Type I diabetes mellitus decreases in vivo macrophage-to-feces reverse cholesterol transport despite increased biliary sterol secretion in mice

Jan Freark de Boer1,* Wijtske Annema1,2*, Marijke Schreurs1,3, Jelske N. van der Veen1, Markus van der Giet4, Niels Nijstad1, Folkert Kuipers1,5, and Uwe J.F. Tietge1,2

1Department of Pediatrics, Center for Liver, Digestive, and Metabolic Diseases, 3 Department of Pathology and Medical Biology, Medical Biology Section, Molecular Genetics, 5Department of Laboratory Medicine, University of Groningen, University Medical Center Groningen, Groningen, The Netherlands
2Top Institute Food and Nutrition, Wageningen, The Netherlands
4 Medizinische Klinik IV–Nephrology, Charite–Campus Benjamin Franklin, Berlin, Germany

* These authors contributed equally to this study

Short title: Type I diabetes mellitus decreases RCT

Address Correspondence to:
Dr. Uwe Tietge
Department of Pediatrics
Center for Liver Digestive and Metabolic Diseases
University Medical Center Groningen
Hanzeplein 1
9713 GZ Groningen
The Netherlands
Tel. +31-50-3636762
Fax +31-50-3611746
email: u_tietge@yahoo.com
Abstract

Type I diabetes mellitus (T1DM) increases atherosclerotic cardiovascular disease, while the underlying pathophysiology is still incompletely understood. We investigated whether experimental T1DM impacts HDL-mediated reverse cholesterol transport (RCT). C57BL/6J mice with alloxan-induced T1DM had higher plasma cholesterol levels (P<0.05), particularly within HDL, and increased hepatic cholesterol content (P<0.001). T1DM resulted in increased bile flow (2.1-fold; P<0.05) and biliary secretion of bile acids (BA, 10.5-fold; P<0.001), phospholipids (4.5-fold; P<0.001), and cholesterol (5.5-fold; P<0.05). Hepatic cholesterol synthesis was unaltered, while BA synthesis was increased in T1DM (P<0.001). Mass fecal BA output was significantly higher in T1DM mice (1.5-fold; P<0.05), fecal neutral sterol excretion did not change due to increased intestinal cholesterol absorption (2.1-fold; P<0.05). Overall in vivo macrophage-to-feces RCT, using [3H]cholesterol-loaded primary mouse macrophage foam cells, was 20% lower in T1DM (P<0.05), mainly due to reduced tracer excretion within BA (P<0.05). In vitro experiments revealed unchanged cholesterol efflux towards T1DM HDL, while SR-BI-mediated selective uptake from T1DM HDL was lower in vitro and in vivo (HDL kinetic experiments) (P<0.05), conceivably due to increased glycation of HDL-associated proteins (+65%, P<0.01). In summary, despite higher mass biliary sterol secretion T1DM impairs macrophage-to-feces RCT, mainly by decreasing hepatic selective uptake, a mechanism conceivably contributing to increased cardiovascular disease in T1DM.

Supplementary key words: high density lipoproteins, bile acids, cardiovascular disease, atherosclerosis, neutral sterols, glucose, efflux, selective uptake, liver, bile
Introduction

Atherosclerotic cardiovascular disease (CVD) is a predominant cause of morbidity and mortality in type 1 diabetes mellitus (T1DM) patients (1, 2). Compared with subjects without diabetes, T1DM confers a 7-fold increase in the risk of fatal CVD (2). However, the mechanisms underlying accelerated atherosclerosis in T1DM are still incompletely understood.

Plasma high density lipoprotein (HDL) cholesterol levels are inversely related to the incidence of CVD (3, 4). The role of this lipoprotein in promoting reverse cholesterol transport (RCT) is currently regarded the main established atheroprotective property of HDL (5, 6). The critical steps in RCT comprise initial efflux of excess cholesterol from lipid-laden macrophages within atherosclerotic lesions towards HDL for transport through the plasma compartment, followed by the subsequent uptake of cholesterol into the liver for excretion into bile and feces (7, 8).

Although T1DM has been associated with changes in sterol metabolism (9-13), no data are currently available addressing the impact of T1DM on RCT. Therefore, this study explored the pathophysiological consequences of experimental T1DM on overall RCT as well as the individual steps involved in this process. Our data demonstrate that macrophage-specific RCT is decreased in T1DM despite increased biliary sterol secretion as well as increased fecal excretion of bile acids. Mechanistically, we identified decreased hepatic selective uptake of cholesterol from glycated HDL as a major underlying factor for reduced RCT in T1DM.
Materials and Methods

Animals

C57BL/6J mice were obtained from Charles River (Sulzfeld, Germany). The animals were caged in animal rooms with alternating 12-hour periods of light (from 7.00 a.m. to 7.00 p.m.) and dark (from 7.00 p.m. to 7.00 a.m.), with ad libitum access to water and mouse chow diet (Arie Blok, Woerden, The Netherlands). Animal experiments were performed in conformity with PHS policy and in accordance with the national laws. All protocols were approved by the responsible ethics committee of the University of Groningen.

Induction of type 1 diabetes mellitus

To induce experimental T1DM, wild-type C57BL/6J were injected intravenously with a single dose of alloxan (65 mg/kg body weight, Sigma, St. Louis, MO, USA), while control mice received an equivalent volume of phosphate-buffered saline (PBS). Blood glucose levels were assessed by tail bleeding using a Onetouch Ultra glucosemeter (LifeScan Benelux, Beerse, Belgium). Plasma insulin levels were determined using an ultrasensitive mouse insulin ELISA kit (Alpco Diagnostics, Salem NH, USA).

