Diabetologia (2005) 48: 1207–1215
DOI 10.1007/s00125-005-1753-z

ARTICLE

Anne Hiukka · Jamila Fruchart-Najib · Eeva Leinonen · Hannele Hilden · Jean-Charles Fruchart · Marja-Riitta Taskinen

Alterations of lipids and apolipoprotein CIII in very low density lipoprotein subspecies in type 2 diabetes

Received: 22 September 2004 / Accepted: 27 January 2005 / Published online: 30 April 2005

Abstract *Aims/hypothesis:* Very low density lipoprotein (VLDL) particles are heterogeneous, comprising two main subspecies, VLDL 1 (Sf 60-400) and VLDL 2 (Sf 20-60). The aim of the study was to examine the distribution and composition of VLDL subspecies in type 2 diabetes. *Subjects, materials and methods:* We studied the composition and concentration of triglyceride-rich lipoproteins (TRLs) in 217 type 2 diabetic patients and 93 control subjects between 50 and 75 years of age. Lipoprotein subspecies were separated by density-gradient ultracentrifugation. Apolipoprotein (apo) CIII and apo E in plasma and apo CIII in TRL subspecies were measured by nephelometry and apo CII in serum by a commercial kit using a single radial immunodiffusion method. *Results:* The concentrations of VLDL 1, VLDL 2 and intermediate density lipoprotein were significantly increased in type 2 diabetes subjects, the change being most marked for VLDL 1. There was a strong linear correlation between VLDL 1 triglycerides and plasma triglycerides in both groups (r=0.879, p< 0.001 and r=0.899, p<0.001). Diabetic subjects had markedly higher plasma ratios of apo CII:apo CIII and apo CIII: apo E. Despite elevated plasma apo CIII, type 2 diabetic subjects had a relative deficiency of apo CIII in all TRL subspecies, suggesting profound disturbances of apo CIII metabolism. *Conclusions/interpretation:* The elevation of VLDL 1 triglycerides is the major determinant of plasma triglyceride concentration in normal subjects and in type 2 diabetic individuals. Both apo CIII and apo E metabolism are disturbed in type 2 diabetes.

Keywords Apolipoprotein CIII · Triglycerides · Type 2 diabetes · VLDL subspecies

Abbreviations apo: apolipoprotein · CVD: cardiovascular disease · FIELD: Fenofibrate Intervention and Event Lowering in Diabetes · GGE: gradient gel electrophoresis · IDL: intermediate-density lipoprotein · IQR: interquartile range · OHA: oral antihyperglycaemic agents · Sf: Svedberg flotation rate · TG: triglycerides · TRL: triglyceride-rich lipoproteins · VLDL: very low density lipoprotein

A. Hiukka · E. Leinonen · H. Hilden · M.-R. Taskinen (✉)
Department of Medicine, Division of Cardiology,
Helsinki University Hospital and Biomedicum,
Haartmaninkatu 8,
00029 Helsinki, Finland
e-mail: Marja-Riitta.Taskinen@helsinki.fi
Tel.: +358-9-47171990
Fax: +358-9-47171992

J. Fruchart-Najib · J.-C. Fruchart
Department of Atherosclerosis, INSERM UR 545,
Pasteur Institute of Lille and Faculty of Pharmacy,
University of Lille 2,
Lille, France

Introduction

A rapidly rising epidemic of type 2 diabetes worldwide portends a gloomy increase of cardiovascular disease (CVD). In type 2 diabetes, dyslipidaemia is a powerful modifiable CVD risk factor [1, 2]. Diabetic dyslipidaemia is a cluster of atherogenic lipid alterations characterised by raised fasting triglycerides (TG), excessive postprandial lipaemia, small dense LDL and low HDL cholesterol [3]. Whether triglycerides are an independent CHD risk factor has been an ongoing topic of debate [4–6]. Recent data from the Hoorn study by Bos and co-workers clarified the issue showing that high triglycerides are a risk factor of CVD in subjects with abnormal glucose metabolism, but not in those with normal glucose metabolism [7]. VLDL particles are heterogeneous and can be separated into two major subspecies, large buoyant VLDL 1 particles (Sf 60–400) and smaller dense VLDL 2 particles (Sf 20–60) [8]. Substantial evidence demonstrates that VLDL 1 and VLDL 2 particles are metabolically diverse and regulated independently of each other [8, 9]. Although VLDL particles are the major carriers of triglycerides, there is little information on the distribution and composition of VLDL subspecies in people with type 2 diabetes.

Apolipoproteins (apo) CIII, CII and E are key functional proteins of VLDL metabolism. Apo CIII is synthesised in the liver and intestine and is a component of triglyceride-rich lipoproteins (TRLs), LDL and HDL [10]. Apo CIII is the most abundant apolipoprotein in VLDL particles and is correlated closely with concentrations of serum total and VLDL triglycerides [11, 12]. Apo CIII modulates metabolism of TRLs at two subsequent steps [13]. Firstly, apo CIII is an inhibitor of lipoprotein lipase activity, and secondly it interferes with apolipoprotein E-mediated receptor binding, delaying removal of remnant particles. So far little attention has been paid to the distribution of apo CIII between VLDL subspecies. Likewise only a few small studies have reported apo CIII levels in a diabetic population and the data are inconclusive [14–17]. In the context of CHD risk, current evidence suggests that the increase of apolipoprotein CIII in TRLs may be an independent risk factor for coronary heart disease [18–21].

In contrast to apo CIII, apo CII is a specific activator of lipoprotein lipase [10] and patients with mutations of the *apo CII* gene display variable elevation of serum triglycerides [22]. The balance between apo CII and apo CIII is considered to be critical for the efficacy of TRL hydrolysis by lipoprotein lipase [10]. Apo E acts as a primary ligand for LDL receptor and LDL receptor-related protein [23, 24] and consequently apo E plays a central role in the removal process of VLDL remnants. Emerging evidence suggests that apo E also has a critical role in intracellular lipid metabolism [25, 26]. These functions of apo E should be protective against atherosclerosis. So far the available data on apo CII and apo E in type 2 diabetic subjects are surprisingly limited.

