Effects of High-Density Lipoprotein Elevation With Cholesteryl Ester Transfer Protein Inhibition on Insulin Secretion


Rationale: High-density lipoprotein cholesterol elevation via cholesteryl ester transfer protein (CETP) inhibition represents a novel therapy for atherosclerosis, which also may have relevance for type 2 diabetes mellitus.

Objective: The current study assessed the effects of a CETP inhibitor on postprandial insulin, ex vivo insulin secretion, and cholesterol efflux from pancreatic β-cells.

Methods and Results: Healthy participants received a daily dose of CETP inhibitor (n=10) or placebo (n=15) for 14 days in a randomized double-blind study. Insulin secretion and cholesterol efflux from MIN6N8 β-cells were determined after incubation with treated plasma. CETP inhibition increased plasma high-density lipoprotein cholesterol, apolipoprotein AI, and postprandial insulin. MIN6N8 β-cells incubated with plasma from CETP inhibitor–treated individuals (compared with placebo) exhibited an increase in both glucose-stimulated insulin secretion and cholesterol efflux over the 14-day treatment period.

Conclusions: CETP inhibition increased postprandial insulin and promoted ex vivo β-cell glucose-stimulated insulin secretion, potentially via enhanced β-cell cholesterol efflux. (Circ Res. 2013;113:167-175.)

Key Words: cholesterol homeostasis ■ high-density lipoprotein cholesterol ■ insulin secretion ■ lipoproteins ■ oxidized low-density lipoprotein ■ type 2 diabetes mellitus

Recent clinical studies indicate that high-density lipoprotein (HDL) cholesterol exerts beneficial actions beyond cardiovascular protection, which may extend to type 2 diabetes mellitus. We previously have shown that reconstituted HDL infusion in individuals with type 2 diabetes mellitus can increase plasma insulin and lower circulating glucose. The actions of HDL and its major protein, apolipoprotein AI (apoAI), to enhance insulin secretion and to stimulate glucose uptake in skeletal muscle suggest that HDL-increasing agents may have efficacy in improving β-cell function and glucose homeostasis. These findings raise the possibility that HDL-increasing agents currently undergoing development for treatment of atherosclerotic coronary disease may have therapeutic relevance for the management of type 2 diabetes mellitus.

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Pancreatic β-cell dysfunction is a hallmark of late-stage type 2 diabetes mellitus. Underlying mechanisms include lipid accumulation with subsequent inflammation, oxidative stress, lipid peroxidation, and endoplasmic reticulum stress. HDL may protect against β-cell dysfunction and increase glucose-stimulated insulin secretion (GSIS) via cellular cholesterol removal and direct signaling actions. Evidence for the emerging role of HDL in glucose metabolism has been recently reviewed. Previous cellular, animal, and human studies suggest that increasing plasma HDL cholesterol may modulate insulin secretion. Cholesteryl ester transfer protein (CETP) inhibition increases plasma HDL cholesterol via a reduction in the transfer of neutral lipids (triglycerides and cholesteryl esters) between HDL and triglyceride-rich lipoprotein particles. The efficacy of this strategy to improve cardiovascular outcomes has not yet been determined, and there is controversy regarding whether CETP inhibition might produce a dysfunctional form of HDL cholesterol, which is less efficient at promoting reverse cholesterol transport than native HDL. This controversy has arisen, in part, because of the early termination of 2 CETP inhibitor trials resulting from adverse events in the case of torcetrapib and, more recently, lack of efficacy with...
Nonstandard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ABCA1</td>
<td>ATP-binding cassette transporter A1</td>
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<tr>
<td>ApoA</td>
<td>apolipoprotein A</td>
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<tr>
<td>CETPi</td>
<td>cholesteryl ester transfer protein inhibitor</td>
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<tr>
<td>GSIS</td>
<td>glucose-stimulated insulin secretion</td>
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<tr>
<td>HDL</td>
<td>high-density lipoprotein</td>
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<tr>
<td>LDL</td>
<td>low-density lipoprotein</td>
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<tr>
<td>oxLDL</td>
<td>oxidized low-density lipoprotein</td>
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dalcetrapib.25 Despite this, the CETP inhibitor (CETPi) class of HDL-increasing agents remains the most advanced therapeutic strategy for long-term HDL elevation with 2 agents currently in late-phase clinical development (phase III) for the management of coronary artery disease.8,26

Although the primary focus of CETP inhibition has been on cardiovascular outcomes, a substudy of the Investigation of Lipid Level Management to Understand Its Impact in Atherosclerotic Events (ILLUMINATE) trial reported favorable effects on glucose metabolism in patients with type 2 diabetes mellitus when treated with torcetrapib.26 Although the mechanisms leading to improved glycemic control were not investigated, we hypothesize that enhanced β-cell function is a likely contributor.

The current study investigated whether increasing HDL cholesterol via CETP inhibition can promote insulin secretion in humans. In a randomized, placebo-controlled, parallel-design clinical trial of a 14-day CETPi intervention, plasma indices of glycemic control were measured. Subsequent ex vivo analyses of MIN6N8 pancreatic β-cell function were performed, including insulin secretion and cholesterol efflux after treatment with plasma from CETPi compared with placebo-treated individuals.

Methods

Human CETPi Trial

To examine the effects of HDL increase on insulin secretory function, a randomized, placebo-controlled, parallel-design clinical trial was performed. The CETPi intervention period was 14 days. Twenty-five healthy men (n=10 CETPi, n=15 placebo) gave written informed consent to participate in the study, which was approved by the Ethics Committee of the Two Basels (Switzerland). A fasting blood sample was taken before a standardized breakfast (10.4 g fat, 18.5 g protein, ≤80 g carbohydrate; total energy 1989 kJ) and a 15-minute postprandial blood sample also was taken immediately before a once-daily oral dose (420 mg) of CETPi (RG7232, Patient Cooperation Treaty No. WO2007090748) or matching placebo.

