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Breakfast partly restores the anti-inflammatory function of high-density lipoproteins from patients with type 2 diabetes mellitus

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ABSTRACT

Background and aims: High-density lipoproteins (HDL) of patients with type 2 diabetes mellitus (T2DM) have impaired anti-inflammatory activities. The anti-inflammatory activity of HDL has been determined *ex vivo* after isolation by different methods from blood mostly obtained after overnight fasting. We first determined the effect of the HDL isolation method, and subsequently the effect of food intake on the anti-inflammatory function of HDL from T2DM patients.

Methods: Blood was collected from healthy controls and T2DM patients after an overnight fast, and from T2DM patients 3 h after breakfast ($n = 17$ each). HDL was isolated by a two-step density gradient ultracentrifugation in iodixanol (HDL_{DGUC2}), by sequential salt density flotation (HDL_{SEQ}) or by PEG precipitation (HDL_{PEG}). The anti-inflammatory function of HDL was determined by the reduction of the TNFα-induced expression of VCAM-1 in human coronary artery endothelial cells (HCAEC) and retinal endothelial cells (REC).

Results: HDL isolated by the three different methods from healthy controls inhibited TNFα-induced VCAM-1 expression in HCAEC. With apoA-I at 0.7 μM, HDL_{DGUC2} and HDL_{SEQ} were similarly effective (16% versus 14% reduction; $n = 3$; $p > 0.05$) but less effective than HDL_{PEG} (28%, $p < 0.05$). Since ultracentrifugation removes most of the unbound plasma proteins, we used HDL_{DGUC2} for further experiments. With apoA-I at 3.2 μM, HDL from fasting healthy controls and T2DM patients reduced TNFα-induced VCAM-1 expression in HCAEC by 58 ± 13% and 51 ± 20%, respectively ($p = 0.35$), and in REC by 42 ± 13% and 25 ± 18%, respectively ($p < 0.05$). Compared to preprandial HDL, postprandial HDL from T2DM patients reduced VCAM-1 expression by 56 ± 16% (paired test: $p < 0.001$) in HCAEC and by 34 ± 13% (paired test: $p < 0.05$) in REC.

Conclusions: The *ex vivo* anti-inflammatory activity of HDL is affected by the HDL isolation method. Two-step ultracentrifugation in an iodixanol gradient is a suitable method for HDL isolation when testing HDL anti-inflammatory function. The anti-inflammatory activity of HDL from overnight fasted T2DM patients is significantly impaired in REC but not in HCAEC. The anti-inflammatory function of HDL is partly restored by food intake.

Introduction

Plasma high-density lipoprotein (HDL)-cholesterol levels are inversely linked with the risk of cardiovascular disease [1]. Accumulating evidence points to a relationship between cardiovascular risk and impaired HDL function rather than the HDL-cholesterol concentration [2,3]. In general, HDL has anti-inflammatory

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activities, which include the ability to downregulate endothelial and monocyte activation and to reduce the migration of monocytes through the endothelium [4–6]. These anti-inflammatory effects are disturbed under pathological conditions such as type 2 diabetes mellitus (T2DM), acute myocardial infarction, and end-stage renal disease [7–10]. HDL from individuals with T2DM increase the expression of vascular adhesion molecules by macrovascular human umbilical vein endothelial cells [11,12]. Further, HDL from T2DM patients is less able to reduce the low-density lipoprotein (LDL)-induced monocyte chemotactic activity in macrovascular human aortic endothelial cells [8]. Hyperglycemia, hyperinsulinemia, (postprandial) hypertriglyceridemia, inflammation, and oxidative stress appear to modify the levels and activity of HDL surface proteins, thereby decreasing the functionality of HDL in T2DM [13–16].

Most if not all *ex vivo* studies on the anti-inflammatory function of HDL used HDL from participants after an overnight fast. However, the largest part of the day the postprandial state applies, and the functionality of HDL in the postprandial state may differ from that in the fasting state. A recent study showed that the cholesterol efflux capacity of HDL was improved after a high-fat meal intake [17]. Food intake may lead to the production of more functional HDL, for example via changes in lipid or protein composition or increased preβ-HDL [17,18]. Whether the anti-inflammatory function also differs between pre- and postprandial HDL is unknown. We hypothesized that the anti-inflammatory function of HDL improves upon food intake, and thereby may diminish the impairment seen in patients with T2DM.

The primary aim of this study is to determine whether food intake affects the anti-inflammatory function of HDL from T2DM patients on macrovascular coronary artery and microvascular retinal endothelial cells. HDL was isolated from plasma of patients with T2DM, after an overnight fast and 3 h after breakfast. HDL composition [19–25] or size distribution [26], and potentially also the HDL functionality, are affected by commonly used methods for HDL isolation [27,28]. Our secondary aim was therefore to compare HDL isolated from plasma by density gradient ultracentrifugation (DGUC) in iodixanol, a non-ionic, non-toxic medium [29,30], with HDL isolated by sequential salt density flotation and polyethylene glycol (PEG) precipitation.