The purpose of this study was to assess the composition and concentration of VLDL 1 and VLDL 2 subspecies, specifically apo CIII concentration in VLDL species in type 2 diabetic and non-diabetic subjects, to gain insight into the metabolism and atherogenicity of VLDL subspecies.

Subjects, materials and methods

Subjects

The FIELD (Fenofibrate Intervention and Event Lowering in Diabetes) study is a multinational, double-blind study started in 1998 in Australia, New Zealand and Finland [27]. In the Helsinki Centre 270 type 2 diabetic patients were recruited to the FIELD study. Of these patients, 217 (69 women) volunteered for this substudy. A group of 93 healthy subjects (44 women) was recruited by letters to the spouses of study patients, through advertisements in the hospital magazine and on the intranet, and by contacting retired pilots. The subjects were between 50- and 75-years-old, with no signs or history of clinical CVD except mild hypertension and no other major chronic diseases. We performed an oral 75-g glucose tolerance test to ensure normal glucose tolerance in accordance with WHO criteria [28]. Subjects with (1) impaired glucose tolerance or impaired fasting glucose, (2) cholesterol >6.0 mmol/l, (3) LDL cholesterol >4.0 mmol/l, (4) S-triglycerides >2.5 mmol/l, (5) transaminase levels over twice the upper limit of normal values, or (6) abnormal thyroid stimulating hormone concentrations, were not eligible.

Sixteen control subjects (17%) and 100 diabetes patients (46%) had medication for arterial hypertension. The median duration of diabetes was 6 years. The mode of diabetes treatment in the study subjects was (1) diet only in 29 (13%), (2) oral antihyperglycaemic agents (OHA) in 135 (62%), (3) insulin only in ten (5%), and (4) a combination of OHA and insulin in 43 (20%) subjects. There were 31 current and 95 former smokers among the diabetes patients, 91 of the patients had never smoked. Among the control subjects there were seven current and 33 former smokers, 52 control subjects had never smoked.

All patients and control subjects signed informed consent forms. The Ethical Committee of the Helsinki University Central Hospital approved the protocol.

Laboratory analyses

All examinations were performed during the placebo run-in period of the FIELD study before any fenofibrate intervention. All lipid measurements were performed in the research laboratory of the Helsinki University Central Hospital, Division of Cardiology, Helsinki, Finland. Blood samples were obtained after an overnight fast. Serum and EDTA plasma were separated by centrifugation and stored at −80°C until analysed. Fasting serum lipoproteins were isolated from fresh serum by sequential ultracentrifugation as described previously [29]. TRL subfractions were isolated by density gradient ultracentrifugation as previously described [30]. Enzymatic colorimetric assays were used to measure cholesterol (Unimate 7 CHOL, Hoffman-La Roche, Basel, Switzerland), triglyceride (Unimate 7 TRIG, Hoffman-La Roche), free cholesterol (Boehringer Mannheim, Mannheim, Germany) and phospholipid (Wako Chemicals, Neuss, Germany) concentrations in whole sera and in lipoprotein fractions using Cobas Mira automatic analyser (Hoffman-La Roche). Protein concentrations in the lipoprotein subfractions were measured by modification of the method of Lowry [31] (DC protein assay, Bio-Rad, Hercules, CA, USA). Immunoturbidimetric kits were used to measure total apo B (Orion Diagnostica, Espoo, Finland). Serum apo CII and apo CIII were measured using commercially available kits using a single radial immunodiffusion method (Daiichi Pure Chemicals, Tokyo, Japan). Due to discontinued production of the apo CII and CIII kits, the levels of only 77 control subjects were determined. Apo E phenotype was determined by a method based on isoelectric focusing of delipidated serum followed by immunoblotting [32]. Plasma glucose concentrations were analysed by a glucose dehydrogenase method (Precision-G Blood Glucose Testing System; Medisense, Abbott, IL, USA). HbA_1c was measured using a commercially available kit (DCA 2000 Analyzer; Bayer Diagnostics, NY, USA). Serum-free insulin concentrations were determined by ra-

dioimmunoassay using the Phadeseph Insulin RIA kit (Pharmacia, Uppsala, Sweden).

Plasma samples and fractions of VLDL 1, VLDL 2 and IDL (intermediate-density lipoprotein) were frozen to −80°C and sent to the laboratory of Dr Fruchart (Department of Atherosclerosis, Pasteur Institute of Lille). Plasma apo CIII and apo E, as well as apo CIII in TRL fractions were measured by nephelometry. The polyclonal antibodies against apo CIII and apo E were generated in rabbit, using total synthetic apo CIII and purified apo E. An ELISA system was used to screen the antisera for antibody activity. The pooled antisera were precipitated with PEG and then dialysed. The specificity of the antibodies was tested by Western blotting against VLDL and HDL. For each antibody, only one band in VLDL and HDL lanes was detected at the expected weight. To confirm the specificity of the assays, the reactivity of the antisera was tested by nephelometry against the purified apolipoproteins and no signal was detected except with the corresponding purified protein against which the antibody was generated. The quantitation assays of apo CIII and apo E were performed on the Beckman immunochemistry system (IMMAGE), which measures the rate of change in light scatter induced by the formation of the immunoprecipitation complex between the polyclonal antibody and the corresponding apolipoproteins. The control and the standard used for this study were purchased from Behring. Samples (15 and 40 μl) were used for the quantitation of apo CIII and apo E, respectively, 200 μl of commercialised potassium phosphate polymer-enhancing buffer (buffer 1, ref: 447650, Beckman Coulter) and 20 μl of antisera were mixed in a reaction cell. The maximum rate of change of light scatter was measured at an optimal gain setting (gain 4 for apo CIII and 3 for apo E quantitation). The inter-assays CV were 5.2 and 4.2%, respectively, for apo E and apo CIII measurements. The recovery of apo CIII in lipoprotein fractions (VLDL, IDL, LDL, HDL and the bottom) was measured in eight samples and averaged 82±17%. The amount of apo CIII found in the density fraction >1.21 g/ml averaged 1.7±1.3 μg/dl, (i.e 0.06±0.05%). Plasma apo CIII levels measured by the two assays were closely correlated (r=0.772, p<0.001) although the absolute values obtained by nephelometry were lower.