Plasma Analyses

Fasting and 15-minute postprandial blood was collected on the first (day 1) and last day (day 14) of the study and the plasma was stored at −80ºC until analysis. Total, HDL, and low-density lipoprotein (LDL) cholesterol and triglyceride concentrations were measured in fasting plasma samples on a Roche Modular System using either enzymatic methods or direct homogeneous assays (Modular Analytics D2400 module; Roche Diagnostics). ApoAI and apopAII were measured using an automated nephelometer (Dade Behring BNII; Siemens Healthcare Diagnostics) in fasting plasma after depletion of apolipoprotein B–containing lipoproteins by a precipitation method using 0.1% 1.1 dextralip/MgCl2 (Sigma-Aldrich). HDL-associated free cholesterol and triglyceride concentration were measured using an enzymatic colorimetric assay on a chemistry immuno-analyzer (Olympus AU 2700; Olympus). Human enzyme-linked immunosorbent assay kits were used to measure plasma insulin (EZHIAFS-14K; Millipore Australia), gastric inhibitory polypeptide (EZGHIP-54K; Millipore Australia), and glucagon-like peptide 1 (EZGLPIT-36K; Millipore Australia) concentrations in postprandial samples taken on day 1 and day 14. Postprandial C-peptide and fasting and postprandial glucose concentrations were measured on an automated analyzer (Architect ci8200; Abbott Diagnostics). To evaluate the efficacy of the CETPi, plasma CETP activity was measured using a homogeneous fluorometric assay kit (Roar Ex-vivo CETP Activity Assay; Roar Biomedical).

Plasma Preparation for Ex Vivo Analyses

To study the effects of HDL, plasma was prepared for ex vivo studies by precipitating out apolipoprotein B–containing lipoproteins via incubation with 0.1% 1.1 dextralip/MgCl2 (Sigma-Aldrich) followed by centrifugation at 20,817g for 30 minutes to remove very-LDL and LDL particles.27 Supernatant was dialyzed against phosphate-buffered saline/EDTA using tube-o-dialyzers (G biosciences) to remove all molecules <15,000 Da, including insulin and glucose, and subsequently was referred to as CETPi plasma or placebo plasma.

LDL Oxidation

LDL (density=1.019–1.063 g/mL) was isolated from the plasma of healthy individuals by sequential ultracentrifugation, as previously described.1 The lipoproteins were then dialyzed overnight against phosphate-buffered saline/EDTA at 4°C. For the ex vivo cholesterol efflux and insulin secretion assays, LDL was oxidized by incubating 1.5 mg/mL LDL protein with 10 μmol/L CuSO4 for 110 minutes at RT in the dark. Oxidation was stopped by adding EDTA at 60 μmol/L at room temperature (RT). The extent of lipoperoxide formation was quantitated by measuring thiobarbituric acid reactive substances in a validated assay.28 Duplicate standards and oxidized LDL (oxLDL) samples were mixed with 2 volumes of ice-cold 10% (wt/vol) trichloroacetic acid (Sigma-Aldrich) and 3 volumes of 1% (wt/vol) thiobarbituric acid (Sigma-Aldrich) and incubated at 95ºC for 30 minutes. Samples were centrifuged (16,200 g, 5 minutes) at RT before 200 μL supernatant was transferred to a 96-well plate and absorbance measured at 532 nm using a Bio-Rad plate reader (Bio-Rad Laboratories). An 8-point calibration curve was prepared using 1,1,3,3-tetramethoxypropane (0–100 μmol/L) and results are expressed as nmol of malondialdehyde equivalents per mg of LDL protein. The oxLDL used for the ex vivo assays was not fully oxidized as indicated by the thiobarbituric acid reactive substances assay (27 nmol malondialdehyde equivalents/mg LDL protein) and compared with previous studies.29,30

Ex Vivo Insulin Secretion From MIN6N8 Pancreatic β-Cells

Insulin secretion was assessed in murine MIN6N8 pancreatic β-cells provided by Jun-ichi Miyagaki (Osaka University, Osaka, Japan) using a modified protocol.11 MIN6N8 pancreatic β-cells were grown in 5 mmol/L glucose DMEM supplemented with 10% fetal calf serum plus 1% penicillin/streptomycin (37°C, 95% O2/5% CO2) to subconfluence and then placed in 5 mmol/L glucose DMEM with 2% fetal calf serum. Cells were incubated with 3.5% CETPi plasma, placebo plasma, lipoprotein-free plasma, LDL-spiked (1.8 mmol/L) plasma, DMSO, or 100 ng/mL CETPi for 72 hours along with oxLDL (50 μg/mL) to simulate the pathophysiology of type 2 diabetes mellitus.31,32 This is a well-validated technique to cholesterol-load cells11 and an appropriate model to examine whether plasma HDL elevation in individuals treated with the CETPi increased GSIS and cholesterol efflux. Treatment of MIN6N8 pancreatic β-cells with oxLDL (50 μg/mL) had no adverse effect on cell morphology, culture confluence, or key apoptotic (Bax, Casp3, Bclx, Ptp2, Bim, and Puma) or antiapoptotic (Bcl2) gene markers involved in different cell death pathways (data not shown). Basal insulin secretion was determined after incubation in low (2.8 mmol/L) glucose Krebs-Ringer bicarbonate buffer for 30 minutes, whereas first-phase (20 minutes) and second-phase (40 minutes) GSIS were measured in response to separate 20 minutes glucose boluses. Cells were lysed in water and sonicated for 15 minutes at 4°C to determine intracellular insulin. Secreted and intracellular insulin were measured using a rat/mouse insulin enzyme-linked immunosorbent assay kit (EZRMI-13K; Millipore Australia).
Total insulin was calculated as the sum of insulin secreted during the basal, first, and second phases, plus intracellular insulin at the end of the experiment. Insulin secretion data are expressed as a percentage change in either absolute insulin secretion at day 14 compared with day 1 (Figure 1) or insulin secretion rate (ng/mL per hour; Table 4).

