimentin negative (data not shown) (45) but were accompanied by increased RhoA activity (46). These characteristics have been described to differentiate cell blebs from lamellipodia, and may be the consequence of the concomitant activation of caspase 3 (47; 48). A modulation of these four major pathways has been described in the pathogenesis of NASH and it has been related to the accumulation of intracellular cholesterol (42), a known modulator of actin cytoskeleton remodeling (49). Interestingly, cholesterol accumulation was also described in the kidney from experimental models of diabetes that resemble T1D and T2D (19; 20), in a similar fashion to what was initially reported for glycosphingolipids in rodents with DKD (50). We therefore studied the intracellular cholesterol content in podocytes exposed to the sera of patients
with DKD and we found an increase of both total and esterified cholesterol (Figure 3). This finding is different from the accumulation of lysosomal free cholesterol as observed when Niemann-Pick C proteins 1 and 2 (NPC1/2) are mutated (51). Interestingly, the accumulation of cholesterol in podocytes treated with DKD+ sera was likely the consequence of reverse cholesterol transport, as a strong down-regulation of ABCA1 mRNA was observed. In addition, experiments using $^3$H-cholesterol showed impaired cholesterol efflux in human podocytes treated with 0.5% DKD- and DKD+ sera for 6 hours (Figure 4) which may partially account for the differences we observed in the cellular cholesterol content in DKD+ sera treated human podocytes. Downregulation of ABCA1 was furthermore confirmed in mRNA transcripts isolated from the glomeruli of 70 patients with T2D and early DKD. In diabetes, accumulation of cholesterol in peripheral tissue targets of diabetic complications has been described and linked to insulin resistance (52). Increased fat fraction has also been described in the pancreas of obese adolescent and diabetic patients (53), and has been shown to impair pancreatic beta cell function and insulin granule exocytosis (54).

How cholesterol accumulation occurs, irrespective of hypercholesterolemia, has been studied in humans and experimental animals. In the NOD mouse model of T1D, ABCA1 proteins are downregulated in both kidneys and circulating macrophages (18). In humans, ABCA1 genetic variants are strongly associated with the risk of coronary artery disease (16) and cholesterol efflux capacity of macrophages inversely correlates with carotid and coronary artery lesions (36). Elegant studies utilizing the sera of patients with and without diabetic nephropathy in a cell-based assay to determine cholesterol efflux capacity in macrophages have demonstrated that the capacity of the sera to induce ABCA1 mediated cholesterol efflux was impaired in patients with T2D and incipient or overt nephropathy (17). As systemic downregulation of ABCA1 also results in impaired pancreatic β-cell function (54), strategies that maintain cellular cholesterol
homeostasis may be beneficial for the treatment of diabetes and its related complications. Supporting these observations and our results presented here, a recent study showed that inhibition of miR-33a, which leads to increased ABCA1 expression is sufficient to normalize insulin secretion and cellular cholesterol levels in pancreatic islets (55).

In order to demonstrate that increased intracellular cholesterol is necessary for DKD+ sera to cause actin remodeling and apoptosis, we utilized pharmacological interventions targeting cellular cholesterol homeostasis prior to exposure to sera from patients. Statins were used to decrease intracellular cholesterol synthesis in podocytes and CD was used to remove cholesterol from cultured podocytes prior to exposure to patient sera. While statins did not protect podocytes in our model (Supplementary Figure S1), CD significantly protected podocytes from apoptosis, insulin resistance, inflammation and cell blebbing (Figure 4), an observation that is consistent with the fact that cells exposed to DKD+ sera showed cholesterol accumulation due to an impairment of reverse cholesterol transport rather than increase in intracellular cholesterol synthesis. As strategies that raise HDL cholesterol have been proven ineffective in reducing events(56) and HDL levels may not correlate to cholesterol efflux (36), we utilized CD as a potent cholesterol acceptor. We administered CD in vivo to BTBR ob/ob mice to test if this would result in preservation of kidney function and reduction of albuminuria. We found that CD administered in vivo for 5 months partially reduced albuminuria and prevented mesangial expansion (Figure 5). Impairment of liver function and hemolytic anemia were not observed. CD treatment effectively reduced the total kidney cholesterol content but did not affect ABCA1 mRNA expression in kidney cortexes. As CD was administered systemically and cells other than kidney cells are characterized by accumulation of intracellular cholesterol, we investigated if CD had any effect on the metabolic profile of BTBR ob/ob mice. Indeed, CD treated diabetic mice experienced a significant reduction of their body weight and of their glycemia after 4 months of
treatment which was associated with an upregulation of ABCA1 expression in pancreatic islets and with the improvement of glucose stimulated insulin release in human islets in vitro (Figure 6). Further studies are needed to identify the factor(s) primarily responsible for intracellular cholesterol accumulation in diabetes and the exact mechanisms of CD protection in diabetes and DKD.