Statistical analysis

Statistical analysis was performed using SPSS 11.0 for Windows (SPSS, Chicago, IL, USA). Results are shown as means and standard deviation or medians and interquartile range (IQR) in the case of skewed distribution of the variable. Factors that were not normally distributed were log transformed. Samples were compared using the general linear model, univariate analysis of variance, adjusted for age, BMI and sex. In the matched-pairs study, samples were compared using the paired samples t-test. The frequency distribution of apo E phenotype between the groups was compared with the Fisher's exact test and the means of plasma apo E concentration in the groups of apo E phenotype were compared with the F-test and further with Scheffe's method. Significant correlations were studied using two-tailed Pearson correlation for normally distributed variables and two-tailed Spearman correlation for non-normally distributed variables. Linear regression slopes for patients and controls were compared using GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego, CA, USA). A p value of <0.05 was considered significant in all analyses.

Results

Subject characteristics

As expected, control subjects were leaner with lower BMI and waist circumference (Table 1). Diabetic patients were in good glycaemic control with median HbA_1c of 7.1%. The lipid profile of the diabetic patients was unfavourable, with higher triglyceride and lower HDL cholesterol concentrations. Expectedly, plasma total apo B was higher in the patients. The concentrations of plasma total apo CIII and apo CII were significantly elevated in diabetic patients (Table 1). Notably, the ratio of serum apo CII:apo CIII was elevated in type 2 diabetic patients (0.50±0.33 vs 0.40±0.10, p<0.001). Plasma apo E levels were reduced, resulting in markedly higher ratio of apo CIII:apo E in diabetic patients compared to control subjects (1.35±0.42 vs 0.73±0.21, p<0.001). The distribution of apo E phenotype did not differ between the groups. Plasma apo E concentrations were comparable between the groups of different apo E phenotypes in type 2 diabetic patients (data not shown). Likewise apo E phenotype did not have significant effects on triglyceride and cholesterol contents in VLDL subspecies (data not shown).

There was a close correlation between plasma apo CIII and triglycerides in non-diabetic subjects but the correlation was much weaker in type 2 diabetic subjects (r=0.762, p<0.001 vs r=0.473, p<0.001, p=0.001 for the difference between the slopes). In contrast the correlation between serum apo CII and triglycerides was stronger in type 2 diabetic subjects than in control subjects (r=0.641, p<0.001 vs r=0.431, p<0.001). Plasma apo E correlated positively with triglycerides in non-diabetic subjects but negatively in diabetic patients (r=0.513, p<0.001 vs r=−0.278, p<0.001, p<0.001 for the difference between the slopes). The levels of serum apo CII and apo CIII were closely correlated in control subjects (r=0.727, p<0.001) as well as in type 2 diabetic patients (r=0.601, p<0.001). Plasma apo CIII and apo E were strongly related in non-diabetic subjects (r=0.502, p<0.001) but this relationship was only marginal in the diabetic subjects (r=0.197, p<0.005).

Lipid and apolipoprotein composition of TRL subspecies

The percentage compositions of VLDL 1, VLDL 2 and IDL particles are presented in Table 2. In type 2 diabetic subjects VLDL 1 contained relatively more cholesterylester and

Table 1 Basic characteristics, metabolic variables, lipoprotein concentration and apolipoprotein concentration in type 2 diabetic patients and control subjects

	Type 2 diabetic patients (*n*=217)	Control subjects (*n*=93)	Adjusted[a] *p* value
Age (years)	62 (56–67)	59 (54–63)	
Sex (men/women)	148/69	49/44	
BMI (kg/m^2)	29.4 (26.7–32.6)	24.9 (23.3–27.4)	
Waist circumference (cm)	99 (93–108)	88 (78–97)	NS
Glycaemic control			
HbA_1c (%)	7.1 (6.3–8.0)	5.2 (5.0–5.4)	<0.001
F-glucose (mmol/l)	7.6 (6.5–9.1)	5.0 (4.6–5.5)	<0.001
HOMA-IR	4.0 (2.5–5.8)	1.3 (1.0–1.8)	<0.001
Blood pressure			
Systolic (mmHg)	142 (133–152)	134 (124–143)	0.041
Diastolic (mmHg)	88 (81–93)	82 (78–87)	0.001
Lipoprotein concentration			
Total cholesterol (mmol/l)	5.0 (4.6–5.6)	5.1 (4.6–5.5)	NS
Total triglyceride (mmol/l)	1.6 (1.2–2.1)	1.0 (0.8–1.4)	<0.001
HDL cholesterol (mmol/l)	1.1 (1.0–1.3)	1.6 (1.3–1.9)	<0.001
LDL cholesterol (mmol/l)	3.1 (2.8–3.6)	3.1 (2.7–3.5)	NS
Apolipoprotein concentration			
Total apo B (mg/dl)	99 (87–114)	93 (81–105)	0.049
Total apo CII (mg/dl)[b]	4.3 (3.4–5.0)	3.1 (2.4–4.0)	<0.001
Total apo CIII (mg/dl)	4.7 (3.9–5.4)	2.8 (2.2–3.4)	<0.001
Total apo E (mg/dl)	3.6 (3.0–4.2)	3.8 (3.2–4.8)	0.001
Apo E phenotype (*N* and %)			
4/4	3 (1.4%)	2 (2.2%)	
4/3	64 (29.4%)	29 (31.2%)	
4/2	4 (1.8%)	0 (0%)	NS
3/3	118 (54.3%)	54 (58.1%)	
3/2	28 (12.9%)	8 (8.6%)	

Data are presented as median (IQR)
[a]Adjusted for age, sex, BMI
[b]*n*=77 for control subjects

phospholipids but less TG and free cholesterol than that of non-diabetic subjects. Consistently, VLDL 2 in type 2 diabetic patients was relatively enriched with cholesterylester and poor in free cholesterol and protein compared with control subjects. The composition of IDL particles was comparable in the two groups. Overall, the concentrations of all lipid components in both VLDL 1 and VLDL 2 were markedly higher in type 2 diabetic subjects than in non-

Table 2 VLDL 1, VLDL 2 and IDL percentage composition in type 2 diabetic patients and control subjects