Ex Vivo Cholesterol Efflux From MIN6N8 Pancreatic β-Cells

Cholesterol efflux assays were performed using a modified protocol as described previously. MIN6N8 pancreatic β-cells were grown in 5 mmol/L glucose DMEM with 10% fetal calf serum to subconflu-ence and then labeled with [3H]cholesterol (GE Healthcare) in ethanol vehicle (<0.4%) and oxLDL (50 μg/mL) to facilitate cholesterol loading for 48 hours at 37°C (95% O2/5% CO2). Cells were washed with phosphate-buffered saline and then treated with the liver X–receptor agonist (TO-901317, 1 μmol/L) to stimulate ATP-binding cassette transporter A1 (ABCA1) expression in serum-free DMEM for 18 hours. Human HDL (10, 25, and 50 μg/mL) and apoAI (20 μg/mL) were used as positive controls. Experimental treatments included CETPi plasma (n=5) and placebo plasma (n=5) collected on day 1 and day 14 of the treatment period at a concentration of 3.5% v/v. This plasma dilution was selected on the basis of preliminary experiments showing that 3.5% plasma elicited cholesterol efflux of a magnitude similar to 50 μg/mL of HDL, a concentration in the mid range of the cholesterol efflux dose–response curve. This was the same dilution used in the insulin secretion experiments. [3H]cholesterol was measured in quadruplicate samples after a 3-hour cholesterol efflux assay into serum-free DMEM, which was centrifuged at 15700×g for 5 minutes. Cells were lysed in 0.1 mol/L NaOH/0.1% SDS and assayed for protein content. [3H]cholesterol in the medium and cells was quantified by liquid scintillation counting, from which the percentage of cholesterol released was determined. Cholesterol efflux data are expressed as the proportion of [3H]cholesterol transferred from cells to the medium.

Statistical Analysis

Data were analyzed using unpaired 2-tailed Student t tests when only 2 experimental groups were compared and for >2 groups by 1-way ANOVA or repeated-measures 1-way ANOVA as appropriate and, when significant (P<0.05), comparison of individual means was performed with a Tukey Honestly Significant Difference test. Correlation analysis was performed using the Pearson coefficient. Results are expressed as mean±SD. All analyses were conducted using SPSS (version 16) and Sigmastat (version 3.5). P<0.05 was deemed significant.

Results

Effect of CETPi on Human Plasma Parameters

There were no differences in age (CETPi: 32±9 vs placebo: 35±9 years) or body mass index (CETPi: 23.0±1.0 vs placebo: 24.5±0.5 kg/m2) between groups. There also were no predosing differences in CETP activity at baseline between the treatment groups (Table 1). The efficacy of the CETPi was confirmed with >80% inhibition of plasma CETP activity achieved for ≤12 hours postdose on both day 1 and day 14 of the treatment period (Table 1).

CETP inhibition increased plasma HDL cholesterol by 46%, HDL-associated apoAI by 22%, apoAII by 10% (P<0.05), and free cholesterol by 70%, and decreased plasma LDL.
Table 1. Human CETPi Trial: Mean CETP Activity

<table>
<thead>
<tr>
<th>Day</th>
<th>CETPi Activity, pmol/mL per minute (n=15)</th>
<th>Placebo CETP Activity, pmol/mL per minute (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1, 0 h</td>
<td>11.1±5.9</td>
<td>12.9±5.6</td>
</tr>
<tr>
<td>Day 1, 12 h</td>
<td>16.1±6.2</td>
<td>10±4.4</td>
</tr>
<tr>
<td>Day 14, 0 h</td>
<td>9.3±5.4</td>
<td>10.0±6.4</td>
</tr>
<tr>
<td>Day 14, 12 h</td>
<td>16.4±7.6</td>
<td>-0.8±1.2</td>
</tr>
</tbody>
</table>

Data are presented as mean±SD; measures on day 1, 0 h are baseline data; CETPi indicates cholesteryl ester transfer protein inhibitor.

*Significant (P<0.05) difference vs placebo.

cholesterol by 32% (P<0.0001 vs placebo unless otherwise stated; Table 2). Circulating triglycerides also were lower after CETPi treatment (P<0.05), but not as a constituent of HDL (Table 2). The increase in postprandial insulin (+75%) after the 14-day CETPi intervention was greater than with placebo (+42%) treatment (P<0.05; Figure 1A). The change in plasma C-peptide concentration mirrored that of insulin, although it Failed to reach statistical significance (P=0.08; Figure 1B). Despite the increase in insulin, there was no difference between groups in fasting or postprandial glucose measured 15 minutes after a standardized breakfast (Table 2). Importantly, there was no Effect of the CETPi on the change in postprandial plasma gastric inhibitory polypeptide (Figure 1C) or glucagon-like peptide 1 (Figure 1D) concentrations.

Over the 14-day intervention period there was a strong correlation between the change in plasma HDL cholesterol and apoAI (r=0.90; P<0.0001). The change in both plasma HDL and apoAI correlated significantly with the change in postprandial insulin (HDL: r=0.53, P<0.01; apoAI: r=0.41; P<0.05; Table 3).

Effect of CETPi Plasma on Insulin Secretion From Mouse Pancreatic β-Cells
There was no difference between groups (CETPi plasma vs placebo plasma) in the absolute rate (Table 4) or percentage change (day 14−day 1) in basal insulin secretion (Figure 2A). GSIS was higher after treatment with the CETPi plasma. The percentage change (from day 1) in both first-phase (+41±91%) GSIS and second-phase (+32±49%) GSIS were greater after exposure to CETPi plasma compared with placebo plasma (P<0.05; Figure 2B and 2C). Additionally, when the data were expressed as a rate, the absolute change (day 14−day 1) in first-phase (P=0.05; Table 4) GSIS and second-phase (P<0.05; Table 4) GSIS were higher after treatment with CETPi plasma compared with placebo plasma. The change in total insulin remaining in lysed pancreatic β-cells after 14 days of treatment was correspondingly lower after incubation with CETPi plasma compared with placebo plasma (P<0.05; Table 4). The change in GSIS between days 1 and 14 in the placebo group was not significant. There was no significant correlation between the change in either plasma HDL or apoAI and the change in first-phase or second-phase GSIS (Table 3).