Materials and methods

Study participants

Patients with T2DM of the outpatient clinic of the Máxima Medical Center (Eindhoven and Veldhoven, NL) were recruited through advertisements and invitations sent by the Dutch Diabetes Association; region Peel & Kempen, to participate in a one-day trial at the Medical Center involving the consumption of three main meals including breakfast. In the study presented here, data before and after breakfast is analyzed. Inclusion criteria were: 1) diagnosis of T2DM by a physician at least 1 year before recruitment, 2) age $\geq$18 years and $\leq$85 years, 3) the ability to ingest three main meals on the trial day. Exclusion criteria were: 1) known malignancy, immunological disease or allergy, or the use of immunomodulatory drugs other than acetylsalicylic acid, 2) the use of medication that may alter glucose metabolism other than insulin and metformin 3) food allergies that prevent the patient from eating the prescribed meals, and 4) renal dysfunction (estimated glomerular filtration rate (eGFR) < 50 ml/min). Patients were asked to take their medication as usual. Healthy controls were recruited from the personnel at the Erasmus University Medical Center (Rotterdam, NL). The single inclusion criterion was: age $\geq$18 years. Exclusion criteria were: 1) (family) history of diabetes, 2) known malignancy, immunological disease or allergy, or use of immunomodulatory drugs or antioxidants other than acetylsalicylic acid, 3) the use of medication that may alter glucose metabolism, including but not limited to sulfonylurea-derivatives, systemic or inhaled glucocorticoids or non-selective beta-blockers, and 4) renal dysfunction (eGFR <50 ml/min). All participants provided written informed consent (MEC-1327, MEC-2016-202, MEC-2014-066). All experiments were performed following relevant institutional and national guidelines and regulations. All experimental protocols were approved by an Erasmus University Medical Center institutional and/or licensing committee.

Experimental design and plasma samples

Ethylene Diamine Tetra-Acetate (EDTA) blood was collected from the healthy controls after an overnight fast, and from the individuals with T2DM after an overnight fast and 3 h after breakfast consumption. Breakfast was offered to the patients in the hospital. Patients were allowed to choose between four different mixed-meal breakfasts (range 400–700 kcal; Table S1). After collection, blood was immediately centrifuged at 3000×*g* for 10 min and plasma was stored at −80 °C until HDL isolation. After isolation, HDL was stored at 4 °C and tested for its anti-inflammatory function within seven days.

Materials

Optiprep™ (60% w/v iodixanol) and polyethylene glycol (PEG)-6000 were obtained from Sigma-Aldrich (Gillingham, UK). Human coronary artery endothelial cells (HCAEC) (CC-2585) were purchased from Lonza (Basel, Switzerland), and retinal endothelial cells (REC) (ACBRI 181) from Cell Systems Kirkland (Kirkland, USA). HCAEC were cultured in endothelial basal medium (EBM)-2 (Lonza, Basel, Switzerland) supplemented with endothelial growth medium (EGM)-2-MV SingleQuots (Lonza, Basel, Switzerland) and 5% fetal calf serum (FCS). REC were cultured in EBM (Lonza, Basel, Switzerland) supplemented with EGM-MV SingleQuots (Lonza, Basel, Switzerland) and 5% FCS. Mouse anti-human vascular cell adhesion molecule 1 (VCAM-1) conjugated to phycoerythrin fluorochrome (CD106-PE, clone STA) was obtained from eBioscience (San Diego, USA). Tumor necrosis factor α (TNFα) was purchased from R&D Systems (Minneapolis, USA).

Biochemical assays

Lipids and apolipoprotein (apo) levels were measured with commercially available kits (DiaSys Diagnostic Systems GmbH, Holzheim, Germany) on an automated Selectra-E analyzer (Vital Scientific, Spankeren, the Netherlands), and protein concentrations were measured with the Pierce™ bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific, Rockford, USA).

Isolation of HDL

Two-step iodixanol density gradient ultracentrifugation (DGUC2)
HDL was first separated from the apoB-containing lipoproteins and then separated from non-lipoprotein-bound plasma proteins. After 1.5 ml of plasma was mixed with Optiprep™ to a final iodixanol concentration of 14%, it was overlayered with 9% iodixanol (w/v) in saline and centrifuged for 2 h (246,000×*g* and 4 °C) using Quickseal polyallomer centrifugation tubes, a Beckman NVTi65.2 rotor, and an Optima XL-100K ultracentrifuge (Beckman Coulter, Palo Alto, USA). After centrifugation, the tubes were fractionated from the bottom (aspiration speed 1.15 ml/min, 44 fractions, 0.1 ml/fraction) and the first 16 fractions were pooled. This pool was mixed with the

Optiprep™ solution to a final iodixanol concentration of 38% (w/v), overlayered with a 38% (w/v) iodixanol solution in saline, and again centrifuged for 2 h at 246,000×*g* and 20 °C. The tubes were fractionated from the bottom, and the fractions 36–44 were pooled. This HDL pool was denoted as HDL_{DGUC2}.