Plasma lipid and lipoprotein analysis

Plasma total cholesterol, triglycerides, free fatty acids, and phospholipids were measured enzymatically using commercially available reagents (Roche Diagnostics, Basel, Switzerland and Wako Pure Chemical Industries, Neuss, Germany). Pooled
plasma samples from mice of the same experimental group were subjected to fast protein liquid chromatography (FPLC) gel filtration using a Superose 6 column (GE Healthcare, Uppsala, Sweden) as described (14). Samples were chromatographed at a flow rate of 0.5 mL/min, and fractions of 500 µl each were collected. Individual fractions were assayed for cholesterol concentrations as described above. Plasma plant sterol levels were measured by gas chromatography exactly as previously published (15).

**Analysis of liver lipid composition**

To determine hepatic cholesterol, phospholipid, and triglyceride content, liver tissue was homogenized, and lipids were extracted following the general procedure of Bligh and Dyer as described (16). Triglycerides and cholesterol were measured using commercial kits as detailed above. Phospholipid content of the liver was determined essentially as published previously (16).

**Bile collection and assessment of biliary excretion of cholesterol, phospholipids, and bile acids**

Continuous bile cannulation was performed on day 10 after injection with either alloxan or PBS. Bile was collected during 30 minutes under Hypnorm (fentanyl/fluanisone; 1 ml/kg) and diazepam (10 mg/kg) anesthesia using a humidified incubator to maintain body temperature. Bile production was determined gravimetrically. Biliary bile salt, cholesterol, and phospholipid concentrations were determined, and the respective biliary excretion rates were calculated as previously described (17).
Fecal bile acid and neutral sterol analysis

Feces of individually housed mice were collected over a period of 24 h, starting on day 8. Fecal samples were dried, weighed, and thoroughly ground. Aliquots were used for determination of bile acids and neutral sterols by gas liquid chromatography as described (17).

In vivo reverse cholesterol transport study

In vivo macrophage-to-feces RCT studies were performed from day 9-11 after either alloxan or saline injection following a previously described protocol (18). Briefly, wild-type C57BL/6J donor mice were injected intraperitoneally with 1.0 ml of 4% Brewer thioglycollate medium (Becton Dickinson, Le Point de Claix, France). On day 4 after thioglycollate injection, peritoneal macrophages were harvested as described (19). Macrophages were plated in RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, HyClone, Logan, UT, USA) and penicillin (100 U/ml)/streptomycin (100 µg/ml) (Invitrogen) and were allowed to adhere for 4 h at 37 °C under 5% CO₂ humidified air. Then non-adherent cells were removed by washing twice with PBS followed by loading of the macrophages with 50 µg/ml acetylated low density lipoprotein (LDL) and 3 µCi/ml [³H]cholesterol (Perkin Elmer, Boston, MA, USA) for 24 h. After washing twice with PBS, the macrophages were equilibrated for 18 h in RPMI 1640 medium containing penicillin (100 U/ml)/streptomycin (100 µg/ml) and 2% bovine serum albumin (BSA, Sigma). Immediately before injection, cells were harvested and resuspended in RPMI 1640 medium. The resuspended [³H]cholesterol-loaded macrophage foam cells (2 million per
mouse) were injected intraperitoneally into individually housed recipient mice. Plasma was collected at the indicated time points after macrophage injection by retroorbital puncture and for the final blood draw by heart puncture. At the end of the experimental period, livers were harvested, snap-frozen in liquid nitrogen and stored at -80 °C. Feces were collected continuously up to 48 h. Counts in plasma were assessed directly by liquid scintillation counting (Packard 1600CA Tri-Carb, Packard, Meriden, CT, USA). Counts from a respective piece of liver were determined following solubilization of the tissue (Solvable, Packard, Meriden, CT, USA) exactly as previously reported (20) and were related to total liver mass. Fecal samples were dried, weighed, and thoroughly ground. Aliquots were separated into bile acid and neutral sterol fractions as previously published (17). Briefly, samples were first heated for 2 h at 80 °C in alkaline methanol and then extracted three times with petroleum ether. In the top layer, counts within the neutral sterol fraction were determined by liquid scintillation counting, while counts incorporated into bile acids were assessed from the bottom layer. Counts recovered from the respective aliquots were related to the total amount of feces produced over the whole experimental period. All obtained counts were expressed relative to the administered tracer dose.

**Determination of fractional intestinal cholesterol absorption**

Fractional cholesterol absorption was determined using a dual isotope method essentially as described (21). Briefly, mice received an intravenous (i.v) dose of 0.3 mg cholesterol-D_{7} dissolved in 20% Intralipid (Fresenius Kabi, Den Bosch, The Netherlands) and an oral dose of 0.6 mg cholesterol-D_{5} dissolved in medium-chain triglyceride oil. Blood spots were collected from the tail on filter paper at t = 0, 3, 6, 12, 24, 48 and 72 h.
Cholesterol was extracted from blood spots using 95% ethanol/acetone (1:1). After an overnight derivatization with N,O-bis-(trimethyl)trifluoroacetamide and trimethylchlorosilane, isotope enrichments were determined by gas chromatography / mass spectrometry (GC/MS). Fractional cholesterol absorption was calculated as the ratio of the area under the enrichment curves (AUC) derived from the oral (cholesterol-D₅) and i.v. (cholesterol-D₇) administration, corrected for the respective administered doses.