To test whether HDL can stimulate insulin release, MIN6N8 pancreatic β-cells were treated with lipoprotein-free plasma and plasma spiked with human HDL at a concentration (1.8 mmol/L) equivalent to that achieved after CETPi treatment in study participants. The data show a 7.9±0.6-fold increase in first-phase insulin secretion (n=6; P<0.0001) and a 2.7±0.3-fold increase in second-phase insulin secretion (n=6; P<0.0001) in the HDL-spiked plasma relative to lipoprotein-free plasma.

Importantly, direct incubation with the CETPi drug at concentrations equivalent to those found in plasma after the 14-day treatment period resulted in no change in either basal insulin secretion or first-phase and second-phase GSIS (Table 5). There also was no change in total insulin remaining in the lysed cells after direct incubation with the drug (Table 5).

Effect of CETPi Plasma on Cholesterol Efflux From Mouse Pancreatic β-Cells

Incubation of cholesterol-loaded MIN6N8 pancreatic β-cells with human HDL stimulated net cholesterol efflux with a linear dose-dependent relationship (Figure 3A). ApoAI also acted as a cholesterol acceptor, resulting in a 2-fold increase in cholesterol efflux at a relatively low concentration (20 μg/mL; Figure 3A). There was a 9-fold increase in cholesterol efflux at the highest dose of HDL (50 μg/mL) when compared with control with no acceptor (Figure 3A), which is equivalent to the magnitude of efflux achieved with 3.5% CETPi treatment.

Table 2. Human CETPi Trial: Plasma Parameters

<table>
<thead>
<tr>
<th>Measure</th>
<th>Placebo Day 1 (n=15)</th>
<th>Placebo Day 14 (n=15)</th>
<th>Change</th>
<th>CETPi Day 1 (n=10)</th>
<th>CETPi Day 14 (n=10)</th>
<th>Change</th>
<th>P Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol, mmol/L</td>
<td>4.8±0.8</td>
<td>4.6±0.7</td>
<td>-0.03±0.48</td>
<td>4.4±0.8</td>
<td>4.1±0.5</td>
<td>-0.29±0.49</td>
<td>0.196</td>
</tr>
<tr>
<td>HDL cholesterol, mmol/L</td>
<td>1.3±0.3</td>
<td>1.2±0.2</td>
<td>-0.10±0.12</td>
<td>1.2±0.2</td>
<td>1.8±0.3</td>
<td>0.55±0.18</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>LDL cholesterol, mmol/L</td>
<td>2.9±0.8</td>
<td>3.0±0.6</td>
<td>0.03±0.44</td>
<td>2.7±0.6</td>
<td>1.8±0.4</td>
<td>-0.86±0.39</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Triglycerides, mmol/L</td>
<td>1.0±0.3</td>
<td>1.4±0.6</td>
<td>0.39±0.47</td>
<td>1.0±0.3</td>
<td>0.9±0.2</td>
<td>-0.06±0.3</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Fasting glucose, mmol/L</td>
<td>4.9±0.3</td>
<td>4.4±0.3</td>
<td>-0.47±0.20</td>
<td>4.8±0.5</td>
<td>4.5±0.3</td>
<td>-0.29±0.29</td>
<td>0.081</td>
</tr>
<tr>
<td>Postprandial glucose, mmol/L</td>
<td>7.1±1.2</td>
<td>7.3±1.0</td>
<td>0.15±0.23</td>
<td>7.8±1.2</td>
<td>7.8±1.0</td>
<td>-0.03±0.18</td>
<td>0.705</td>
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<tr>
<td>HDL compositional analysis</td>
<td></td>
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<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ApoAI, mg/dL</td>
<td>131±14</td>
<td>128±13</td>
<td>-3.3±11.9</td>
<td>119±19</td>
<td>146±19</td>
<td>26.7±14.8</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>ApoAII, mg/dL</td>
<td>32±3.9</td>
<td>31±5.4</td>
<td>-1.0±3.7</td>
<td>30±4.5</td>
<td>33±4.2</td>
<td>3.1±4.6</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Free cholesterol, mg/dL</td>
<td>9.3±2.5</td>
<td>8.7±2.6</td>
<td>-0.65±0.91</td>
<td>8.3±1.0</td>
<td>14.1±1.9</td>
<td>5.8±1.7</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Triglycerides, mg/dL</td>
<td>9.0±2.5</td>
<td>10.4±2.8</td>
<td>1.4±2.6</td>
<td>8.2±1.7</td>
<td>7.7±2.1</td>
<td>-0.5±2.5</td>
<td>0.085</td>
</tr>
</tbody>
</table>

Data are presented as mean±SD. P values from Student t test. Measures on day 1 are baseline characteristics. ApoAI indicates apolipoprotein A; CETPi, cholesteryl ester transfer protein inhibitor; HDL, high-density lipoprotein; LDL, low-density lipoprotein.
plasma (Figure 3B). The absolute change (day 14−day 1) in cholesterol efflux to CETPi plasma (1.23±0.39%) was >12-fold for placebo plasma (0.10±0.13%; P<0.001; Figure 3B). The change in both plasma HDL cholesterol and apoAI concentrations correlated strongly with the change in cholesterol efflux (r=0.78 and 0.70, respectively; P<0.05; Table 3).

**Discussion**

We report here for the first time that CETPi inhibition in healthy humans increases postprandial insulin and ex vivo GSIS. These observations provide clinical context to previous work linking HDL with insulin secretion in cell and animal models, as well as human studies of both a single HDL elevation1,3,5 and HDL-increasing therapies, such as CETPi inhibition, there-
fur may augment β-cell function and postprandial insulin secretion in individuals with type 2 diabetes mellitus.2,20 Enhanced GSIS and improved glucose tolerance without exacerbating the incretin response. The insulin secretory effect is most likely because of the CETPi inhibition because basal insulin secretion was not altered.

The insulin secretory effect is most likely because of the HDL elevation induced by the CETPi because there was no direct effect of the CETPi on insulin secretion and lipoprotein-free plasma spiked with HDL at the concentration (1.8 mmol/L) equivalent to that achieved after CETPi treatment in study participants stimulated significant insulin secretion. HDL-increasing therapies, such as CETPi inhibition, therefore may augment β-cell sensitivity to glucose, resulting in enhanced GSIS and improved glucose tolerance without exacerbating the characteristic fasting hyperinsulinemia observed in those individuals with type 2 diabetes mellitus.