Sequential salt density flotation HDL was isolated from plasma as described previously [31]. In the first step, 2.1 ml of a KBr solution (density 1.063 g/ml) was underlayered with 3 ml of plasma brought to a density of 1.063 g/ml by dissolving KBr and centrifuged for 20 h at 220,000×*g* and 4 °C in an NVTi65.2 rotor. The floating lipid layer, containing very-low-density (VLDL), intermediate-density (IDL), and low-density lipoproteins (LDL), was discarded. Next, the density of the remaining HDL-containing solution was adjusted to 1.23 g/ml by adding KBr. This HDL solution was layered underneath the same volume of a 1.21 g/ml KBr-solution and centrifuged for 44 h at 220,000×*g* and 4 °C. The HDL-containing floating layer was collected into a fresh tube, adjusted to a density of 1.23 g/ml, layered under a 1.21 g/ml KBr solution, and re-centrifuged (44 h, 220,000×*g*, 4 °C). Thereafter, the HDL-containing floating layer was collected and dialyzed overnight against phosphate-buffered saline (PBS). The obtained HDL was denoted as HDL_{SEQ}.

PEG precipitation ApoB-containing lipoproteins were removed by mixing 0.6 ml plasma with 0.3 ml PEG-solution (36% PEG-6000 in 10 mM HEPES, pH 8.0), followed by 30 min incubation on ice and centrifugation (30 min, 2000×*g*), as described previously [23]. The HDL-containing supernatant was collected and was denoted as HDL_{PEG}.

Electrophoresis of HDL proteins

The proteins in the HDL_{DGUC2} preparations were separated by denaturing SDS-polyacrylamide gel electrophoresis using the Mini-PROTEAN system and 15-wells 4–20% gradient precast gels from Bio-Rad (Lunteren, The Netherlands). Of each HDL_{DGUC2} preparation, apoA-I content was measured, and a volume containing 2.8 μg of apoA-I was loaded onto the gel. After electrophoresis, the gels were Coomassie-stained and analyzed by densitometry using ImageQuant TL, version 8.1 (GE Healthcare Life Sciences, Amersham, UK). The densities of the bands were converted to μg based on the density of the apoA-I band in the corresponding lane. Bands with molecular sizes of 32k, 23k, 18.5k and 9k were taken to represent apoE, apoA-I, apoA-II and apoC-II + apoC-III, respectively.

TNFα-induced VCAM-1 expression by HCAEC and REC

HCAEC and REC were grown to confluence in gelatin-coated culture flasks with medium containing 5% FCS in a humidified incubator (37 °C, 5% CO_2). For the experiments, HCAEC and REC (passage 7–13) were seeded in gelatin-coated 12 well plates (200,000 cells per well) in 750 μl supplemented basal media containing 1% FCS and cultured overnight (37 °C, 5% CO_2). Cells were pre-incubated for 3 h with isolated HDL; the amount of HDL added was based on the apoA-I content. As assay controls, cells were incubated in parallel either with 1) supplemented basal medium only, 2) PBS (pH 7.4), or 3) isolation material solution without HDL. After pre-incubation, TNFα was added and the cells were cultured for an additional 17 h (37 °C, 5% CO_2). Subsequently, cells were prepared for analysis of VCAM-1 protein expression by fluorescence-activated cell sorting (FACS). All conditions were tested in triplicate unless stated otherwise. The amount of TNFα used was chosen to elicit a 10-fold increase in fluorescence intensity in the FACS analysis (range 0.5–10 ng/ml).

Quantification of VCAM-1 expression by flow cytometry

Adherent cells were washed with PBS, collected by gentle scraping in PBS containing 2 mM EDTA (pH 6.4), and centrifuged (3 min, 729×*g*). Cell pellets were suspended in staining buffer (PBS containing 1% bovine serum albumin) and incubated with anti-VCAM-1 antibodies (1:10 and 1:20 for HCAEC and REC, respectively) for 20 min in the dark at room temperature. Thereafter, cells were washed with staining buffer and analyzed for VCAM-1 protein expression levels through measurement of the fluorescence intensity using flow cytometry (1×10^4 events per sample) on a FACSCanto-II (Becton Dickinson, Franklin Lakes, USA). Viable cells were gated and data were analyzed with Infinicyt software (Cytognos SL, Salamanca, Spain).

Statistical analysis

Data are expressed as mean ± SD or n (%) unless stated otherwise. Differences between patients and controls were tested by independent samples *t*-test for continuous data and by $\chi 2$ test for categorical data. In *ex vivo* experiments, the mean VCAM-1 expression (assessed by measuring the mean fluorescence intensity) induced by TNFα without HDL was used as a reference value. The mean VCAM-1 expression in the parallel incubations was expressed as a percentage of this reference value. Outliers were identified with the ROUT method (Q set at 1%). Differences between experimental conditions were analyzed by one-way ANOVA followed by Tukey's test. These analyses were performed in GraphPad Prism, version 6. We compared the anti-inflammatory effects of HDL from T2DM patients and nondiabetic control individuals on REC and HCAEC with two linear regression models (model 1: no adjustments for potential confounders; model 2: adjusted for age and sex). Within the patient group, the effect of breakfast consumption on HDL anti-inflammatory function was assessed by paired-samples t-tests. Associations between the HDL anti-inflammatory activity and potential contributing factors were evaluated with bivariate Pearson correlation analysis. The normality of the data was confirmed with the Shapiro-Wilk test. These analyses were carried out using SPSS version 24.0. Results with p values of ≤ 0.05 were considered statistically significant.