In vitro efflux assay

HDL for efflux and cellular cholesterol uptake studies described below was isolated from mouse plasma by density gradient ultracentrifugation as described previously (20).

THP-1 human monocytes (ATCC via LGC Promochem, Teddington, UK) were grown in suspension culture in RPMI 1640 medium supplemented with 10% FBS and penicillin (100 U/ml)/streptomycin (100 µg/ml) until differentiation into macrophages by the addition of 100 nM phorbol myristate acetate (PMA, Sigma). Differentiated THP-1 macrophages were loaded with 50 µg/ml acetylated LDL and 1 µCi/ml [³H]cholesterol for 24 h followed by equilibration for 18 h as previously published (18). Then cells were washed with PBS and 50 µg protein/ml of isolated mouse HDL was added. After 24 h radioactivity within the medium was determined by liquid scintillation counting. The cell layer was washed twice with PBS, whereafter 0.1 M NaOH was added. Plates were incubated 30 minutes at room temperature, and the radioactivity remaining within the cells was assessed by liquid scintillation counting. Wells incubated with RPMI without added HDL were used as blanks to determine HDL-independent efflux, and these values
were subtracted from the respective experimental values. Efflux is given as the percentage of counts recovered from the medium in relation to the total counts present on the plate (sum of medium and cells).

In vitro selective cholesterol uptake assay

HDL was labelled with the non-hydrolyzable trap label \(^{3}\text{H}\)cholesteryl ether (Perkin Elmer) essentially as described previously (22). Cholesteryl ether behaves metabolically as cholesteryl ester (14), however, because of the ether bond resecretion by the cells is prevented. To assess selective uptake ldlA[mSR-BI] cells (kindly provided by Dr. Monty Krieger, Dept. of Biology, Massachusetts Institute of Technology, Cambridge, MA, USA) were used, a CHO-derived cell line lacking LDL receptor expression that was in addition stably transfected with the mouse SR-BI cDNA. Cells were cultured as described except that 24 h before adding the labeled HDL preparations (50 \(\mu\text{g HDL cholesterol/ml}\) 10\% FBS was substituted by 10\% lipoprotein-depleted serum (16). Labeled HDL preparations were then added to the cells in serum-free DMEM, and incubations were continued for 6 h. Supernatants and cells were processed as detailed above for macrophages, and radioactivity within medium and cells was determined. Cellular uptake is expressed as the percentage of counts recovered from the cells in relation to the counts present on the plate (sum of medium and cells).

Determination of HDL glycation

The extent of HDL glycation was determined using an enzymatic fructosamine assay (Diazyme, Dresden, Germany) according to the manufacturer’s protocol. Mouse
HDL was isolated by ultracentrifugation as described above, and 40 µg of total protein from respective HDL preparations was used in the assay.

In vivo HDL kinetics studies

HDL kinetics studies were performed essentially as published previously (16, 20, 22). Autologous HDL was isolated by ultracentrifugation from pooled plasma of either control or T1DM mice (density 1.063 < d < 1.21) and dialyzed extensively against sterile PBS containing 0.01% EDTA. HDL was then labeled with the respective trap labels $^{125}$I-tyramine-cellobiose (TC) and cholesteryl hexadecyl ether (cholesteryl-1,2,3$^3$H; Perkin Elmer Life Sciences). Then 0.4 µCi of $^{125}$I and 0.7 million dpm of the $^3$H tracer were injected into the tail veins of fasted control and T1DM mice. Blood samples were obtained by retroorbital bleeding at 5 min, 1 h, 3 h, 6 h, 11 h, and 24 h after injection. Plasma decay curves for both tracers were generated by dividing the plasma radioactivity at each time point by the radioactivity at the initial 5-minute time point after tracer injection and used to calculate fractional catabolic rates (FCRs) after fitting to a bicompartamental model using the SAAM II program (16). Hepatic uptake of HDL apolipoproteins ($^{125}$I) and HDL-CEs ($^3$H-cholesteryl ether) was calculated by expressing the counts recovered in liver as a percentage of the injected dose, which was calculated by multiplying the initial plasma counts (5-minute time point) with the estimated plasma volume (3.5% of total body weight). Selective uptake into liver was determined by subtracting the percentage of the injected dose of $^{125}$I-HDL recovered in liver from the percentage of the injected dose of $^3$H-HDL-CE.
Western blotting

Western blots for SR-BI were carried out on total liver homogenates as well as on hepatic membrane fractions prepared essentially as described (16). Protein concentrations were determined with the bicinchoninic acid (BCA) assay (Pierce Biotechnology, Inc., Rockford, IL, USA). Equal amounts of protein were separated by SDS-PAGE electrophoresis and blotted onto nitrocellulose. SR-BI was visualized using a commercially available goat anti-mouse SR-BI antibody (Novus Biologicals, Littleton, CO, USA), followed by the appropriate HRP-conjugated secondary antibody. HRP was detected using chemiluminescence (ECL, GE Healthcare). Quantitation was carried out using the freely available ImageJ software (http://rsb.info.nih.gov/ij/), adjusting the background for the area size of each band and subtracting it from the respective bands. Results were normalized for the average of the control mice.