Although plasma HDL cholesterol and apoAI positively correlated with the change in postprandial insulin, the weaker relationships between the change in first-phase and second-phase GSIS and plasma HDL/apoAI would be anticipated given the more complex and variable nature of the insulin-secretion assay compared with direct measures of plasma infusion1 as well as HDL elevation >6 months.20 These data are consistent with the hypothesis that HDL elevation via CETPi inhibition may improve β-cell function and postprandial insulin secretion in individuals with type 2 diabetes mellitus to mitigate harmful postprandial glycemic excursions.3,16,38,39

This possibility requires testing in large CETPi clinical trials involving patients with type 2 diabetes mellitus. After CETPi inhibition for 14 days, postprandial insulin and C-peptide increased 2-fold when compared with placebo. Furthermore, the change in plasma HDL cholesterol and apoAI positively correlated with the change in postprandial insulin over that same treatment period. The postprandial sample was taken 15 minutes after a standardized meal when glucose concentrations in the placebo and CETPi-treated groups were equivalent. This suggests that for a given postprandial increase in glucose (2–3 mmol/L in this study) in these healthy individuals, CETPi treatment resulted in a greater increase in plasma insulin. Importantly, this was not because of an effect on the major incretins, gastric inhibitory polypeptide and glucagon-like peptide 1.

It is anticipated that the higher circulating insulin would enhance postprandial glucose clearance after CETPi-induced HDL elevation, although blood was not sampled at later time points. Increased postprandial insulin could benefit those with established type 2 diabetes mellitus, who typically have high fasting insulin, but impaired β-cell sensitivity and insulin secretory response to a glucose load. This latter effect generally manifests itself as a blunted postprandial first-phase insulin response resulting in a greater plasma glucose excursion and delayed postmeal recovery.36,38,39 CETPi inhibition thus has the potential to improve postprandial glycemic control without disturbing the incretin response.

In a previous study we reported that HDL at a concentration of 50 μg/mL, which equates to an apoAI concentration of ≈40 μg/mL, rescues oxLDL-induced reductions in MIN6N8 pancreatic β-cell GSIS but has no effect alone.1 The current study develops a therapeutic context, showing that apolipoprotein B–depleted dialysed plasma taken from CETPi-treated (vs placebo-treated) individuals increases first-phase and second-phase GSIS from MIN6N8 pancreatic β-cells pretreated with oxLDL. Importantly, this effect was shown to be specific to glucose-stimulated insulin responses because basal insulin secretion was not altered.

**Table 3. Relationship Between the Change in Plasma HDL Cholesterol/ApoAI and Key Parameters Over the 14-Day Treatment Period**

<table>
<thead>
<tr>
<th></th>
<th>Plasma HDL Cholesterol</th>
<th>Plasma ApoAI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>r</td>
<td>P</td>
</tr>
<tr>
<td>Postprandial insulin</td>
<td>0.53</td>
<td>0.007*</td>
</tr>
<tr>
<td>First-phase GSIS</td>
<td>0.46</td>
<td>0.086</td>
</tr>
<tr>
<td>Second-phase GSIS</td>
<td>0.43</td>
<td>0.110</td>
</tr>
<tr>
<td>Cholesterol efflux</td>
<td>0.78</td>
<td>0.008*</td>
</tr>
</tbody>
</table>

ApoA indicates apolipoprotein A; GSIS, glucose-stimulated insulin secretion; HDL, high-density lipoprotein; r, Pearson correlation coefficient.

*Significant (P<0.05) correlation between parameters.

**Table 4. Effect of CETPi Plasma on GSIS From oxLDL-Treated MIN6N8 β-Cells**

<table>
<thead>
<tr>
<th></th>
<th>Placebo Plasma</th>
<th>CETPi Plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal insulin secretion for 30 min, ng/mL per hour</td>
<td>(n=5)</td>
<td>(n=10)</td>
</tr>
<tr>
<td>Day 1</td>
<td>299±148</td>
<td>357±165</td>
</tr>
<tr>
<td>Day 14</td>
<td>289±68</td>
<td>342±146</td>
</tr>
<tr>
<td>Change</td>
<td>−10±126</td>
<td>−15±106</td>
</tr>
<tr>
<td>First-phase GSIS for 20 min, ng/mL per hour</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 1</td>
<td>740±581</td>
<td>400±361</td>
</tr>
<tr>
<td>Day 14</td>
<td>512±440</td>
<td>523±406</td>
</tr>
<tr>
<td>Change</td>
<td>−228±174</td>
<td>122±337</td>
</tr>
<tr>
<td>Second-phase GSIS for 40 min, ng/mL per hour</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 1</td>
<td>205±57</td>
<td>118±24</td>
</tr>
<tr>
<td>Day 14</td>
<td>145±43</td>
<td>154±66</td>
</tr>
<tr>
<td>Change</td>
<td>−61±40</td>
<td>37±62*</td>
</tr>
<tr>
<td>Intracellular insulin content, ng/mL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 1</td>
<td>2256±865</td>
<td>2408±1111</td>
</tr>
<tr>
<td>Day 14</td>
<td>2679±973</td>
<td>2358±942</td>
</tr>
<tr>
<td>Change</td>
<td>423±176</td>
<td>−50±362*</td>
</tr>
</tbody>
</table>

Data are presented as mean±SD. CETPi indicates cholesteryl ester transfer protein inhibitor; GSIS, glucose-stimulated insulin secretion; oxLDL, oxidized low-density lipoprotein.