Results

Effect of HDL isolation method on the anti-inflammatory activity

HDL was isolated from plasma of healthy volunteers by a two-step density gradient ultracentrifugation in iodixanol (HDL_{DGUC2}), sequential salt density flotation (HDL_{SEQ}), or PEG precipitation (HDL_{PEG}). When tested at an HDL concentration of 0.71 μM apoA-I, HDL_{DGUC2}, HDL_{SEQ} and HDL_{PEG} significantly suppressed the TNFα-induced VCAM-1 expression in HCAEC ($p < 0.001$) (Fig. 1A). At concentrations present in the HDL preparation, iodixanol slightly decreased TNFα-induced VCAM-1 expression, while PEG did not. When we corrected for this, HDL_{DGUC2} was as effective as HDL_{SEQ} (16% versus 14%; $p > 0.05$) but less effective than HDL_{PEG} (28%, $p < 0.05$) in inhibiting the TNFα-induced VCAM-1 expression in HCAEC (Fig. 1B). HDL_{PEG} still contains most of the free, non-lipoprotein plasma proteins. These free proteins as well as apoB-containing lipoproteins were removed in the first and second step of HDL isolation by density gradient ultracentrifugation in iodixanol (Figs. S1A and S1B). Therefore, we selected HDL_{DGUC2} to be used in all further experiments.

When tested in a concentration ranging from 0.7 to 3.0 μM apoA-I, HDL_{DGUC2} dose-dependently suppressed the TNFα-induced VCAM-1 expression in HCAEC (Fig. 2). When corrected for the

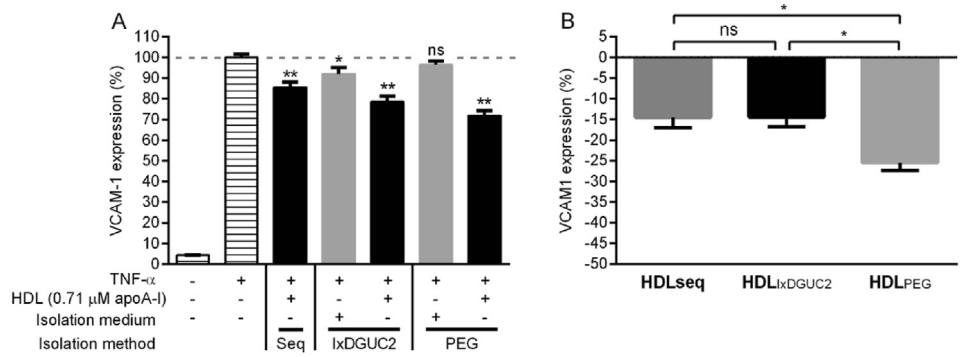

Fig. 1. Comparison of effects of HDL_{SEQ}, HDL_{DGUC2}, and HDL_{PEG} on TNFα-stimulated VCAM-1 expression by HCAEC. The concentration of HDL added was based on the apoA-I concentration (0.71 μM). A) VCAM-1 expression (mean ± SEM) is shown in the absence or in the presence of TNFα (10 ng/ml) and PBS, iodixanol (3.9 vol % isolation medium), and PEG (2 vol % isolation medium) as controls for HDL_{SEQ}, HDL_{DGUC2} and HDL_{PEG}, respectively. *p* values compared to TNFα-stimulated PBS control: $*p \leq 0.05$; $**p \leq 0.001$. B) Inhibition of TNFα-stimulated VCAM-1 expression (mean ± SEM) in the presence of HDL_{SEQ}, HDL_{DGUC2}, and HDL_{PEG} relative to their respective controls; $*p \leq 0.05$. Results are the mean of three independent experiments with pooled HDL isolated from plasma of three healthy volunteers.

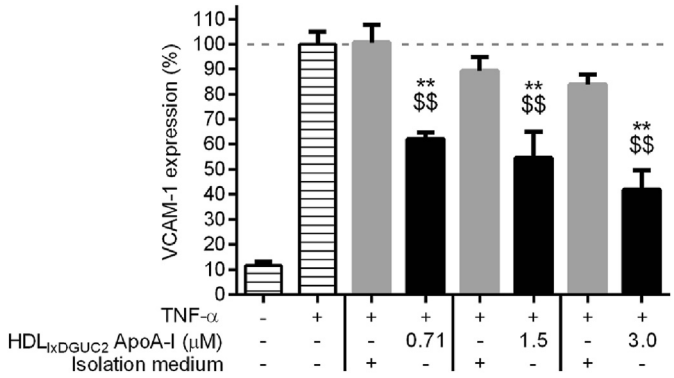

Fig. 2. Dose-response effect of HDL_{DGUC2} on TNFα-induced VCAM-1 expression by HCAEC. VCAM-1 expression (mean ± SEM) is shown induced by TNFα (2 ng/ml). The TNFα-induced VCAM-1 expression in the presence of PBS was set at 100%. Results are the average of three independent experiments with pooled HDL isolated from plasma of three healthy volunteers. As a control, the iodixanol isolation solution was added corresponding to the volume of the HDL_{DGUC2}. $**p \leq 0.01$ versus TNFα-stimulated PBS control; $^{\$\$}p \leq 0.01$ versus corresponding isolation material control.

inhibitory effect of the iodixanol, the inhibition of VCAM-1 expression was approximately 45% at all three HDL concentrations tested.