Analysis of gene expression by real-time quantitative PCR

Total RNA from mouse livers was isolated using Trizol (Invitrogen) and quantified with a NanoDrop ND-100 UV-Vis spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). cDNA synthesis was performed from 1 µg of total RNA using reagents from Invitrogen. Real-time quantitative PCR was carried out using an ABI-Prism 7700 (Applied Biosystems, Foster City, CA, USA) sequence detector with the default settings (16). Multi-exon spanning PCR primers and fluorogenic probes were designed with the Primer Express Software (Applied Biosystems) and synthesized by Eurogentec (Seraing, Belgium). The mRNA expression levels presented were calculated
relative to the average of the housekeeping gene cyclophilin and further normalized to the relative expression levels of the respective controls.

Statistical analysis

Statistical analysis was carried out using the Statistical Package for the Social Sciences (SPSS, Inc., Chicago, IL, USA). Values are expressed as means ± SEM. Unpaired Student’s t test was used to assess statistical differences between groups. Statistical significance for all comparisons was assigned at P<0.05.
Results

Plasma cholesterol levels and liver cholesterol content are increased in type 1 diabetic mice.

Mice treated with alloxan became severely hyperglycemic within 2 days after injection and remained diabetic throughout the 11 days of the experiment (P<0.001, Table 1). Consistent with these results, blood HbA1c levels were significantly increased in alloxan-injected mice on day 10 (P<0.001, Table 1). The striking decrease in plasma insulin levels (P<0.001, Table 1) confirmed alloxan-induced destruction of pancreatic beta cells.

Plasma total cholesterol was increased in diabetic mice (P=0.01, Table 1). FPLC profiles showed an overall increase in HDL cholesterol levels and also the appearance of larger HDL particles in the diabetic group (Figure 1). Plasma triglycerides were higher in diabetic mice compared to controls (P<0.01, Table 1), while phospholipid and free fatty acid levels remained unchanged (Table 1).

Body weight in diabetic mice was lower than in controls (P<0.001, Table 1). Although absolute liver weight was not different between the groups, liver weight relative to body weight was increased in T1DM mice (P<0.001, Table 1). Hepatic cholesterol content was 22% higher in the diabetic group (P<0.001, Table 1), while liver triglycerides and phospholipids were not affected (Table 1).

Biliary cholesterol and bile acid secretion are increased in type 1 diabetic mice.
Continuous bile cannulation was performed to assess the impact of T1DM on biliary sterol secretion. Bile flow was 2.1-fold increased (2.67 ± 0.36 vs 1.25 ± 0.11 µl/min, P<0.01, Figure 2A) in diabetic mice. Furthermore, biliary bile acid secretion (2333 ± 354 vs 223 ± 13 nmol/min/100 g BW, P<0.001) was 10.5-fold increased, while biliary phospholipid (103.2 ± 14.9 vs 23.0 ± 1.6 nmol/min/100 g BW, P<0.001) and cholesterol secretion (11.73 ± 2.36 vs 2.15 ± 0.17 nmol/min/100 g BW, P<0.01) were 4.5-fold and 5.5-fold higher in the T1DM group, respectively (Figures 2B-D).

Fecal bile acid excretion was significantly higher in diabetic mice (2.85 ± 0.20 vs 2.01 ± 0.12 µmol/day, P<0.01, Figure 3A). However, fecal neutral sterol excretion did not change significantly (4.04 ± 0.28 vs 3.46 ± 0.23 µmol/day, P=0.13, Figure 3B), conceivably attributable to increased intestinal cholesterol absorption as indicated by higher plasma campesterol/cholesterol ratios in T1DM mice compared with controls (12.5 ± 1.1 vs 8.4 ± 0.4 x 10⁻³, P<0.01) as well as by a 2.1-fold higher intestinal fractional cholesterol absorption in direct measurements (74 ± 12 vs 35 ± 8 %, P<0.05).

*Hepatic gene expression analysis indicates increased bile acid synthesis in type I diabetic mice.*

The hepatic expression of the major transporters for biliary cholesterol, ATP-binding cassette transporter G5 (*Abcg5*) and *Abcg8* was increased by 34% (P<0.05) and 33% (P<0.01), respectively (Table 2). The expression of the biliary phospholipid transporter *Abcb4* (*Mdr2*) was increased by 19% (P<0.05), whereas the expression of the bile acid transporter *Abcb11* (*Bsep*) was not different (Table 2). The expression of HMG Coenzyme A reductase (*Hmgcr*) remained unchanged indicating that cholesterol
synthesis rates were not altered in T1DM mice. Identical plasma lathosterol/cholesterol ratios in T1DM mice and controls as measure of endogenous cholesterol synthesis rates further supported this conclusion (1.6 x 10^{-4} ± 0.2 x 10^{-4} vs 1.6 x 10^{-4} ± 0.2 x 10^{-4}). However, a 9-fold (P<0.01) increase in the expression of Cyp7a1 and a 1.7-fold elevated expression of Cyp8b1 (P<0.05) indicated increased bile acid synthesis in diabetic mice.

**Macrophage-to-feces RCT is decreased in type I diabetic mice.**

Biliary sterol secretion, either as free cholesterol or after metabolic conversion to bile acids, is a critical step in RCT (7). Therefore, a macrophage-to-feces RCT experiment was performed to investigate whether increased biliary sterol secretion in diabetic mice would translate into increased RCT. Following i.p. injection with [3H]cholesterol-loaded macrophage foam cells, counts recovered from plasma tended to be higher in diabetic mice compared to controls (3.13 ± 0.46 vs 2.21 ± 0.19 % of injected dose, P = 0.07 at 48 h, Figure 4A). The amount of label present within the liver at time of sacrifice did not differ between the groups (Figure 4B). Surprisingly, despite the increased mass biliary sterol secretion, overall macrophage-to-feces RCT was reduced by 20% in diabetic mice compared to controls (9.23 ± 0.79 vs 11.54 ± 0.61 % of injected dose, P<0.05, Figure 4C). This difference was mainly due to a decreased amount of label excreted within the fecal bile acid fraction of T1DM mice (7.08 ± 0.77 vs 9.11 ± 0.46 % of injected dose, P<0.05, Figure 4C), while label recovered within the fecal neutral sterol fraction was not different between groups (Figure 4C).