*Significance (P<0.05) vs placebo plasma.
Mechanisms

There are a number of mechanisms that may explain the observed increase in GSIS from MIN6N8 pancreatic β-cells after incubation with plasma from individuals treated with the CETPi (CETPi plasma). Given the higher plasma HDL cholesterol concentrations after CETP inhibition and the important role of HDL and apoAI in reverse cholesterol transport, a reduction in β-cell lipid content is a possible explanation. Our results show functional cholesterol efflux from MIN6N8 pancreatic β-cells to human plasma. Most importantly, efflux to CETPi plasma was increased over the 14-day treatment period with no change in efflux to placebo plasma. Interestingly, the change in plasma HDL cholesterol and apoAI across the treatment period strongly correlated with the change in εx vivo cholesterol efflux. Consistent with previous literature linking HDL to insulin secretion from β-cells,2,4,14-16 our observations suggest that CETP inhibition may modulate insulin secretion via changes in net cholesterol efflux. Cholesterol accumulation has been identified as an important precursor to β-cell dysfunction and failure, particularly in the context of type 2 diabetes mellitus.3,16,40,41 These pathologies can be largely rescued in vitro by reducing β-cell cholesterol content with statins or methyl-β-cyclodextrin.41,42 Combined, these data suggest that abnormalities in cholesterol homeostasis may contribute to impaired β-cell function typical of type 2 diabetes mellitus and that this may be rescued by CETP inhibition.

It is also possible that HDL and apoAI may increase insulin secretory capacity via direct signaling actions independent of lipid removal. Using apoAI at a concentration ≈20-fold higher (864 μg/mL) than that in our previous study,1 Fryirs et al.3 reported an increase in basal insulin secretion and GSIS from mouse pancreatic β-cells in the absence of cholesterol loading with oxLDL. This effect was observed without depletion of intracellular cholesterol, although functional cholesterol efflux was not measured. These results suggest that at these higher doses, apoAI may act as an insulin secretagogue, perhaps acting via direct signaling pathways linked to the HDL transporters (ABCA1) and ABCG1.3,14,15,17 A critical role for ABCA1 in regulating insulin granule fusion events in pancreatic β-cells recently has been reported for patients with loss-of-function mutations in the ABCA1 gene with low circulating HDL.14,40,44 Furthermore, the absence of both ABCA1 and ATP-binding cassette transporter G1 induces greater defects in β-cell function than deficiency of either transporter individually, suggesting that these transporters provide important and complementary contributions to β-cell function by maintaining islet cholesterol homeostasis in vivo.45 At a cellular level, cholesterol content can affect membrane fluidity and permeability,46 as well as cell signaling via membrane microdomains or sterol regulatory element-binding proteins.3,47

Clinical Context

Although no large clinical trials have directly examined the long-term effects of HDL-increasing agents on postprandial
glycemic control, circumstantial evidence of a beneficial effect is emerging. Post hoc analyses after the ILLUMINATE trial showed that patients treated with torcetrapib in conjunction with atorvastatin had lower fasting glucose (−0.34 mmol/L) and hemoglobin A1C (−0.33%) at 3 months compared with those using the statin alone. This was observed in addition to conventional management of type 2 diabetes mellitus with hypoglycemic agents and despite lower fasting insulin in the CETPi-treated group. The improved glucose control in these patients may be because of a combination of factors, including increased circulating HDL cholesterol and apoAI with a concomitant reduction in LDL cholesterol, improvement in β-cell function, or enhanced insulin sensitivity and skeletal muscle glucose uptake, as we and others have shown previously. Because glycemic control was not the primary end point in the ILLUMINATE trial, no postprandial glucose or insulin data were collected. Current large clinical trials examining the effects of potent second-generation CETP inhibitors, including anacetrapib and evacetrapib, on cardiovascular events provide an ideal opportunity to study the effects of HDL elevation on glucose homeostasis in more detail.

Limitations
Ideally, a more comprehensive time-course study of CETPi actions on circulating insulin and glucose should have been undertaken. In vivo assessment of insulin secretion also would have been informative in these individuals. However, this clinical trial was not designed to assess these parameters and either the necessary investigations were not performed or the plasma was not available. Despite these shortcomings, the current study provides evidence that HDL elevation via CETP inhibition can influence postprandial insulin responses and therefore supports a rationale for more detailed investigations.

Conclusion
The current study links HDL cholesterol to insulin secretion via novel actions of CETP inhibition in increasing postprandial insulin in healthy individuals in association with enhanced ex vivo cholesterol efflux and GSIS from MIN6N8 pancreatic β-cells. These data provide clinical context for investigation of HDL-increasing agents as potential therapies for the management of type 2 diabetes mellitus. Of particular interest is the possibility that CETP inhibition, via effects on β-cell insulin secretion, may reduce harmful postprandial glycemic excursions and associated vascular complications.

Acknowledgments
F. Hoffman La Roche (Basel, Switzerland) provided plasma from their clinical trial using the cholesteryl ester transfer protein inhibitor (RG7232, Patent Cooperation Treaty No. WO2007090748) but provided no funding for the study.

Sources of Funding
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### Table 5. Direct Effect of the CETPi on GSIS From oxLDL-Treated MIN6N8 β-Cells

<table>
<thead>
<tr>
<th></th>
<th>DMSO Vehicle (n=6)</th>
<th>100 ng/mL CETPi (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal insulin secretion for 30 min, ng/mL per hour</td>
<td>317±35</td>
<td>343±28</td>
</tr>
<tr>
<td>First-phase GSIS for 20 min, ng/mL per hour</td>
<td>222±130</td>
<td>419±83</td>
</tr>
<tr>
<td>Second-phase GSIS for 40 min, ng/mL per hour</td>
<td>75±28</td>
<td>84±19</td>
</tr>
<tr>
<td>Intracellular insulin content, ng/mL</td>
<td>374±17</td>
<td>360±10</td>
</tr>
</tbody>
</table>

Data are presented as mean±SD. CETPi indicates cholesteryl ester transfer protein inhibitor; GSIS, glucose-stimulated insulin secretion; oxLDL, oxidized low-density lipoprotein.