	Type 2 diabetic patients (*n*=217) Percentage composition (%)	Control subjects (*n*=93)	Adjusted[a] *p* value
VLDL 1			
Triglyceride	67.5 (65.5–69.8)	69.9 (68.2–71.3)	<0.001
Cholesterylester	4.9 (3.5–5.9)	2.5 (81.5–3.5)	<0.001
Free cholesterol	4.0 (3.6–4.7)	4.2 (3.8–4.7)	NS
Phospholipid	14.9 (14.4–15.6)	14.6 (13.9–15.2)	0.005
Protein	8.7 (8.1–9.4)	8.9 (7.5–9.7)	NS
VLDL 2			
Triglyceride	45.7 (42.7–48.3)	46.6 (44.1–48.9)	NS
Cholesterylester	14.0 (11.6–16.3)	12.5 (10.0–14.5)	<0.001
Free cholesterol	7.5 (7.0–8.2)	7.8 (7.2–8.5)	0.032
Phospholipid	20.0 (19.3–20.8)	19.8 (19.4–20.3)	NS
Protein	12.8 (12.0–13.5)	13.6 (12.3–14.5)	0.002
IDL			
Triglyceride	22.3 (19.3–26.1)	21.0 (18.6–24.6)	NS
Cholesterylester	28.4 (24.6–31.1)	28.1 (24.6–31.5)	NS
Free cholesterol	10.2 (9.6–10.9)	11.0 (10.5–11.9)	<0.001
Phospholipid	22.6 (21.8–23.2)	22.3 (21.8–22.8)	NS
Protein	16.4 (15.4–17.2)	16.8 (15.6–18.5)	NS

Data are presented as median +IQR
[a]Adjusted for age, sex and BMI

diabetic subjects (data not shown). Consequently the mass concentrations of VLDL 1 and VLDL 2 were increased in type 2 diabetic compared with non-diabetic subjects by 125 and 82% ($p<0.001$). Apo CIII in VLDL1 particles was slightly higher in diabetic patients than in control subjects (0.52±0.33 vs 0.47±0.39 mg/dl). In contrast type 2 diabetic subjects had significantly less apo CIII in VLDL 2 (0.21± 0.13 vs 0.31±0.22 mg/dl, $p<0.001$) and IDL particles (0.07± 0.05 vs 0.11±0.07 mg/dl, $p<0.001$) than non-diabetic subjects.

We calculated the ratio of apo CIII:phospholipids and free cholesterol, the surface components of TRL particles. Notably, the ratio of apo CIII:phospholipids and free cholesterol in both VLDL 1 (0.037 vs 0.070, $p<0.001$) and in VLDL 2 (0.017 vs 0.040, $p<0.001$) as well as in IDL (0.005 vs 0.009, $p<0.001$) were lower in type 2 diabetic subjects than in control subjects. We also calculated the ratio of apo CIII:cholesterylester and triglyceride. Diabetic patients had markedly reduced ratios of apo CIII:cholesterylester and triglyceride in VLDL 1 (0.008 vs 0.014, $p<0.001$), VLDL 2 (0.006 vs 0.015, $p<0.001$) and IDL (0.003 vs 0.006, $p<0.001$). This implies that apo CIII does not increase in concert with core lipids in type 2 diabetes.

The relationship of VLDL triglycerides and serum triglycerides

Strong positive correlations existed between triglycerides in VLDL 1 and VLDL 2 and serum total triglycerides in both diabetic subjects and in control subjects (Fig. 1a, b). However, the slope for VLDL 1 was much steeper than that for VLDL 2 in both groups ($p<0.001$). The data indicate that for each increment of serum triglycerides there is a higher increase of triglycerides in VLDL 1 than in VLDL 2 particles. The regression lines of VLDL 1 TG and total TG were comparable between diabetic and the control group (Fig. 1c). Similarly, the regression lines of VLDL 2 TG and total TG were comparable between the two groups (Fig. 1d).

Relationship between triglycerides and apolipoproteins in VLDL subspecies

We observed strong correlations between apo CIII and triglycerides in both VLDL 1 and in VLDL 2 in control subjects and in type 2 diabetic patients. Notably the slope for apo CIII in VLDL 1 in non-diabetic subjects was con-