In conclusion, using an assay in which normal human podocytes are cultured with the sera of patients with DKD, we have shown that circulating factors other than glucose may directly affect podocyte function in vitro. We uncovered a novel mechanism in which intracellular cholesterol accumulation due to impaired efflux plays a central role in podocyte injury in vitro. These data suggest that this mechanism may contribute to the development and/or the progression of DKD. Furthermore, using CD in our in vitro and in vivo studies, we were able to protect podocytes from injury caused by intracellular cholesterol accumulation, indicating that CD may represent a safe, FDA approved, effective way to target reverse cholesterol transport in diabetes and DKD. These observations underline the need to design safety and efficacy clinical trials to study the use of hydroxypropyl-β-cyclodextrin in DKD.

**AUTHOR CONTRIBUTIONS**

A.F. and S.M. conceived the project, designed and supervised the study, analyzed the data, and wrote the manuscript.

G.W.B conceived one of the techniques utilized;

S.M., J.G., R.A.-P., T.Y., C.E.P., R.V., D.M. performed the in vitro and in vivo experiments.

M.L., C.F., M.I.L., P.H.G performed some of the experiments and collected patients samples and clinical information.
A.M., C.E.P, R.G. designed and performed experiments related to cholesterol determination and cholesterol efflux.

M.K., R.G.N., V.N., A.R. DKD microarray data and collected the patient clinical data.

K.J. processed all the histology.

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TABLES:

Table 1. Clinical characteristics of the patients with T1D (with or without DKD), and their respective controls.

|                        | C (N=10) | DKD- (N=10) | DKD+ (N=10) |
|------------------------|----------|-------------|-------------|
| Age                    | 44±11    | 48±7        | 49±9        |
| Gender                 | M/F (10/0) | M/F (10/0) | M/F (10/0)  |
| Diabetes duration      | -        | 29±11       | 32±10       |
| HbA1c (%) (mmol/mol)   | 5.5±0.3  | 7.9±0.8     | 7.9±1.2     |
|                        | 37±3.3   | 63±8.8      | 63±13       |
| FPG (mg/dl)            | 81±19    | 102±12      | 92±26       |
| Serum creatinine (mg/dl)| 0.9±0.1 | 0.8±0.1     | 1.7±0.9**   |
| Total chol (mg/dl)     | 182±23   | 170±23      | 166±54      |
| HDL chol (mg/dl)       | 56±12    | 74±14       | 64±16       |
| LDL chol (mg/dl)       | 105±24   | 79±14       | 82±48       |
| Triglyceride (mg/dl)   | 96±37    | 67±23       | 103±44      |

Data are presented as mean ± SD. FPG=fasting plasma glucose. **p<0.01 when comparing DKD+ to DKD- and C.
Table 2. Serology of BTBR heterozygous and homozygous mice treated with CD

|                | Het    | Het+CD | Homo   | Homo+CD |
|----------------|--------|--------|--------|---------|
| Hb (g/dl)      | 15.9±0.9 | 16.5±0.6 | 14.5±3.7 | 15.1±1.2 |
| AST (U/l)      | 146±25  | 111±44  | 143±78  | 157±26  |
| ALT (U/l)      | 12±7    | 14±9    | 22±13   | 37±22   |
| GGT (U/l)      | 5.2±2.5 | 4.6±0.3 | 6.5±3.5 | 5.2±2.3 |
| Cholesterol (mg/dl) | 46±7 | 105±9### | 95±4*** | 197±17### |