Anti-inflammatory activity of preprandial HDL from T2DM patients and healthy controls

The characteristics of the patients with T2DM and the healthy controls are presented in Table 1. In the T2DM group, age and body mass index (BMI) were higher than in the control group. As expected, fasting triglyceride and glucose levels were higher and HDL-cholesterol was lower in the T2DM group than in the healthy controls. Total cholesterol and LDL-cholesterol were lower as well, possibly related to the use of cholesterol-lowering drugs.

Both patient and control HDL significantly decreased the TNFα-induced VCAM-1 expression by HCAEC and REC (all $p < 0.001$; Fig. 3). HDL from T2DM patients and healthy controls similarly decreased the VCAM-1 expression in HCAEC (51 ± 20% versus 58 ± 13%, $p = 0.353$ (model 2, Table 2)). In contrast, HDL from T2DM patients decreased VCAM-1 expression in REC significantly less than HDL from healthy controls (25 ± 18% versus 42 ± 13%, $p < 0.01$; model 1, Table 2). This difference was still significant after correction for age and sex ($p < 0.05$; model 2, Table 2). No correlation was detected between BMI and anti-inflammatory function of HDL from healthy controls or T2DM patients (Pearson's correlation test: $p > 0.05$).

Because the triglyceride and cholesterol content in HDL has been reported to affect its stability and functioning [15], we determined the triglyceride/apoA-I ratio and the cholesterol/apoA-I ratio. The triglyceride/apoA-I ratio in HDL_{DGUC2} of the T2DM patients was significantly higher than in HDL_{DGUC2} of the healthy controls (0.15 ± 0.04 versus 0.10 ± 0.03, $p < 0.01$), the cholesterol/apoA-I ratio in HDL_{DGUC2} of the T2DM patients was significantly lower than in HDL_{DGUC2} of the healthy controls (1.03 ± 0.19 versus 1.27 ± 0.20, $p < 0.01$). This indicates that the HDL particles from the T2DM patients contained on average relatively more triglycerides and less cholesterol. However, no associations were detected between the triglyceride/apoA-I ratio or the cholesterol/apoA-I ratio and the anti-inflammatory function of HDL in either REC or HCAEC. The apoA-II, apoC-II + apoC-III, and apoE content of the HDL particles from the healthy controls and T2DM patients did not significantly differ, however, the HDL apoA-II content was higher in T2DM patients than in healthy controls, which was almost statistically significant (Table 3). The anti-inflammatory function of HDL from the healthy controls correlated with relative apoA-II content ($r = 0.647$, $p = 0.023$) and apoE-content ($r = -0.759$, $p = 0.007$) when tested in HCAEC, but not in REC; no associations were found for HDL from the T2DM patients or from the combined study population.

Effect of breakfast on the anti-inflammatory activity of HDL from T2DM patients

The T2DM patients were free to choose one out of four different types of breakfast that were offered. Thirteen of the 16 patients chose for the two 400–500 kcal breakfast types (Table S1). Three hours after consuming the breakfast, a trend towards increased plasma triglyceride levels was observed, but not significantly ($p = 0.058$). Plasma glucose, total cholesterol, HDL-cholesterol, and LDL-cholesterol levels were not affected by breakfast consumption (Table 4).

Breakfast consumption significantly improved the ability of HDL to reduce the TNFα-induced VCAM-1 expression both in HCAEC and in REC. In HCAEC, the VCAM-1 expression was inhibited 56 ± 16% and 49 ± 19% by post- and preprandial HDL, respectively (difference 7 ± 6%, $p < 0.001$). In REC, this was 34 ± 16% and 26 ± 18%, respectively (difference 8 ± 13%, $p < 0.05$) (Fig. 3). Breakfast consumption did not affect the triglyceride/apoA-I ratio (0.15 ± 0.04

Table 1
Participant characteristics.

	Healthy controls	T2DM patients	*p* value
n	17	17	
Age (years)	43.5 ± 12.8	62.5 ± 9.3	<0.001
Sex (M) (n, %)	3 (18)	8 (47)	0.067
Smokers (n, %)	0 (0)	3 (18)	0.089
BMI (kg/m^2)	23.0 ± 2.9[a]	31.6 ± 4.8	<0.001
Diabetes duration (years)	–	16.6 ± 7.7	
Previous macrovascular event (n, %)	0	7 (41)	
Ischemic heart disease (n, %)	–	4 (24)	
Ischemic brain disease (n, %)	–	5 (29)	
Peripheral artery disease (n, %)	–	2 (12)	
Previous microvascular event (n,%)	0	9 (53)	
Retinopathy (n, %)	–	6 (35)	
Neuropathy (n, %)	–	4 (24)	
Nephropathy (n, %)	–	1 (6)	
Medication (n, %)	0		
Acetylsalicylic acid (n, %)	–	7 (41)	
Metformin (n, %)	–	11 (65)	
Insulin (n, %)	–	15 (88)	
Statins (n, %)	–	12 (71)	
Anti-hypertensive drugs (n, %)	–	12 (71)	
Fasting glucose (mmol/L)	5.01 ± 0.88	7.56 ± 2.34	<0.001
Total cholesterol (mmol/L)	4.98 ± 0.91	4.11 ± 0.84	0.007
HDL-cholesterol (mmol/L)	1.61 ± 0.33	1.09 ± 0.40	<0.001
LDL-cholesterol (mmol/L)	2.79 ± 0.69	2.20 ± 0.61	0.014
Triglyceride (mmol/L)	0.89 ± 0.32	1.67 ± 0.90	0.002