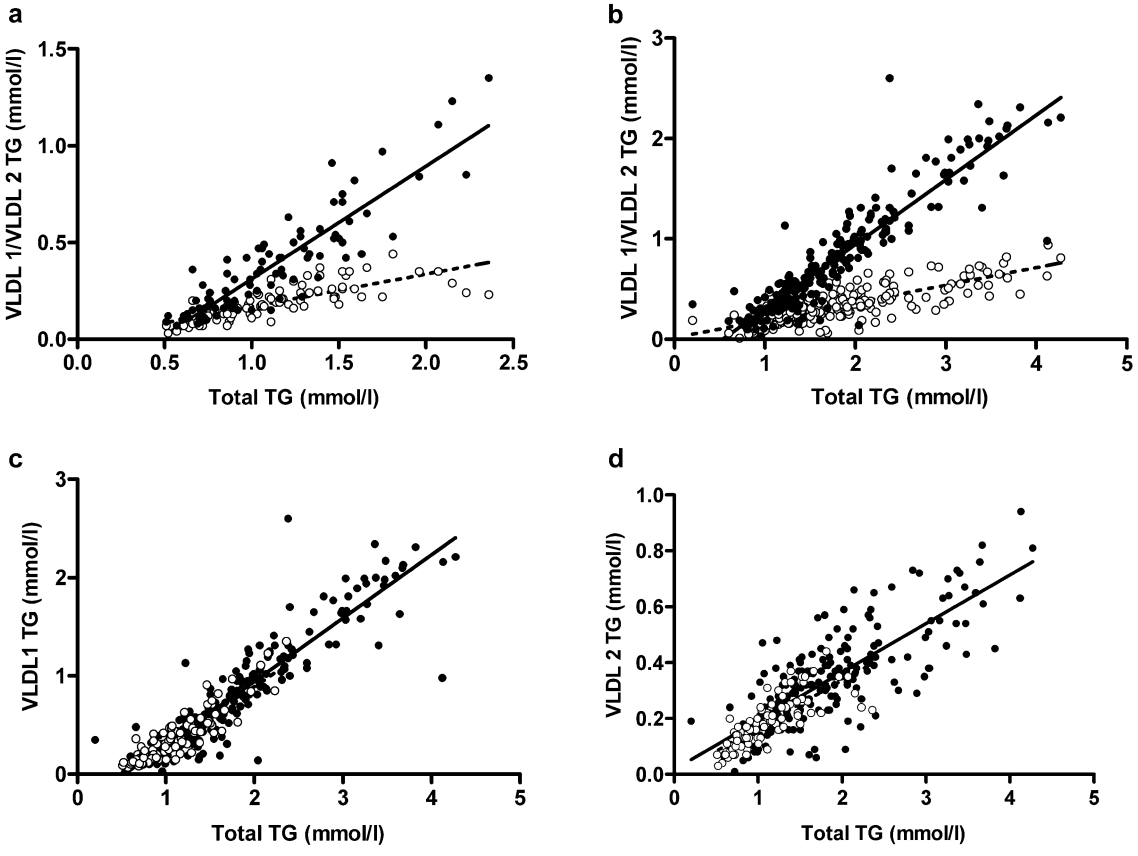

Fig. 1 The relationship of VLDL 1 and VLDL 2 triglyceride and serum triglyceride concentrations in type 2 diabetic patients and control subjects. **a** *Closed symbol*: VLDL 1 TG; *open symbol*: VLDL 2 TG. $r=0.879$, $p<0.001$ for VLDL 1 TG; $r=0.870$, $p<0.001$ for VLDL 2 TG in the control group. Difference between the slopes $p<0.001$. **b** *Closed symbol*: VLDL 1 TG; *open symbol*: VLDL 2 TG. $r=0.899$, $p<0.001$ for VLDL 1 TG; $r=0.771$, $p<0.001$ for VLDL 2 TG in the patient group. Difference between the slopes $p<0.001$. **c, d** *Closed symbol*: type 2 diabetic patients; *open symbol*: control subjects. Difference between the slopes **c** $p=0.27$, **d** $p=0.85$

siderably steeper than in type 2 diabetic subjects (Fig. 2a, $p<0.001$). Likewise the slope for apo CIII in VLDL 2 was considerably steeper in normal subjects than in diabetic patients (Fig. 2b, $p<0.001$). Thus, for each increment of TG in VLDL 1 and VLDL 2 there was a smaller increase of apo CIII in VLDL 1 and VLDL 2 of diabetic subjects than in control subjects. The association between TG and apo CIII in IDL was significant in diabetic subjects ($r=0.616$, $p<0.001$) but not in control subjects ($r=0.204$, $p=0.051$).

The matched-pairs study

The diabetic patients and control subjects were paired for sex, BMI (mean intra-pair difference 1.14 kg/m^2) and serum TG levels (mean intra-pair difference 0.09 mmol/l). Altogether we found 52 matched pairs (31 male and 21 female pairs). Despite similar serum TG levels (1.2 mmol/l in both groups) diabetic subjects had a lower concentration of HDL cholesterol (1.5±0.4 vs 1.2±0.5 mmol/l, $p=0.004$). Notably the concentrations of both plasma apo B (96.0±13 vs 90.5±15 mg/dl, $p=0.005$) and apo CIII (4.37±0.8 vs 3.12±0.8 mg/dl, $p<0.001$) were higher in diabetic subjects than in control group. The concentrations of plasma apo CII and apo E did not differ between the groups. The concentration of apo CIII in VLDL 1 (0.34±0.18 vs 0.57±0.42 mg/dl, $p=0.001$), VLDL 2 (0.17±0.08 vs 0.35±0.25 mg/dl, $p<0.001$) and IDL (0.05±0.02 vs 0.12±0.08 mg/dl, $p<0.001$) was significantly lower in diabetic patients than in normal subjects.

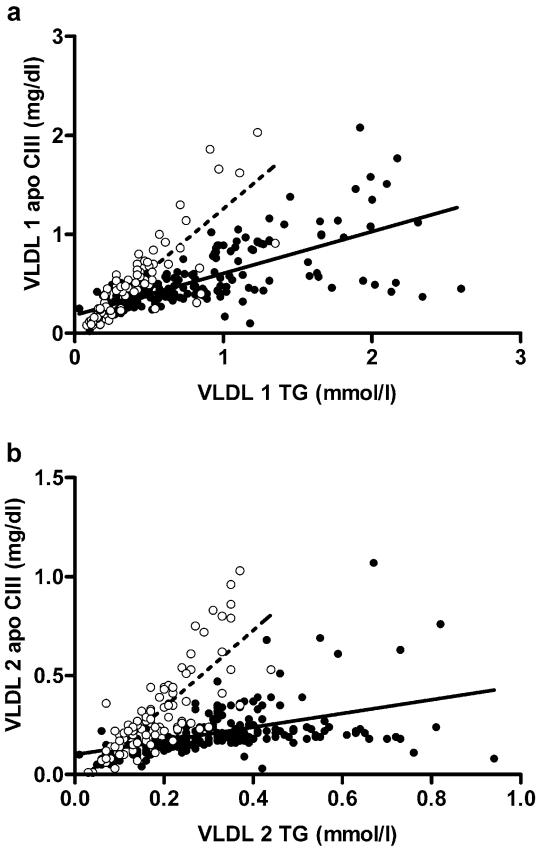

Fig. 2 The relationship of apo CIII and triglycerides in **a** VLDL 1 and **b** VLDL 2. *Closed symbols*: type 2 diabetic patients; *open symbols*: control subjects. **a** $r=0.900$, $p<0.001$ for control group; $r=0.756$, $p<0.001$ in type 2 diabetic patients. **b** $r=0.803$, $p<0.001$ for control group; $r=0.575$, $p<0.001$ for type 2 diabetic patients. Difference between the slopes: $p<0.001$ in both panels

Discussion

In this study we demonstrate that the elevation of VLDL 1 triglycerides is the major determinant of serum triglyceride concentration in healthy normolipidaemic subjects as well as in patients with type 2 diabetes. Both plasma apo CII and apo CIII levels were elevated but apo E levels were decreased in type 2 diabetes. Paradoxically we detected a relative deficiency of apo CIII in all TRL species in type 2 diabetic subjects, providing evidence that the concentration of apo CIII does not increase in concert with core lipids. Since apo CIII has a critical role of in the hepatic clearance of VLDL remnants, lack of apo CIII may result in an impaired removal of these particles and a prolonged residence time in circulation. Thus, the elevation of VLDL particles in type 2 diabetes will have adverse consequences on other lipoprotein subspecies like LDL and HDL subclasses, and together these changes will comprise a highly atherogenic cluster [3].