Figure 3. Effect of cholesteryl ester transfer protein inhibitor (CETPi) plasma on cholesterol efflux from oxidized low-density lipoprotein (oxLDL)-treated MIN6N8 pancreatic β-cells. MIN6N8 pancreatic β-cells were incubated with oxLDL (50 μg/mL) and (A) different doses of human high-density lipoprotein (HDL; 10, 25, and 50 μg/mL; black bars) and apolipoprotein AI (20 μg/mL; hatched bar) or (B) 3.5% placebo plasma (n=5; white bar) vs CETPi plasma (n=5; black bar) for 3 hours. Data are expressed as the change (day 14−day 1) in the percentage of cholesterol released into the media (mean±SD). *Significance (P<0.05) between the change (day 14−day 1) in placebo plasma vs CETPi plasma. **Significant (P<0.001) difference compared with control.
None.

References


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**Novelty and Significance**

**What Is Known?**

- Protective effects of high-density lipoprotein (HDL) against cardiovascular disease risk have been linked with increased cholesterol efflux and reverse cholesterol transport.
- HDL more recently has been shown to modulate insulin secretion and to stimulate glucose uptake into skeletal muscle.
- Cholesteryl ester transfer protein (CETP) inhibitors are HDL-increasing agents currently in phase III clinical trials, but the effects of these agents on insulin secretion have not been examined.

**What New Information Does This Article Contribute?**

- Increasing plasma HDL cholesterol by 48% and apolipoprotein AI by 24% via CETP inhibitor therapy for 14 days increases postprandial plasma insulin concentration, in comparison with placebo treatment.
- Mouse pancreatic β-cells incubated with plasma from individuals treated with a CETP inhibitor exhibited an increase in both first-phase and second-phase glucose-stimulated insulin secretion and cholesterol efflux, with no effect on basal insulin secretion.
- The effects of CETP inhibitors on glucose-stimulated insulin secretion are likely mediated by HDL elevation.

HDL-increasing agents currently are being tested for efficacy in reducing the risk of atherosclerotic cardiovascular disease; however, they also may be effective in the management of type 2 diabetes mellitus, which is commonly associated with dyslipidemia. The use of 1 CETP inhibitor is associated with improved glycemic control in patients with type 2 diabetes mellitus, but effects on insulin secretion were not examined. The current study shows that in healthy adults HDL elevation via CETP inhibition increases postprandial plasma insulin concentration in healthy individuals and that plasma from these individuals can increase glucose-stimulated insulin secretion and cholesterol efflux from mouse pancreatic β-cells. Furthermore, the elevations in plasma HDL cholesterol and apolipoprotein AI positively correlated with the change in postprandial plasma insulin. These observations provide clinical therapeutic context to previous work linking HDL with insulin secretion in cell culture and animal models, as well as human studies of both single HDL infusion and longer-term HDL elevation over the course of months. Raising HDL via CETP inhibition thus may improve β-cell function and postprandial insulin secretion in individuals with type 2 diabetes mellitus to minimize harmful postprandial glycemic excursions.
Effects of High-Density Lipoprotein Elevation With Cholesteryl Ester Transfer Protein Inhibition on Insulin Secretion

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Supplemental Material

Detailed Methods

**Human CETPi trial**
To examine the effects of high-density lipoprotein (HDL)-elevation on insulin secretory function, a randomized, placebo-controlled parallel design clinical trial was performed. The cholesteryl ester transfer protein inhibitor (CETPi) intervention period was 14 days. Twenty-five healthy males (n=10 CETPi, n=15 placebo) gave written informed consent to participate in the study, which was approved by the Ethics Committee of the Two Basels (Switzerland). A fasting blood sample was taken prior to a standardized breakfast (10.4g fat, 18.5g protein, ≤80g carbohydrate; total energy 1989kJ) and a 15min postprandial blood sample was also taken immediately before a once daily oral dose (420mg) of CETPi (RG7232, PCT #WO2007090748) or matching placebo.

**Plasma analyses**
Fasting and 15min postprandial blood was collected on the first (day 1) and last day (day 14) of the study and the plasma was stored at -80°C until analysis. Total, HDL and low-density lipoprotein (LDL) cholesterol and triglyceride concentrations were measured in fasting plasma samples on a Roche Modular System using either enzymatic methods or direct homogeneous assays (Modular Analytics D2400 module, Roche Diagnostics, IN). Apolipoprotein AI (apoAI) and apoAII were measured using an automated nephelometer (Dade Behring BNII, Siemens Healthcare Diagnostics, IL) in fasting plasma after depletion of apoB-containing lipoproteins by a precipitation method using 0.1% 1.1 dextralip/MgCl$_2$ (Sigma-Aldrich, Sydney, NSW, Australia). HDL-associated free cholesterol and triglyceride concentration were measured using an enzymatic colorimetric assay on a chemistry immuno-analyzer (Olympus AU 2700, Olympus, PA). Human ELISA kits were used to measure plasma insulin (EZHIASF-14K, Millipore Australia, Kilsyth, VIC, Australia), gastric inhibitory polypeptide (GIP; EZHGIP-54K, Millipore Australia) and glucagon-like peptide 1 (GLP-1; EZGLP1T-36K, Millipore Australia) concentrations in postprandial samples taken on day 1 and day 14. Postprandial C-peptide, fasting and postprandial glucose concentrations were all measured on an automated analyzer (Architect ci16200, Abbott Diagnostics, IL). To evaluate the efficacy of the CETP inhibitor, plasma CETP activity was measured using a homogeneous, fluorometric assay kit (Roar Ex-vivo CETP Activity Assay, Roar Biomedical, NY).

**Plasma preparation for ex vivo analyses**
To study the effects of HDL, plasma was prepared for *ex vivo* studies by precipitating out apoB-containing lipoproteins via incubation with 0.1% 1.1 dextralip/MgCl$_2$ (Sigma-Aldrich) followed by centrifugation at 20,817 x g for 30min (4°C) to remove very low-density lipoprotein (VLDL) and LDL particles. To prepare lipoprotein-free plasma, all lipoproteins were removed from whole, pooled plasma from healthy individuals by adjusting plasma density (d<1.3g/mL) with KBr followed by ultracentrifugation at 65,000 x g for 24h (4°C). Supernatant was dialyzed against PBS/EDTA using tube-o-dialyzers (G biosciences; St Louis, MO) to remove all molecules less than 15,000Da, including insulin and glucose and subsequently referred to as CETPi-plasma or placebo-plasma.