*The efflux capacity of HDL is not affected in type I diabetic mice.*
Glycation of apolipoprotein (apo) A-I has been associated with reduced functionality in cholesterol efflux assays (23). Thus, we first determined whether alloxan-induced T1DM results in increased glycation of HDL proteins. HDL from diabetic mice was more glycated compared to control HDL as judged by a significant increase in fructosamine residues (83 ± 3 vs. 50 ± 6 nmol/mg protein, P<0.01, Figure 5A). Next, we investigated whether increased glycation of HDL particles would translate into altered cholesterol efflux from \[^3\text{H}\]\text{cholesterol}-loaded macrophage foam cells towards these particles as a potential mechanism explaining decreased macrophage-to-feces RCT in T1DM mice. However, the amount of labeled cholesterol effluxed \emph{in vitro} from macrophage foam cells towards either control HDL or HDL isolated from diabetic mice did not differ (Figure 5B) indicating that changes in the efflux capacity of HDL do not represent the underlying mechanism for decreased RCT in T1DM mice.

\textit{Uptake of HDL cholesterol by the liver is impaired in type I diabetic mice.}

Hepatic uptake of HDL cholesterol is another key process in RCT and impaired uptake of HDL cholesterol by the liver would offer an alternative explanation for decreased macrophage-to-feces RCT in diabetic mice. Therefore, the hepatic expression of the selective uptake transporter for HDL cholesterol, scavenger receptor class BI (SR-BI), was investigated. While the mRNA expression of SR-BI was 22% higher in diabetic mice compared to controls (P<0.05, Figure 6A), neither total nor membrane-associated SR-BI protein expression was different between groups (Figure 6B). Besides the expression of SR-BI, a reduced affinity of the ligand, namely the HDL particle, could also impair hepatic selective uptake of HDL cholesterol. Selective uptake from T1DM
HDL into ldlA[mSR-BI] cells, an *in vitro* model system for SR-BI-mediated selective uptake of cholesterol from HDL, was reduced by 41% (18.3 ± 2.9 vs 31.2 ± 2.8 %, P<0.05, Figure 6C).

To investigate whether reduced hepatic selective uptake als occurs *in vivo* in T1DM mice a series of HDL kinetic studies was performed. While the HDL protein turnover did not differ between controls mice and T1DM (0.098 ± 0.009 vs 0.089 ± 0.006 pools/h, n.s., Figure 6D), the HDL-CE FCR was significantly lower in diabetic animals (0.169 ± 0.005 vs 0.131 ± 0.006 pools/h, P<0.01, Figure 6D) indicating reduced whole body selective uptake. Hepatic uptake of HDL proteins was similar in controls and T1DM mice (26.4 ± 2.6 vs 23.8 ± 2.3 %, n.s., Figure 6E), however, uptake of HDL-CE was lower in T1DM than controls (46.0 ± 2.4 vs 36.6 ± 2.3 %, P<0.05, Figure 6E) translating into a significant reduction of hepatic selective uptake under diabetic conditions (19.6 ± 1.9 vs 12.8 ± 0.9 %, P<0.05, Figure 6E). These combined data demonstrate that impaired hepatic selective uptake of cholesterol from T1DM HDL particles occurs also *in vivo* and indicate that this mechanism contributes to reduced *in vivo* RCT in T1DM mice.
Discussion

Our data demonstrate that experimental T1DM results in a decrease in macrophage-to-feces RCT despite increased biliary sterol secretion rates. To delineate the underlying mechanism of this finding our study explored the impact of T1DM on key steps relevant for RCT. While cholesterol efflux towards glycated HDL was not impaired, the functionality of glycated HDL in SR-BI-mediated selective uptake was significantly decreased, conceivably representing a major contributing factor to reduced RCT in T1DM mice.

The starting point of RCT is cholesterol efflux from macrophage foam cells within the vascular wall. Under conditions of hyperglycemia, HDL-associated proteins readily become glycated (24, 25), which might have important functional implications as glycated apoA-I has been reported to be defective in mediating cholesterol efflux (23). However, our data demonstrate that in the context of a whole HDL particle in vitro cholesterol efflux towards HDL from T1DM mice was unchanged compared with control HDL. This observation is supported by previous studies using isolated total HDL (26, 27). Combined, these data suggest that glycation of HDL proteins might inhibit ABCA1-mediated efflux, whereas ABCG1-mediated efflux is not affected.

Following transport of cholesterol by HDL through the plasma compartment, the next important step of RCT is selective uptake into the liver mediated by SR-BI. Hepatic SR-BI expression remained largely unchanged in T1DM mice. However, HDL kinetic studies revealed that hepatic selective uptake was significantly lower in T1DM mice in vivo, and also in vitro the properties of T1DM HDL to function in selective uptake were
significantly impaired. Thereby reduced selective uptake of cholesterol from diabetic HDL likely contributes significantly to the decrease in RCT observed in T1DM mice in our study by decreasing the input of cholesterol originating from macrophages into the hepatic cholesterol pool.