A potential limitation of this study is that we separated TRL fractions using density-gradient ultracentrifugation and may have lost some apo CIII into the density >1.20 g/ml lipid-free fraction during ultracentrifugation. However, we were able to detect only trivial amounts of apo CIII in the lipid free fraction. Our results are in line with the finding that ultracentrifugation causes less than 5% of total apo CIII to be stripped off in the density >1.21 g/ml fraction [33]. In contrast to apo CIII, the detachment of apo E seems to be more robust and about 20–40% of apo E is lost into the non-lipid fraction [34]. Clearly the lack of apo E measurements in VLDL subspecies prohibits any concise conclusion on the metabolic relevance of plasma apo E reduction in type 2 diabetes. However, in type 2 diabetes the inverse correlation between apo E and triglycerides opposed to the strong positive correlation observed in non-diabetic subjects hints at the derangement of apo E metabolism.

Lipoprotein kinetic studies have shown that VLDL particles are metabolically heterogeneous [8]. Substantial evidence demonstrates that both the production and catabolism of large TG-rich VLDL 1 (Sf 60–400) and smaller cholesterol-rich VLDL 2 (Sf 20–60) are independently regulated [8, 9]. The metabolic processing of VLDL 1 and VLDL 2 in the VLDL-IDL-LDL cascade has parallel but diverse pathways that produce IDL and LDL. So far very little attention has been paid to VLDL subspecies in diabetes although the elevation of serum triglycerides is a distinct feature of diabetic dyslipidaemia. Previous studies have reported a proportionally higher increase of VLDL 1 particles than that of VLDL 2 particles in small groups of type 2 diabetic patients [30, 35]. Our results expand these earlier observations demonstrating clearly that the elevation

of VLDL 1 triglycerides is the major determinant of serum triglyceride concentration both in normal subjects and in type 2 diabetic individuals. In agreement, the NMR profile of VLDL subspecies indicated that the increase of VLDL in type 2 diabetes is primarily due to an increased number of large VLDL particles [36]. The slope for VLDL 1 TG over a substantial range of serum triglycerides was much steeper than that for VLDL 2. Thus, the actual contribution of VLDL 1 triglyceride to serum triglyceride concentration becomes more pronounced when the triglyceride level rises. The fact that both diabetic and non-diabetic subjects fell on the same regression line suggest that the relationship was probably not due to diabetes itself but linked to factors driving the elevation of VLDL 1 triglycerides.

The available data on lipid composition of VLDL particles in diabetic dyslipidaemia are controversial. The studies looking at VLDL particles as a single entity have reported VLDL particles to be triglyceride enriched [37, 38]. However, a relatively larger increase of VLDL 1 mass compared with VLDL 2 mass in type 2 diabetes will be reflected as a relative increase of TG when studying only the whole VLDL particles. In this study the relative content of triglycerides in VLDL 1 was slightly, albeit significantly less, in diabetic subjects than in control subjects. This is consistent with a previous study reporting a relative depletion of triglycerides in VLDL1 particles of diabetic subjects [39]. Overall our results agree with previous data demonstrating that the elevation of serum triglyceride concentration primarily reflects an increase in the number, but not the size, of particles in both normolipidaemic and hypertriglyceridaemic subjects [40].

So far the data on plasma apo CII and apo CIII in type 2 diabetes are limited and controversial. Apo CIII levels have been reported to be elevated in the diabetic population but the limitations of these studies are small cohorts and heterogeneity of the subjects [14–17]. Most studies have reported normal apo CII levels and a normal or slightly decreased apo CII:apo CIII ratio in type 2 diabetic subjects [17, 41, 42]. In this study we observed a significant increase of both apo CII and apo CIII concentrations. The fact that the ratio of apo CII:apo CIII in total plasma was elevated in type 2 diabetic subjects is noteworthy and argues against impaired lipolysis of VLDL particles. As expected, a positive correlation existed between apo CIII and triglycerides in both VLDL 1 and in VLDL 2 in normal subjects. Unexpectedly the regression lines for apo CIII in both VLDL 1 and VLDL 2 across a wide range of triglycerides were less steep in diabetic subjects than in non-diabetic subjects. Thus for each increment of triglycerides in VLDL 1 and VLDL 2 there was less apo CIII in diabetic subjects than in non-diabetic subjects. To explore further this unexpected finding we calculated the ratio of apo CIII over the surface lipids and core lipids. Both ratios confirmed the relative depletion of apo CIII in diabetic subjects as compared with non-diabetic subjects. Likewise, the relative deficiency of apo CIII in both VLDL 1 and VLDL 2 fraction was demonstrated in type 2 diabetic subjects matched for fasting triglyceride level and BMI with non-diabetic subjects. Interestingly a recent study [43] reported that diabetes status was associated with lower concentrations of apo C III containing large and small VLDL particles whereas hypertriglyceridaemia in itself was linked to an increase of apo CIII containing VLDL particles. Likewise in diabetic patients, a lower ratio of apo CIII:triglycerides in VLDL has been reported [16]. Thus, despite elevation of plasma apo CIII, diabetes status is linked with a relative deficiency of apo CIII in all TRL species.

Both apo CIII and apo CII have a broad distribution being components of VLDL and HDL particles and are readily exchangeable between VLDL and HDL depending on the dynamics of lipoprotein metabolism [11, 12, 44]. Further studies are requested to establish if there are abnormal shifts between apo CIII, CII and E between VLDL and HDL particles in type 2 diabetes. A large body of data provides evidence that the plasma apo CIII concentration is increased in hypertriglyceridaemic subjects and there is a close positive correlation between plasma apo CIII and TG concentrations [11, 12, 44]. Likewise, a positive correlation exists between levels of plasma apo CII and TG [33, 45]. This was true also in our type 2 diabetic and non-diabetic subjects, arguing against a major derangement of apo CII metabolism.