Data are presented as mean ± SD. Hb=Hemoglobin; AST= aspartate aminotransferase; ALT= alanine aminotransferase; GGT= gamma-glutamyl transpeptidase; Het=heterozygous; Homo=Homozygous; CD=2-Hydroxypropyl-β-cyclodextrin. ***p<0.001 when comparing Homo to Het. ### p<0.001 when comparing CD treated versus controls.
FIGURE LEGENDS:

Figure 1. Expression analysis of podocytes cultured with patient sera. (a) cRNA beadchip analysis was performed using cRNA isolated from human podocytes exposed to pooled sera from control patients, patients without (DKD-) or with kidney disease (DKD+). Quadruplicate expression data analysis was performed after batch correction. 1015 significant probes were identified as differentially expressed between DKD- and DKD+, and pathway analysis revealed that four major pathways were modulated as shown in the heatmap. (b) Bar graph analysis (mean±SD) and representative western blot analysis of four independent experiments demonstrating increased MyD88 protein expression in DKD+ treated podocytes when compared to C. **p<0.01. (c) Bar graph analysis (mean±SD) of fold changes in phosphorylated AKT/total AKT after insulin stimulation in four different experiments. Data were analyzed with luminex technology and demonstrated decreased cellular insulin sensitivity in DKD+ and DKD- treated cells when compared to C. *p<0.05. (d) Bar graph analysis (mean±SD) of cleaved caspase 3 analyzed in four independent experiments, demonstrating increased cleaved caspase 3 in DKD+ treated cells when compared to C. **p<0.01
Figure 2. **Actin remodeling in DKD+ sera treated podocytes.**

(a) Representative F-actin staining (top) and bright field images (bottom) of podocytes exposed to DKD+ sera when compared to C and DKD- sera, demonstrating cell blebbing in DKD+ serum treated cells. White and black arrows point to areas of cell blebbing. 

(b) Quantitative analysis of cell blebs (mean±SD) observed in podocytes exposed to individual DKD+ sera when compared to DKD- and C. ***P<0.001. 

(c) Representative immunofluorescence images demonstrating the localization of active RhoA (green) at the sites of cell blebbing in DKD+ serum treated podocytes. Lysophosphatidic acid (LPA) induced RhoA activation was utilized as positive control. F-actin is visualized in red (Rhodamine phalloidin) and nuclei in blue (DAPI). 

(d) Bar graph analysis (mean±SD) and representative western blot of four independent experiments demonstrating increased total RhoA in DKD+ serum treated podocytes when compared to C. **p<0.01. 