Notes: Variables are expressed as mean ± SD or *n* (%). Fasting plasma levels are presented. *p* values for continuous variables were calculated with an independent-samples *t*-test, *p* values for categorical variables were calculated with a χ^2 test. Abbreviation: *n*, number of participants.
[a] Data on BMI were available for 13 healthy controls.

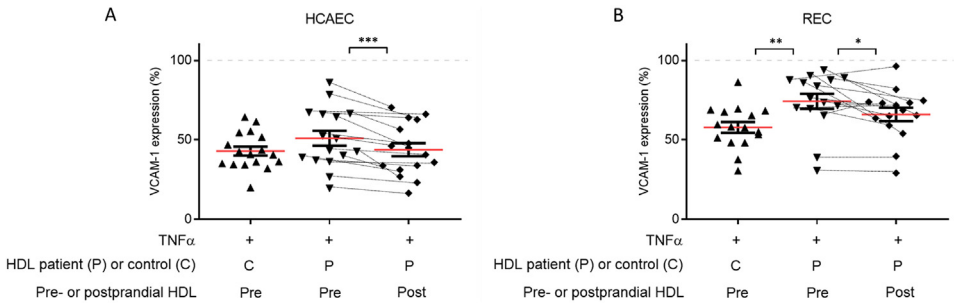

Fig. 3. Anti-inflammatory properties of preprandial HDL from nondiabetic control individuals and pre- and postprandial HDL from T2DM patients. The VCAM-1 expression (mean ± SEM) on HCAEC (A) ($n \geq 15$ per group) and REC (B) ($n \geq 16$ per group) is induced by TNFα (2 ng/ml for HCAEC, 5 ng/ml for REC). HDL was added in an apoA-I concentration of 3.2 μM. VCAM-1 expression of TNFα-incubated cells (tested in sextuplicate) and HDL- and TNFα-incubated cells (tested in triplicate) were corrected for VCAM-1 expression of a negative control (cells incubated with supplemented basal medium only, tested in sextuplicate) (VCAM-1 minus VCAM-1 negative control). The TNFα-induced VCAM-1 expression in the absence of HDL was set at 100%. *p* values for comparison of preprandial HDL from control individuals and T2DM patients were calculated with and corrected for age and sex using linear regression analysis (Table 2: model 2), the *p* values for comparison of pre-and postprandial HDL from T2DM patients were calculated with a paired-samples *t*-test. *$p \leq$ 0.05.

Table 2
Regression analysis of anti-inflammatory properties of preprandial HDL from T2DM patients and controls.

	Variables	HCAEC		REC	
		β (95% CI)	*p* value	β (95% CI)	*p* value
Model 1	T2DM	0.074 (−0.188; 0.041)	0.198	−0.169 (−0.283;-0.055)	0.005**
Model 2	T2DM	−0.074 (−0.233; 0.086)	0.353	−0.177 (−0.334;-0.021)	0.028*
	Age	−0.007 (−0.061; 0.048)	0.802	−0.008 (−0.060; 0.045)	0.768
	Sex	−0.043 (−0.176; 0.090)	0.514	−0.096 (−0.225; 0.033)	0.137

Notes: The dependent variable in the model was the reduction in TNFα-induced VCAM-1 expression by preprandial HDL on HCAEC ($n = 17$ per group) and REC ($n = 16$ per group). The VCAM-1 expression was induced by TNFα (2 ng/ml for HCAEC, 5 ng/ml for REC) and HDL was added in an apoA-I concentration of 3.2 μM. The reduction in VCAM-1 expression is the HDL-promoted reduction of the TNFα-induced VCAM-1 expression (set at 1.0). *p* values were calculated with linear regression analysis (T2DM (0: no, 1: yes); age (10 years); sex (1: male, 2: female)). *$p \leq$0.05, **$p \leq$0.01. **Abbreviations**: β, regression coefficient; CI, confidence interval.

and 0.15 ± 0.04 in pre- and post-prandial condition, respectively, $p = 0.166$), the cholesterol/apoA-I ratio (1.07 ± 0.18 and 1.09 ± 0.24, $p = 0.569$) or the relative apoA-II, apoC-II + apoC-III, or apoE content of HDL_{DGUC2} (Table 3). No association was found between the

Table 3
Apolipoprotein content of HDL_{DGUC2} preparations.