The key question is: how to explain the relative deficiency of apo CIII in TRL species in the context of increased VLDL production in type 2 diabetes? Direct input of apo CIII into VLDL seems to account for about 50% or more of apo CIII production in both normolipidaemic subjects and in patients with elevated serum triglycerides [44]. In agreement with this, the VLDL apo CIII production rate is increased in subjects with hypertriglyceridaemia and there is a close correlation between plasma apo CIII levels and apo B production [46, 47]. Obviously assumptions drawn from these studies in non-diabetic subjects do not necessarily apply to type 2 diabetes. Metabolic kinetic studies are required to establish the mechanism behind the relative deficiency of apo CIII in VLDL species in type 2 diabetes.

In summary, the elevation of VLDL 1 species is a key feature of diabetic dyslipidaemia. Our data also suggest that the associations of apo CIII and apo E with TRL metabolism are profoundly disturbed in type 2 diabetes. Our results emphasise the need to study the distribution and composition of VLDL subclasses separately to capture the atherogenicity of TRL subpopulations in diabetic dyslipidaemia.

Duality of interest M.-R. Taskinen has received lecture fees from Laboratoires Fournier.

Acknowledgements This work was supported by grants from the Finnish Diabetes Association (to E. Leinonen and M.-R. Taskinen), the Jenny and Antti Wihuri Fund (to E. Leinonen), the Helsinki University Central Hospital Research Foundation (to E. Leinonen and M.-R.Taskinen) and Sigrid Juselius Foundation to (M.-R. Taskinen). We thank the volunteers for participating in this study. We also thank Ritva Marjanen, Virve Naatti, Helinä Perttunen-Nio and Tomi Silvennoinen for their excellent technical assistance and Helena Laakkonen for skilful editing.

References

1. Lehto S, Ronnemaa T, Haffner SM, Pyorala K, Kallio V, Laakso M (1997) Dyslipidemia and hyperglycemia predict coronary heart disease events in middle-aged patients with NIDDM. Diabetes 46:1354–1359
2. Turner RC, Millns H, Neil HA et al (1998) Risk factors for coronary artery disease in non-insulin dependent diabetes mellitus: United Kingdom prospective diabetes study (UKPDS: 23) [comment]. BMJ 316:823–828
3. Taskinen MR (2003) Diabetic dyslipidaemia: from basic research to clinical practice. Diabetologia 46:733–749
4. Criqui MH, Heiss G, Cohn R et al (1993) Plasma triglyceride level and mortality from coronary heart disease. N Engl J Med 328:1220–1225
5. Hokanson JE, Austin MA (1996) Plasma triglyceride level is a risk factor for cardiovascular disease independent of high-density lipoprotein cholesterol level: a meta-analysis of population-based prospective studies. J Cardiovasc Risk 3:213–219
6. Avins AL, Neuhaus JM (2000) Do triglycerides provide meaningful information about heart disease risk? Arch Intern Med 160:1937–1944
7. Bos G, Dekker JM, Nijpels G et al (2003) A combination of high concentrations of serum triglyceride and non-high-density-lipoprotein-cholesterol is a risk factor for cardiovascular disease in subjects with abnormal glucose metabolism—The Hoorn study. Diabetologia 46:910–916
8. Packard CJ, Shepherd J (1997) Lipoprotein heterogeneity and apolipoprotein B metabolism. Arterioscler Thromb Vasc Biol 17:3542–3556
9. Malmstrom R, Packard CJ, Caslake M et al (1997) Defective regulation of triglyceride metabolism by insulin in the liver in NIDDM. Diabetologia 40:454–462
10. Jong MC, Hofker MH, Havekes LM (1999) Role of ApoCs in lipoprotein metabolism: functional differences between ApoC1, ApoC2, and ApoC3. Arterioscler Thromb Vasc Biol 19:472–484
11. Marcoux C, Tremblay M, Fredenrich A, Davignon J, Cohn JS (2001) Lipoprotein distribution of apolipoprotein C-III and its relationship to the presence in plasma of triglyceride-rich remnant lipoproteins. Metabolism 50:112–119
12. Campos H, Perlov D, Khoo C, Sacks FM (2001) Distinct patterns of lipoproteins with apoB defined by presence of apoE or apoC-III in hypercholesterolemia and hypertriglyceridemia. J Lipid Res 42:1239–1249
13. van Dijk KW, Rensen PC, Voshol PJ, Havekes LM (2004) The role and mode of action of apolipoproteins CIII and AV: synergistic actors in triglyceride metabolism? Curr Opin Lipidol 15:239–246
14. Briones ER, Mao SJ, Palumbo PJ, O'Fallon WM, Chenoweth W, Kottke BA (1984) Analysis of plasma lipids and apolipoproteins in insulin-dependent and noninsulin-dependent diabetics. Metabolism 33:42–49
15. Stalenhoef AF, Demacker PN, Lutterman JA, van't Laar A (1982) Apolipoprotein C in type 2 (non-insulin-dependent) diabetic patients with hypertriglyceridaemia. Diabetologia 22:489–491
16. Pan XR, Cheung MC, Walden CE, Hu SX, Bierman EL, Albers JJ (1986) Abnormal composition of apoproteins C-I, C-II, and C-III in plasma and very-low-density lipoproteins of non-insulin-dependent diabetic. Chinese Clin Chem 32:1914–1920
17. Ishibashi S, Yamada N, Shimano H, Takaku F, Akanuma Y, Murase T (1989) Composition of very-low-density lipoproteins in non-insulin-dependent diabetes mellitus. Clin Chem 35:808–812
18. Gervaise N, Garrigue MA, Lasfargues G, Lecomte P (2000) Triglycerides, apo C3 and Lp B:C3 and cardiovascular risk in type II diabetes. Diabetologia 43:703–708
19. Luc G, Fievet C, Arveiler D et al (1996) Apolipoproteins C-III and E in apoB- and non-apoB-containing lipoproteins in two populations at contrasting risk for myocardial infarction: the ECTIM study. J Lipid Res 37:508–517
20. Sacks FM, Alaupovic P, Moye LA et al (2000) VLDL, apolipoproteins B, CIII, and E, and risk of recurrent coronary events in the Cholesterol and Recurrent Events (CARE) trial. Circulation 102:1886–1892
21. Onat A, Hergenc G, Sansoy V et al (2003) Apolipoprotein C-III, a strong discriminant of coronary risk in men and a determinant of the metabolic syndrome in both genders. Atherosclerosis 168:81–89
22. Santamarina-Fojo S (1992) Genetic dyslipoproteinemias: role of lipoprotein lipase and apolipoprotein C-II. Curr Opin Lipidol 3:186–195
23. Clavey V, Lestavel-Delattre S, Copin C, Bard JM, Fruchart JC (1995) Modulation of lipoprotein B binding to the LDL receptor by exogenous lipids and apolipoproteins CI, CII, CIII, and E. Arterioscler Thromb Vasc Biol 15:963–971
24. Ji ZS, Dichek HL, Miranda RD, Mahley RW (1997) Heparan sulfate proteoglycans participate in hepatic lipase and apolipoprotein E-mediated binding and uptake of plasma lipoproteins, including high density lipoproteins. J Biol Chem 272:31285–31292
25. Mensenkamp AR, Jong MC, van Goor H et al (1999) Apolipoprotein E participates in the regulation of very low density lipoprotein–triglyceride secretion by the liver. J Biol Chem 274:35711–35718
26. Heeren J, Grewal T, Laatsch A et al (2003) Recycling of apoprotein E is associated with cholesterol efflux and high-density lipoprotein internalization. J Biol Chem 278:14370–14378
27. Keech AC, Field Study Investigators T (2004) The need for a large-scale trial of fibrate therapy in diabetes: the rationale and design of the Fenofibrate Intervention and Event Lowering in Diabetes (FIELD) study ISRCTN64783481. Cardiovasc Diabetol 3:9
28. World Health Organisation (1999) Definition, diagnosis and classification of diabetes mellitus and its complications. Report of a WHO consultation: Part I. Diagnosis and classification of diabetes mellitus. World Health Organisation, Geneva, pp 1–65
29. Taskinen MR, Kuusi T, Helve E, Nikkila EA, Yki-Jarvinen H (1988) Insulin therapy induces antiatherogenic changes of serum lipoproteins in noninsulin-dependent diabetes. Arteriosclerosis 8:168–177
30. Taskinen MR, Packard CJ, Shepherd J (1990) Effect of insulin therapy on metabolic fate of apolipoprotein B-containing lipoproteins in NIDDM. Diabetes 39:1017–1027
31. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the folin phenol reagent. J Biol Chem 193:265–275
32. Havekes LM, de Knijff P, Beisiegel U, Havinga J, Smit M, Klasen E (1987) A rapid micromethod for apolipoprotein E phenotyping directly in serum. J Lipid Res 28:455–463
33. Schonfeld G, George PK, Miller J, Reilly P, Witztum J (1979) Apolipoprotein C-II and C-III levels in hyperlipoproteinemia. Metabolism 28:1001–1010
34. Havel RJ, Kotite L, Vigne JL et al (1980) Radioimmunoassay of human arginine-rich apolipoprotein, apoprotein E: concentration in blood plasma and lipoproteins as affected by apoprotein E-3 deficiency. J Clin Invest 66:1351–1362
35. Guerin M, Le Goff W, Lassel TS, Van Tol A, Steiner G, Chapman MJ (2001) Atherogenic role of elevated CE transfer from HDL to VLDL(1) and dense LDL in type 2 diabetes: impact of the degree of triglyceridemia. Arterioscler Thromb Vasc Biol 21:282–288
36. Garvey WT, Kwon S, Zheng D et al (2003) Effects of insulin resistance and type 2 diabetes on lipoprotein subclass particle size and concentration determined by nuclear magnetic resonance. Diabetes 52:453–462
37. McEneny J, O'Kane MJ, Moles KW et al (2000) Very low density lipoprotein subfractions in type II diabetes mellitus: alterations in composition and susceptibility to oxidation. Diabetologia 43:485–493