Biliary sterol secretion is thought to be a major determinant for the completion of the RCT pathway (7, 8). Our data show enhanced biliary secretion of bile acids as well as cholesterol in T1DM mice. These results are consistent with previously published data demonstrating that alloxan- or streptozotocin-induced diabetes increased biliary sterol secretion rates in rats (13, 28). In addition, concentrations of bile acids and cholesterol in gallbladder bile were increased in T1DM mice injected with alloxan as well as in non-obese diabetic (NOD)-mice (11, 29). Since biliary bile acid secretion is a major driving force for biliary cholesterol secretion (30), the primary point of dysregulation in T1DM is likely in the metabolism of bile acids. Hepatic gene expression analysis indicated increased de novo bile acid synthesis in T1DM animals, for which cholesterol serves as the substrate (31). In contrast to fecal bile acid excretion, fecal neutral sterol excretion was similar between groups despite the 5.5-fold higher biliary cholesterol secretion in the diabetic mice. These data are conceivably explained by 2.1-fold increased cholesterol absorption rates observed in the T1DM mice in our study. In addition, also food intake was almost 2-fold higher in T1DM mice (data not shown), overall resulting in a substantial increase in cholesterol supply from diet. Increased intestinal cholesterol absorption has previously been observed by others in insulin-deficient diabetic rodent models as well as in humans (9, 10, 12, 32). However, it is important to point out that our study specifically identified decreased SR-BI-mediated selective uptake, which
represents the point of entry for macrophage-derived cholesterol into the entero-hepatic system, as the major block in RCT affected in T1DM.

Certain methodological issues have to be considered in the interpretation of our results. HDL kinetic studies using trap labels for HDL proteins as well as HDL cholesteryl ester clearly demonstrated reduced hepatic selective uptake rates in vivo in T1DM mice. The hepatic tracer data in the RCT experiment, however, were obtained 48 h after injection of labeled macrophages using a freely distributable label. The methodology of the in vivo RCT assay is therefore not suitable to allow any conclusion on selective uptake. In addition, the data from the macrophage RCT assay only indicate, whether the tracer deloaded from the macrophages enters better or less good the RCT system and is finally deposited in the feces. Overall cholesterol fluxes through the body can not be derived from these data, since then different pool sizes of cholesterol are important and would have to be taken into account.

Besides mediating RCT, HDL particles have additional anti-atherosclerotic properties such as inhibiting endothelial inflammation (33), promoting vascular nitric oxide generation (34) as well as protecting LDL against oxidative modification (35, 36). Increasing evidence suggests that also these atheroprotective functions of HDL particles are impaired in T1DM. The anti-oxidative properties of HDL appear to be diminished in T1DM (37, 38) and the ability of HDL from T1DM patients to counteract the inhibitory effects of oxidized LDL on endothelium-dependent vasorelaxation is also reduced (39). In addition, glycation of apoA-I was recently shown to decrease the potency of HDL to inhibit neutrophil infiltration and adhesion molecule expression using the carotid artery collar model in rabbits (40). In addition to decreased RCT, these alterations are expected
to contribute to an overall reduced capacity of diabetic HDL to protect against atherosclerotic CVD.

Figure 7 summarizes our current working model on the effects of T1DM on sterol metabolism and RCT. Biliary secretion of bile acids and cholesterol is significantly increased, while hepatic cholesterol synthesis remains largely unaffected. Nevertheless, hepatic cholesterol levels do not decrease, since the liver cholesterol pool in diabetic mice receives more input via increased food intake and higher intestinal cholesterol absorption. These events result in an enhanced cycling of cholesterol between the liver and the intestine without having a net effect on fecal neutral sterol excretion. To compensate for increased fecal bile acid loss, also hepatic bile acid synthesis from cholesterol is upregulated. With respect to RCT, less cholesterol originating from macrophages enters the hepatic cholesterol pool, since SR-BI-mediated selective uptake from diabetic HDL is impaired. Thereby a smaller fraction of this cholesterol is then used for bile acid synthesis. These changes are reflected by decreased overall RCT, primarily due to reduced tracer excretion within the fecal bile acid fraction.

In conclusion, insulin-deficient T1DM mice exhibit decreased RCT despite increased biliary sterol secretion. These unfavorable changes are conceivably due to decreased properties of glycated HDL to function in hepatic selective uptake. Impaired RCT is expected to contribute to the increased risk for atherosclerotic CVD morbidity and mortality in patients with T1DM.
Acknowledgements

We are grateful to Dr. Monty Krieger (Dept. of Biology, Massachusetts Institute of Technology, Cambridge, MA, USA) for providing the ldlA[mSR-BI] cells used in this study. This work was supported by grants from the Netherlands Organization for Scientific Research (VIDI Grant 917-56-358), the Top Institute (TI) Food and Nutrition and the Groningen Expert Center for Kids with Obesity (all to UJFT).
References


mediated selective uptake from HDL but not biliary cholesterol secretion. *J Lipid Res* **49**: 563-571.


Normal high density lipoprotein inhibits three steps in the formation of mildly oxidized low density lipoprotein: step 1. *J Lipid Res* **41**: 1481-1494.


Figure legends

Figure 1: T1DM mice have increased plasma HDL cholesterol levels. Pooled plasma samples (n = 6 mice per group) from mice of the same experimental group were subjected to fast protein liquid chromatography (FPLC) gel filtration using a Superose 6 column as detailed in materials and methods. Subsequently, individual fractions were assayed for cholesterol concentrations. The black line shows the cholesterol profile of the PBS control mice and the dashed line the cholesterol profile of the alloxan-injected mice.