	Healthy controls	T2DM patients Preprandial	*p* value*	T2DM patients Postprandial	*p* value**
ApoA-II	0.29 ± 0.20 (12)	0.64 ± 0.56 (12)	0.058	0.60 ± 0.52 (11)	0.283
ApoC-II + ApoC-III	0.81 ± 0.19 (12)	0.94 ± 0.37 (12)	0.300	0.84 ± 0.29 (11)	0.102
ApoE	0.05 ± 0.03 (11)[$]	0.04 ± 0.02 (11)[$]	0.354	0.05 ± 0.04 (11)	0.276

Variables are expressed as μg protein/2.8 μg apoA-I (mean ± SD (*n*)). *: T2DM versus healthy controls, by unpaired *t*-test; **: postprandial versus preprandial, by paired-samples *t*-test; [$]: outlier detected with the ROUT method (Q: 1%) and removed from dataset.

Table 4
Effect of breakfast on plasma glucose and lipid levels of T2DM patients.

	Fasted	Postprandial	*p* value
Glucose (mmol/L)	7.47 ± 2.39	7.93 ± 2.24	0.435
Total cholesterol (mmol/L)	4.11 ± 0.87	4.15 ± 1.02	0.695
HDL-cholesterol (mmol/L)	1.08 ± 0.40	1.05 ± 0.36	0.456
LDL-cholesterol (mmol/L)	2.21 ± 0.63	2.17 ± 0.64	0.435
Triglyceride (mmol/L)	1.71 ± 0.92	1.87 ± 0.84	0.058

Notes: $n = 16$. Variables are expressed as mean ± SD. *p* values were calculated with a paired-samples *t*-test.

improvement of the HDL anti-inflammatory function and the triglyceride/apoA-I ratio, the cholesterol/apoA-I ratio, the apoA-II, apoC-II + apoC-III or apoE content of HDL_{DGUC2}, plasma glucose, plasma lipid levels, or BMI (Pearson's correlation tests: $p > 0.05$).

Discussion

Here we show that food intake partially restores the impaired anti-inflammatory function of HDL from T2DM patients. The consumption of breakfast was found to improve the anti-inflammatory effects of HDL from T2DM patients on both human macrovascular coronary artery and microvascular retinal endothelial cells. Since several T2DM features modify the HDL function [15,16,32], circulating HDL may be structurally and functionally modified over time. HDL has been shown to inhibit inflammatory responses by monocytes driven by non-HDL apo-CIII [33]. Upon a meal, HDL-bound apo-CIII increases at the expense of non-HDL apo-CIII, thereby further reducing its pro-inflammatory response [34]. We did not observe a meal-induced change in the relative apoC-II + apoC-III content of HDL, which may suggest that HDL-bound apoC-II is displaced by apoC-III [35]. The activities of several enzymes involved in HDL remodeling, such as hepatic lipase and cholesteryl ester transfer protein (CETP) [36], change, thereby improving the HDL function. Also, in the postprandial state, both the liver and intestine contribute to the plasma HDL pool, and intestinal-derived HDL may differ from liver-derived HDL [37]. Moreover, HDL that is newly synthesized, for instance following the consumption of a meal, may have preserved anti-inflammatory activities. This hypothesis is supported by studies showing that oral consumption of polyunsaturated fatty acids improves the anti-inflammatory properties of HDL [38], while these are impaired with intravenous infusion of 20% intra-lipid containing polyunsaturated fatty acids [39]. This may render the intestine an attractive target for new therapeutic strategies aiming to improve the HDL functionality in patients with T2DM.

In line with our finding that the functionality of HDL was improved in the postprandial condition compared to the preprandial condition, a higher cholesterol efflux capacity of post- compared to preprandial HDL has been reported [17,18]. Most *ex vivo* studies addressing the HDL anti-inflammatory function use HDL that was isolated after an overnight fast. However, most people spend a large part of their day in the postprandial state. Therefore, the function of HDL may be more representative in a postprandial state than after an overnight fast.

In line with previous studies [8,11,12,40], we observed a diminished anti-inflammatory function of HDL from T2DM patients compared to that of healthy controls. HDL derived from T2DM patients had a higher apoA-II content relative to apoA-I, which may affect the interaction of HDL particles with plasma enzymes or triglyceride-rich lipoproteins [41]. HDL function is also impaired in kidney disease [9]. Nephropathy was present in only one of the included patients, suggesting that the dysfunction is rather related to T2DM. The anti-inflammatory function of HDL from T2DM patients and healthy controls did not differ significantly when tested on HCAEC. This contrasts with reported data on other macrovascular endothelial cells [8,11,12]. Most of our patients were on statin treatment, which has been shown to improve the anti-inflammatory function of HDL [12,40,42]. This may partly explain why we observed no difference in the effect of patient and control HDL on HCAEC. Our data suggest that the effects of HDL vary between macro- and microvascular endothelial cells, but also among macrovascular endothelial cells derived from different organs, underlining the known heterogeneity in the structure and function of endothelial cells [43].