(e) Quantitative analysis (mean±SD) of the number of podocytes showing cell blebbing after exposure to the sera of five patients with DKD+ and decreased glomerular filtration rate (GFR) when compared to the sera collected from the same five patients six years prior when they had normal GFR. No significant differences were observed. A representative F-actin staining of cell blebbing is also shown. Clinical characteristics of the patients are shown in Supplementary Table 2.
**Figure 3. Cholesterol accumulation in podocytes exposed to DKD+ sera.** (a) Representative Oil Red O staining of podocytes exposed to DKD+ sera when compared to C and DKD- sera. Black arrows point to spots of major lipid droplet accumulation. (b) Representative filipin staining (orange) and phosphorylated caveolin staining (green) of podocytes exposed to DKD+ sera when compared to C and DKD-. (c) Bar graph quantitative analysis (mean±SD) of Oil Red O positive cells in podocytes exposed to the pools of sera from 10 patients with DKD-, DKD+ or to pools of the sera from controls, demonstrating that exposure to both, DKD- and DKD+ sera, cause significant lipid droplet accumulation in cultured human podocytes. *p<0.05, ***p<0.001. (d, e, f) Bar graph analysis (mean±SD) of total cholesterol (Tot C), free cholesterol (Free C) and esterified cholesterol (Est C) as determined via enzymatic reaction in podocytes exposed to pools of DKD+ sera when compared to C and DKD-. *p<0.05. (g, h, i) Quantitative RT PCR analysis (mean±SD) of LDL receptor, HMG-CoA reductase and ABCA1 expression in podocytes exposed to individual patient sera. ***p<0.001. (j). Expression analysis of glomerular gene expression of lipid related genes in 70 patients with early DKD, 21 patients with membranous nephropathy (MN) and 18 patients with focal segmental glomerulosclerosis (FSGS) when compared to 32 living donors. Numbers reflect fold change in disease when compared to living donors. Genes passing an FDR correction (qvalue) for multiple testing below 5% were considered significantly regulated genes with significant changes in gene expression and are highlighted by blue or red background colors. (k) Cholesterol efflux assay demonstrating decreased cholesterol efflux after 6 hours in podocytes treated with DKD- (p<0.01) and DKD+ (p<0.001) sera when compared to C.
Figure 4. CD protects podocytes from changes observed after exposure to DKD+ sera. (a) Representative phalloidin (red) and phosphorylated caveolin (green) confocal images of normal human podocytes exposed to DKD+ sera when compared to C and DKD- sera in the presence (CD) or absence (control) of CD. DAPI (blue) was used to identify nuclei (b, c) Bar graph analysis (mean±SD) of the effect of CD on total (B) and esterified cholesterol (C) in CD treated (+) versus untreated (-) podocytes exposed to DKD+ sera when compared to C and DKD- sera. *p<0.05 when comparing DKD+ versus C. #p<0.05 when comparing CD treated versus untreated podocytes in the same group. (d, e, f) Bar graph analysis (mean±SD) of cleaved Caspase 3, insulin stimulated AKT phosphorylation and MyD88 expression in CD treated (+) versus untreated (-) podocytes exposed to DKD+ sera when compared to C and DKD- sera. *p<0.05 and **p<0.01 when comparing DKD+ versus C. #p<0.05 when comparing CD treated versus untreated podocytes in the same group.
Figure 5. CD protects from DKD in vivo. (a) CD administered to homozygous and heterozygous BTBR ob/ob mice subcutaneously three times a week (n=6 per group) resulted in a reduction in albumin/creatinine ratios (mean±SD) starting at 3 months after the initiation of the treatment. *p<0.05, **p<0.01, ***p<0.001. (b) Kidney weight (mean±SD) was significantly increased in homozygous mice (**p<0.01), and such an increase was prevented by CD treatment (##p<0.01). (c) Bar graph analysis (mean±SD) of the effect of CD on ABCA1 mRNA expression in kidney cortices of homozygous and heterozygous BTBR ob/ob mice. (d) Bar graph analysis (mean±SD) of the effect of CD on the total cholesterol content in kidney cortices of homozygous and heterozygous BTBR ob/ob mice. (e, f) Bar graph analysis (mean±SD) showing that serum BUN and creatinine concentrations remain unchanged after CD treatment of the mice. Measurements were performed on serum obtained from the mice at sacrifice. (g) Representative PAS staining of kidney sections from homozygous and heterozygous BTBR ob/ob mice after five months of treatment with either CD or vehicle. (h, i) Bar graph analysis (mean±SD) of the scores for mesangial expansion (h) and of the glomerular surface area (i) on PAS stained kidney sections from homozygous and heterozygous BTBR ob/ob mice after five months of treatment with either CD or vehicle were assessed by two blinded, independent investigators. *p<0.05 when comparing DKD+ versus C. #p<0.05 when comparing CD treated versus untreated mice in the same group.
Figure 6. CD improves diabetes in vivo. (a,b) CD administered to homozygous and heterozygous BTBR ob/ob mice subcutaneously three times a week (n=6 per group) resulted in a significant reduction in body weight (mean±SD) starting at four month after the initiation of the treatment. *p<0.05, **p<0.01. (c) CD administered to homozygous BTBR ob/ob mice resulted in a significant reduction in random glycemia (mean±SD) starting at four month after the initiation of the treatment. (d,e) Bar graph analysis (mean±SD) of fasting plasma insulin and glucose concentrations. Fasting plasma insulin (**p<0.01; ***p<0.001)(d) and fasting plasma glucose (***p<0.01)(e) were significantly increased in homozygous mice when compared to heterozygous controls. The increase was prevented by CD treatment (**p<0.01). (f) IPGTT performed at 5 month after the initiation of the CD treatment showed improved glucose tolerance in CD treated BTBR ob/ob mice when compared to untreated BTBR ob/ob mice. (g) CD treatment did not affect the sensitivity to a single dose of short acting insulin (4 mU/g) in BTBR ob/ob mice. (h) Representative perifusion experiment and bar graph analysis of the area under the curve (AUC) demonstrating the effect of 0.5 mM CD on glucose stimulated insulin release in human pancreatic islets from four independent donors (**p<0.01). (i) Immunofluorescence staining for ABCA1 reveals increased ABCA1 expression in pancreata of CD treated BTBR ob/ob mice when compared to untreated littermates (Figures 6i, left). Bar graph analysis (Figure 6i, right) showing that pancreata isolated from homozygous BTBR ob/ob mice are characterized by significantly decreased ABCA1 expression when compared to heterozygous littermates (###p<0.001). CD treatment significantly increased ABCA1 expression in pancreata of homozygous BTBR ob/ob (***p<0.001) and heterozygous BTBR ob/+ mice (**p<0.01).
RAC = 'Regulation of Actin Cytoskeleton'; ISP = 'Insulin Signaling Pathway'; CCRI = 'Cytokine Cytokine Receptor Interaction'; APOP = 'Apoptosis'
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DKD + DKD +
0
20
40
60
80
100
7 ± 2 yrs
Number of blebbing cells (%)
Normal GFR Decreased GFR