38. Taskinen MR, Beltz WF, Harper I et al (1986) Effects of NIDDM on very-low-density lipoprotein triglyceride and apolipoprotein B metabolism: studies before and after sulfonylurea therapy. Diabetes 35:1268–1277
39. James RW, Pometta D (1991) The distribution profiles of very low density and low density lipoproteins in poorly-controlled male, type 2 (non-insulin-dependent) diabetic patients. Diabetologia 34:246–252
40. Steiner G, Tkac I, Uffelman KD, Lewis GF (1998) Important contribution of lipoprotein particle number to plasma triglyceride concentration in type 2 diabetes. Atherosclerosis 137:211–214
41. Manzato E, Zambon A, Lapolla A et al (1993) Lipoprotein abnormalities in well-treated type II diabetic patients. Diabetes Care 16:469–475
42. Gabor J, Spain M, Kalant N (1980) Composition of serum very-low-density and high-density lipoproteins in diabetes. Clin Chem 26:1261–1265
43. Lee SJ, Moye LA, Campos H, Williams GH, Sacks FM (2003) Hypertriglyceridemia but not diabetes status is associated with VLDL containing apolipoprotein CIII in patients with coronary heart disease. Atherosclerosis 167:293–302
44. Batal R, Tremblay M, Barrett PH et al (2000) Plasma kinetics of apoC-III and apoE in normolipidemic and hypertriglyceridemic subjects. J Lipid Res 41:706–718
45. Kashyap ML, Srivastava LS, Chen CY et al (1977) Radioimmunoassay of human apolipoprotein CII: a study in normal and hypertriglyceridemic subjects. J Clin Invest 60:171–180
46. Chan DC, Watts GF, Redgrave TG, Mori TA, Barrett PH (2002) Apolipoprotein B-100 kinetics in visceral obesity: associations with plasma apolipoprotein C-III concentration. Metabolism 51:1041–1046
47. Cohn JS, Patterson BW, Uffelman KD, Davignon J, Steiner G (2004) Rate of production of plasma and very-low-density lipoprotein (VLDL) apolipoprotein C-III is strongly related to the concentration and level of production of VLDL triglyceride in male subjects with different body weights and levels of insulin sensitivity. J Clin Endocrinol Metab 89:3949–3955