Here we show that HDL isolated by iodixanol-DGUC2 is suitable for testing its anti-inflammatory function, using TNFα-induced VCAM-1 expression on HCAEC or REC as readout. The two-step DGUC is a relatively fast and mild method for isolation of HDL free from both apoB-containing lipoproteins and unbound plasma proteins. The HDL isolated by two-step DGUC shows a similar anti-inflammatory function as HDL isolated with sequential salt density flotation. The major advantage of the iodixanol-DGUC2 over sequential salt density flotation is that iodixanol is non-toxic and non-ionic and that isolation requires shorter centrifugation times: 4 versus 108 h, respectively [20]. Also, HDL_{DGUC2} obtained using the two-step iodixanol-DGUC method inhibited TNFα-induced VCAM-1 expression at much lower apoA-I concentrations than routinely reported for testing HDL isolated with other methods [38,44,45]. The advantage of the iodixanol-DGUC2 over the PEG precipitation method is the removal of the majority of unbound plasma proteins [29,46], thereby decreasing the chance that plasma proteins interfere when testing the HDL anti-inflammatory function. Since most plasma proteins are not removed by PEG precipitation, it cannot be excluded that they contribute to the observed inhibition of VCAM-1. This may explain the slightly higher inhibitory effect of HDL_{PEG} as compared to the other isolation methods.

Ex vivo HDL function research is a growing field, and awareness that HDL may be modified by the HDL isolation protocol is crucial for a proper interpretation of the pathophysiological role of HDL in disease. A strength of our study is, therefore, the use of a relatively fast and gentle method of HDL isolation that may have less effect on HDL composition compared to the more commonly used methods. Several limitations apply to our work. We used HDL isolated from plasma obtained from a limited number of healthy volunteers and T2DM patients. Despite this limited number, a difference in the functionality of HDL between T2DM patients and healthy

individuals was detected. However, the limited sample size does not allow corrections for potential confounders other than age and sex. Of particular interest are BMI and statin use, since they differ between healthy and T2DM individuals and some data exists on their effect on HDL anti-inflammatory function. In our study population, no correlation existed between BMI and HDL anti-inflammatory function. Statins have been shown to improve HDL anti-inflammatory function in statin-naive individuals with coronary heart disease or high-risk individuals [40]. However, statin use in primary prevention of cardiovascular disease did not improve HDL anti-inflammatory function compared to placebo after a year of treatment [47], and no association was found between anti-inflammatory function and prevalent simvastatin use in type 2 diabetes [25]. The contribution of statin use to HDL anti-inflammatory function in our T2DM population is unknown. However, if statin use indeed improves the HDL anti-inflammatory function, the difference in HDL function in T2DM patients and healthy individuals might even be larger. It is likely that multiple aspects of altered metabolism in T2DM contribute to some degree to the difference seen in HDL anti-inflammatory function between T2DM patients and healthy individuals, but the individual contributions are difficult to investigate and remain therefore unknown.

The mechanism by which a meal improves the anti-inflammatory capacities of HDL from T2DM patients remains unknown. Since the assessment of the postprandial HDL functionality was limited to only one time point, the dynamics of the changes in HDL functionality after breakfast remain to be investigated. Additionally, we did not test the effect of breakfast on the functionality of HDL from individuals without T2DM. Comparing the postprandial changes in HDL from T2DM with those in healthy individuals could inform us about the mechanisms underlying the functional changes. Furthermore, we used the reduction in TNF-α induced VCAM-1 protein expression on endothelial cells as a single readout for HDL anti-inflammatory function. We therefore cannot comment on the effects of these isolation methods and food intake on other anti-inflammatory properties of HDL. In general, however, the results of this readout are in line with other assays of HDL anti-inflammatory function [32]. Finally, participants were not offered similar breakfasts but were allowed to choose one out of four diets that slightly differed in energy density and composition. The small number of patients per diet makes it impossible to attribute the observed beneficial effect of breakfast on HDL functionality to a particular diet compound. It does show, however, that a regular breakfast can improve HDL anti-inflammatory function.

In conclusion, our findings indicate that the two-step iodixanol-DGUC is a suitable method for HDL isolation when testing HDL anti-inflammatory function. Moreover, consumption of breakfast after an overnight fast can improve the anti-inflammatory function of HDL from T2DM patients. Determinants of this improvement remain to be elucidated.

Disclosure statement

The authors have nothing to disclose.

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Authors' contribution

WAD and MTM designed the study. RFHL, NEMAM, LCvVvdZ, FPJL, and CMGvR performed the experiments and analyzed the data. AJMV and MTM interpreted the HDL isolation data. RFHL, AMM, AGL, and HRH designed the feeding experiment with the patients. MvH and EJGG interpreted the data of the participants. PJML and WAD analyzed and interpreted the data from the anti-inflammatory assays. RFHL and NEMAM performed the statistical analyses. NEMAM, RFHL, WAD, AJMV, and MTM wrote the manuscript. All authors critically read and approved the final manuscript.

Ethics approval and consent to participate

All participants provided written informed consent (Mec-1327, Mec-2016-202, Mec-2014-066).

Consent for publication

All authors have agreed to authorship, read and approved the manuscript, and given consent for submission and subsequent publication of the manuscript.

Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.athplu.2021.08.006.

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