DKD -                  DKD +
DKD -                  DKD +
DKD -                  DKD +
DKD normal GFR    DKD low GFR

C      DKD -                  DKD +
C      DKD -                  DKD +
C      DKD -                  DKD +
DKD + detail

DKD normal GFR    DKD low GFR

RhoA

Gapdh

DKD normal GFR    DKD low GFR
Diabetes
SUPPLEMENTARY MATERIALS

Supplementary Table 1: Clinical characteristics of patients from which kidney biopsies were collected and analyzed by Affymetrix microarray gene expression profiling.

Supplementary Table 2: Clinical characteristics of five patients with DKD from which consecutive serum samples where collected 6 years apart.

Supplementary Figure 1: Simvastatin treatment of DKD+ sera treated podocytes does not protect from actin cytokeleton remodeling.

Supplementary Information 1: The Finnish Diabetic Nephropathy Study Centers

Supplementary Information 2: Else-Kroener Fresenius foundation members
Supplementary Table 1. Clinical characteristics of patients from which kidney biopsies were collected and analyzed by Affymetrix microarray gene expression profiling

|                              | DKD (T2D) | Living Donors |
|------------------------------|-----------|---------------|
| Total number of patients     | 70        | 32            |
| Gender (F / M)               | 52 F / 18 M | 15 F / 17 M   |
| Age (years)                  | 45 ± 10   | 48 ± 12       |
| Urine albumin/creatinine ratio (mg/g) | 292.2 ± 967.3 | NA           |
| Serum creatinine (mg/dl)     | 0.74 ± 0.18 | 0.80 ± 0.26  |
| Serum cholesterol (mg/dl, n = 57) | 169 ± 37   | NA            |
| HbA1c (%)                    | 9.3 ± 2.1 | NA            |

Data are presented as mean ± SD. NA: non available
Supplementary Table 2. Clinical characteristics of five patients with DKD from which two consecutive serum samples where collected 6 years apart.

|                         | Time 0 (n=5) | Time 1 (n=5) |
|-------------------------|-------------|-------------|
| Age                     | 44±13       | 50±13       |
| Gender                  | M/F (5/0)   | M/F (5/0)   |
| Diabetes duration       | 25±17       | 31±18       |
| HbA1C (%)               | 8.1±1.4     | 7.8±1.4     |
|                         | (mmol/mol)  |             |
| Serum creatinine (mg/dl)| 0.84±0.09   | 1.13±0.16   |
| eGFR                    | 109±14      | 75±11       |
| Total chol (mg/dl)      | 229±40      | 165±29      |
| HDL chol (mg/dl)        | 56±10       | 54±14       |
| Triglyceride (mg/dl)    | 160±40      | 103±47      |

Data are presented as mean ± SD.
Supplementary Figure 1. Simvastatin does not protect podocytes from changes observed after exposure to DKD+ sera. (A) Representative confocal images of phalloidin stained normal human podocytes exposed to DKD+ sera when compared to C and DKD- sera in the presence or absence (control) of simvastatin.
Supplementary Information 2

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