Figure 2: T1DM mice have increased biliary sterol secretion. Continuous bile cannulation was performed on day 10 after injection with either alloxan (n = 5) or PBS (n = 6). Bile was collected during 30 minutes. Bile production and biliary bile salt, phospholipid, and cholesterol concentrations were determined. These data were used to calculate bile flow (A) and biliary bile salt (B), phospholipid (C), and cholesterol (D) secretion rates, respectively. Data are given as means ± SEM. ** P<0.01, *** P<0.001 compared to PBS-injected control mice.

Figure 3: Fecal bile acid excretion is increased in T1DM mice, while fecal neutral sterol excretion is unaltered. Feces of individually housed mice (n = 8 per group) were collected over a period of 24 h, starting on day 8 after alloxan or PBS injection. Fecal samples were dried, weighed, and thoroughly ground. Aliquots were used for determination of bile acids (A) and neutral sterols (B) by gas liquid chromatography as
detailed in materials and methods. Data are given as means ± SEM. ** P<0.01 compared to PBS-injected control mice.

**Figure 4: Macrophage-to-feces reverse cholesterol transport is decreased in T1DM mice.** On day 9 after injection with either PBS (n = 8) or alloxan (n = 7), individually housed mice were injected intraperitoneally with 2 million [3H]cholesterol-loaded macrophage foam cells per mouse. Plasma was collected at the indicated time points after macrophage injection, and counts were assessed by liquid scintillation counting at the indicated time points (A). At the end of the experimental period, livers were harvested, snap-frozen in liquid nitrogen and stored at -80 °C. Counts in a weighed liver sample were determined following solubilization of the tissue and related to total liver mass (B). Feces were collected continuously up to 48 h and were dried, weighed, and thoroughly ground. Aliquots were separated into bile acid and neutral sterol fractions as detailed in materials and methods, and counts recovered from the respective aliquots were related to the total amount of feces produced over the whole experimental period (C). All obtained counts were expressed relative to the administered tracer dose. Data are given as means ± SEM. * P<0.05 compared to PBS-injected control mice.

**Figure 5: HDL from T1DM mice is glycated but HDL-mediated efflux from macrophage foam cells remains unchanged.** HDL was isolated from mice injected with either PBS or alloxan, and fructosamine residues were determined (n = 4 per group) as measure of HDL glycation (A). Differentiated THP-1 macrophages were loaded with 50 µg/ml acetylated LDL and 1 µCi/ml [3H]cholesterol as detailed in materials and methods.
Subsequently, cells were washed with PBS and 50 µg protein/ml of isolated mouse HDL was added (n = 4 per group). After 24 h radioactivity within the medium was determined by liquid scintillation counting. The cell layer was washed twice with PBS, whereafter 0.1 M NaOH was added. Plates were incubated for 30 minutes at room temperature, then radioactivity remaining within the cells was assessed. HDL-independent efflux was calculated from wells incubated with RPMI without added HDL and these values were subtracted from the respective experimental values. Efflux is given as the percentage of counts recovered from the medium in relation to the total counts present on the plate (sum of medium and cells) (B). Data are given as means ± SEM. ** P<0.01 compared to HDL isolated from PBS-injected control mice.

**Figure 6: T1DM mice have impaired selective uptake of HDL cholesterol by the liver.** Hepatic mRNA expression of SR-BI in mice that were injected with either PBS (n = 8) or alloxan (n = 7) was determined by real-time quantitative PCR (A). Protein expression of SR-BI in the liver homogenates (n=6 in each group, left panel) and in the hepatic membrane fraction (n=6 in each group, right panel) was determined by Western blot and representative images are shown (B). SR-BI-mediated selective uptake of HDL cholesterol from isolated HDL was determined in vitro using ldlA[mSR-BI] cells as detailed in materials and methods (C). Fractional catabolic rates of HDL protein as well as HDL-CE were determined in control (n=6) and T1DM (n=5) mice after injection of autologous HDL labeled with 125I-tyramine cellobiose and 3H-cholesteryl ether as described in materials and methods (D). Hepatic uptake rates of HDL protein, HDL-CE as well as selective uptake rates in control and T1DM mice determined at the 24h-time
point of the kinetic experiment shown in panel D (E). Experimental conditions and calculations are detailed in materials and methods. All data are given as means ± SEM. * P<0.05, ** P<0.01 compared to controls.

**Figure 7: Working model summarizing the impact of T1DM on sterol metabolism and RCT.** Compared to controls (panel A), biliary secretion of bile acids and cholesterol is significantly increased in T1DM (panel B). While hepatic cholesterol synthesis remains unaltered, bile acid synthesis is up-regulated. Hepatic cholesterol levels do not decrease, since the liver cholesterol pool in diabetic mice receives more input via increased intestinal cholesterol absorption due to both, increased food uptake and higher fractional absorption rates. These events result in an enhanced cycling of cholesterol between the liver and the intestine without having a net effect on fecal neutral sterol excretion. In terms of RCT, less cholesterol originating from macrophages enters the hepatic pool due to impaired hepatic SR-BI-mediated selective uptake from diabetic HDL, and a lower fraction of this cholesterol is subsequently used for bile acid synthesis. These changes are reflected by decreased overall RCT, primarily attributable to reduced tracer excretion within the fecal bile acid fraction. Bold arrows represent increased fluxes in T1DM mice (B) compared to PBS-injected controls (A), while thinner arrows represent decreased fluxes. chol, cholesterol; BA, bile acids.
### Tables