**LDL oxidation**
LDL (density = 1.019-1.063g/ml) was isolated from the plasma of healthy individuals by sequential ultracentrifugation. The lipoproteins were then dialyzed overnight against PBS/EDTA at 4°C. For the *ex vivo* cholesterol efflux and insulin secretion assays, LDL was oxidized by incubating 1.5mg/ml LDL protein with 10μmol/L CuSO$_4$ for 110min at RT in the dark. Oxidation was stopped by adding EDTA at 60μmol/L at RT. The extent of lipoperoxide formation was quantitated by measuring thiobarbituric acid reactive substances (TBARS) in a validated assay. Duplicate standards and oxLDL samples were mixed with two volumes of ice-cold 10% (w/v) trichloroacetic acid (Sigma-Aldrich) and 3 volumes of 1% (w/v) thiobarbituric acid (Sigma-Aldrich) and incubated at 95°C for 30min. Samples were centrifuged (16,200 x g, 5min) at RT before 200μl of the supernatant was transferred to a 96-well plate and absorbance measured at 532nm using a Bio-Rad plate reader (Bio-Rad Laboratories, Gladesville, NSW, Australia). An 8-point calibration curve was prepared using
1.1,3,3-tetramethoxypropane (0-100μM) and results expressed as nmol of malondialdehyde (MDA) equivalents/mg LDL protein. The oxLDL used for the ex vivo assays was not fully oxidized as indicated by the TBARS assay (27nmol MDA equivalents/mg LDL protein) and compared to previous studies.

**Ex vivo insulin secretion from MIN6N8 pancreatic β-cells**

Insulin secretion was assessed in murine MIN6N8 pancreatic β-cells provided by Prof Jun-ichi Miyagaki (Osaka University, Osaka, Japan) using a modified protocol. MIN6N8 pancreatic β-cells were grown in 5mmol/L glucose Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum (FCS) plus 1% penicillin/streptomycin (37°C, 95% O₂/5% CO₂) to sub-confluence then placed in 5mmol/L glucose DMEM with 2% FCS. Cells were incubated with 3.5% CETPi-plasma, placebo-plasma, lipoprotein-free plasma, HDL (1.8mmol/L)-spiked plasma, DMSO or 100ng/ml CETPi for 72h along with oxidized low-density lipoprotein (oxLDL) (50μg/ml) to simulate the pathophysiology of type 2 diabetes. This is a well-validated technique to cholesterol-load cells and an appropriate model to examine whether plasma HDL elevation in individuals treated with the CETPi increased GSIS and cholesterol efflux. Treatment of MIN6N8 pancreatic β-cells with oxLDL (50μg/ml) had no adverse effect on cell morphology, culture confluence or key apoptotic (Bax, Casp3, Bclx, Ptpn2, Bim & Puma) or anti-apoptotic (Bcl2) gene markers involved in different cell death pathways (data not shown). Basal insulin secretion was determined after incubation in low (2.8mmol/L) glucose Krebs-Ringer Bicarbonate buffer (KRBB) for 30min whereas first phase (20min) and second phase (40min) GSIS were measured in response to separate 20mmol/L glucose boluses. Cells were lyzed in water and sonicated for 15min at 4°C to determine intracellular insulin. Secreted and intracellular insulin were measured using a rat/mouse insulin ELISA kit (EZRMI-13K, Millipore Australia). Total insulin was calculated as the sum of insulin secreted during the basal, first and second phases, plus intracellular insulin at the end of the experiment. Insulin secretion data are expressed as either a percentage change in absolute insulin secretion at day 14 compared to day 1 (Figure 1) or insulin secretion rat (ng/ml/hr; Table 4).

**Ex vivo cholesterol efflux from MIN6N8 pancreatic β-cells**

Cholesterol efflux assays were performed using a modified protocol. MIN6N8 pancreatic β-cells were grown in 5mmol/L glucose DMEM with 10% FCS to sub-confluence then labelled with [1α,2α(n)-³H]cholesterol ([³H]cholesterol 1μCi/ml; GE Healthcare, UK) in ethanol vehicle (<0.4%) and oxLDL (50μg/ml) to facilitate cholesterol loading for 48h at 37°C (95% O₂/5% CO₂). Cells were washed with PBS then treated with the liver-X-receptor (LXR) agonist (TO-901317, 1μmol/L) to stimulate ABCA1 expression in serum-free DMEM for 18h. Human HDL (10, 25 and 50μg/ml) and apoAI (20μg/ml) were used as positive controls. Ex vivo cholesterol efflux from MIN6N8 pancreatic β-cells with oxLDL (50μg/ml) had no adverse effect on cell morphology, culture confluence or key apoptotic (Bax, Casp3, Bclx, Ptpn2, Bim & Puma) or anti-apoptotic (Bcl2) gene markers involved in different cell death pathways (data not shown). Basal insulin secretion was determined after incubation in low (2.8mmol/L) glucose Krebs-Ringer Bicarbonate buffer (KRBB) for 30min whereas first phase (20min) and second phase (40min) GSIS were measured in response to separate 20mmol/L glucose boluses. Cells were lyzed in water and sonicated for 15min at 4°C to determine intracellular insulin. Secreted and intracellular insulin were measured using a rat/mouse insulin ELISA kit (EZRMI-13K, Millipore Australia). Total insulin was calculated as the sum of insulin secreted during the basal, first and second phases, plus intracellular insulin at the end of the experiment. Insulin secretion data are expressed as either a percentage change in absolute insulin secretion at day 14 compared to day 1 (Figure 1) or insulin secretion rat (ng/ml/hr; Table 4).

**Statistical analysis**

Data were analyzed using unpaired two-tailed Student’s t-tests when only two experimental groups were compared and for more than two groups by one-way ANOVA or repeated measures one-way ANOVA as appropriate and where significant (p<0.05), comparison of individual means was performed with a Tukey HSD test. Correlation analysis was performed using the Pearson coefficient. Results are expressed as mean ± SD. All analyses were conducted using SPSS (v16) and Sigmastat (v3.5). p<0.05 was deemed significant.
References