**Table 1.** Plasma and liver parameters in mice on day 10 after injection with alloxan or PBS

<table>
<thead>
<tr>
<th></th>
<th>PBS (n= 6-8)</th>
<th>Alloxan (n= 6-8)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Plasma parameters</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td>8.4 ± 0.2</td>
<td>&gt;30</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>5.0 ± 0.1</td>
<td>8.2 ± 0.3 ‡</td>
</tr>
<tr>
<td>Insulin (ng/ml)</td>
<td>1.24 ± 0.06</td>
<td>0.19 ±0.3 ‡</td>
</tr>
<tr>
<td>Cholesterol (mg/dl)</td>
<td>85 ± 3</td>
<td>102 ± 5 §</td>
</tr>
<tr>
<td>Triglycerides (mg/dl)</td>
<td>47 ± 6</td>
<td>85 ± 10 †</td>
</tr>
<tr>
<td>Phospholipids (mg/dl)</td>
<td>199 ± 4</td>
<td>214 ± 11</td>
</tr>
<tr>
<td>Free fatty acids (mmol/l)</td>
<td>0.84 ± 0.17</td>
<td>0.70 ± 0.16</td>
</tr>
<tr>
<td><strong>Body weight and liver parameters</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>24.5 ± 0.4</td>
<td>20.5 ± 0.7 †</td>
</tr>
<tr>
<td>Liver weight (g)</td>
<td>1.13 ± 0.03</td>
<td>1.18 ± 0.06</td>
</tr>
<tr>
<td>Liver / body weight ratio</td>
<td>0.045 ± 0.001</td>
<td>0.060 ± 0.002 ‡</td>
</tr>
<tr>
<td>Liver cholesterol (µmol/g)</td>
<td>4.6 ± 0.1</td>
<td>5.6 ± 0.1 ‡</td>
</tr>
<tr>
<td>Liver triglycerides (µmol/g)</td>
<td>4.9 ± 0.9</td>
<td>2.8 ± 0.9</td>
</tr>
<tr>
<td>Liver phospholipids (µmol/g)</td>
<td>34.0 ± 1.3</td>
<td>34.0 ± 0.5</td>
</tr>
</tbody>
</table>

Data are given as means ± SEM. § P<0.05, † P<0.01, ‡ P<0.001 each compared to PBS controls.
Table 2. Hepatic mRNA expression levels at day 10 after injection with alloxan or PBS

<table>
<thead>
<tr>
<th>Gene</th>
<th>PBS (n = 6)</th>
<th>Alloxan (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abcg5</td>
<td>1.00 ± 0.08</td>
<td>1.34 ± 0.09§</td>
</tr>
<tr>
<td>Abcg8</td>
<td>1.00 ± 0.03</td>
<td>1.33 ± 0.08†</td>
</tr>
<tr>
<td>Abcb4</td>
<td>1.00 ± 0.05</td>
<td>1.19 ± 0.06§</td>
</tr>
<tr>
<td>Abcb11</td>
<td>1.00 ± 0.02</td>
<td>0.93 ± 0.07</td>
</tr>
<tr>
<td>Hmgcr</td>
<td>1.00 ± 0.08</td>
<td>1.08 ± 0.22</td>
</tr>
<tr>
<td>Cyp7a1</td>
<td>1.00 ± 0.29</td>
<td>8.71 ± 2.16†</td>
</tr>
<tr>
<td>Cyp8b1</td>
<td>1.00 ± 0.11</td>
<td>1.69 ± 0.27§</td>
</tr>
</tbody>
</table>

Expression levels were normalized to the housekeeping gene cyclophilin and further normalized to the mean expression levels of the PBS control group. Data are given as means ± SEM. § P<0.05, † P<0.01 each compared to PBS controls.
Figure 1

Lipoprotein profile

VLDL  LDL  HDL

cholesterol (mg/dl)

Fraction

PBS  Alloxan
Figure 2

A  Bile flow

B  Biliary bile acid secretion

C  Biliary phospholipid secretion

D  Biliary cholesterol secretion

PBS  Alloxan
Figure 3

(A) Fecal bile acid excretion

(B) Fecal neutral sterol excretion
Counts in liver

**PBS**

<table>
<thead>
<tr>
<th>% of injected dose</th>
<th>0</th>
<th>2</th>
<th>4</th>
<th>6</th>
<th>8</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Alloxan</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Total NS BA**

<table>
<thead>
<tr>
<th>% of injected dose</th>
<th>0</th>
<th>5</th>
<th>10</th>
<th>15</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PBS</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Alloxan</strong></td>
<td>*</td>
<td>*</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Tracer in plasma

**PBS**

<table>
<thead>
<tr>
<th>% of injected dose</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Alloxan</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Macrophage-to-feces RCT

**PBS**

<table>
<thead>
<tr>
<th>% of injected dose</th>
<th>0</th>
<th>5</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Alloxan</strong></td>
<td>*</td>
<td>*</td>
<td></td>
</tr>
</tbody>
</table>

Figure 4
Figure 5

A  Fructosamine

B  Cholesterol efflux to HDL

** nmol/mg HDL-protein

PBS  Alloxan

percent

PBS  Alloxan
Figure 6

A. SR-BI mRNA expression

B. SR-BI protein expression

C. Cholesterol uptake from HDL

D. HDL catabolic rate

E. Hepatic HDL uptake in vivo
Figure 7