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Effects of dietary cholesterol on plasma lipoproteins and their subclasses in IDDM patients

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Summary To compare the effects of dietary cholesterol supplementation in insulin-dependent diabetic (IDDM) patients and normal subjects, 10 male IDDM patients in good glycaemic control (HbA_{1c} 7.3 ± 0.9 %) (mean ± SD) and normal plasma lipid levels, and 11 control male subjects of similar age, body mass index and lipid plasma levels underwent a double blind, cross-over, sequential study. Cholesterol supplementation of 800 mg/day or placebo were given for consecutive periods of 3 weeks. The concentration of plasma total cholesterol increased significantly with the dietary cholesterol supplementation compared to placebo in IDDM patients by 6 % ($p < 0.05$) and in control subjects by 9 % ($p < 0.05$). No changes were observed in the concentration of plasma triglycerides in either group. The LDL cholesterol level increased by 12 % ($p < 0.01$) in patients and by 7 % ($p < 0.05$) in control subjects. In patients plasma HDL cholesterol concentration remained the same, while in control subjects it tended to increase after cholesterol supplementation (from 1.14 ± 0.26 to 1.23 ± 0.27 mmol/l, $p = 0.06$). During the cholesterol intake period the mean concentration of LDL1, LDL2 and LDL3 subclasses in patients showed a significant increase by 21.0 ($p < 0.05$), 20.4 ($p < 0.001$) and 11.1 % ($p < 0.05$), respectively, resulting in an 18.0 % increase in mean total LDL mass ($p < 0.001$) without major changes in LDL composition. In the control subjects the changes in the concentrations of LDL subclasses during cholesterol intake were less and not significant. In the IDDM patients the cholesterol intake did not affect the concentration or composition of HDL subclasses or total HDL mass. In contrast, in control subjects cholesterol intake increased the mean concentration of HDL2a by 12.2.% ($p < 0.05$) and this increase was significantly different if compared to changes obtained in the patients. In conclusion, compared to normal subjects, in IDDM patients, dietary cholesterol intake increased the LDL particle mass significantly and had no positive effect on HDL. [Diabetologia (1998) 41: 193–200]

Keywords Dietary cholesterol, plasma lipoproteins, lipoprotein subclasses, lipoprotein composition, IDDM patients.

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Abbreviations: LDL, Low density lipoprotein; VLDL, very low density lipoprotein; HDL, high density lipoprotein; IDDM, insulin-dependent diabetes mellitus; CETP, cholesteryl ester transfer protein.

Clinical studies have shown that a high cholesterol intake increases the levels of plasma and low density lipoprotein (LDL) cholesterol, even when dietary fat is kept constant [1]. The elevation of LDL cholesterol level is highly variable (4–58 %) [1, 2] and this may depend on different factors, such as the type of fat utilized in the diet, dietary cholesterol absorption efficiency, ability to reduce endogenous cholesterol synthesis, regulation of the synthesis of bile acids, amount of intracellular cholesterol, genetic background [1–6].

Despite the large number of studies on healthy or hyperlipidaemic individuals [1–7], no data is available

on the effects of cholesterol supplementation in insulin-dependent diabetic (IDDM) patients. For IDDM patients a high intake of cholesterol could be particularly harmful for the following reasons: 1) the removal of LDL particles from circulation by the apo B receptor is often impaired, as a consequence of LDL glycosylation [8]; 2) other steps in cholesterol metabolism have been reported to be altered in diabetic animals (increase in cholesterol absorption and synthesis, increase in liver cholesterol content) [9] and these defects may also be present in human diabetic patients and affect their response to dietary cholesterol.

Epidemiological studies have shown that the relationship between dietary cholesterol intake and cardiovascular mortality is independent of plasma cholesterol concentrations [10]. This suggests that other mechanisms could be involved in the way dietary cholesterol affects cardiovascular mortality, such as alterations in the concentration of lipoprotein subclasses or in their composition. Results from animal studies support this suggestion: in rabbits, for instance, dietary cholesterol has been shown to increase small very low density lipoprotein (VLDL) particles [11, 12], which are considered to be more atherogenic than large VLDL particles [13] and which are already increased in IDDM patients (even normolipidaemic) [14, 15]. The awareness of the effects of dietary cholesterol on lipid metabolism in IDDM patients – who are also characterized by other subtle abnormalities [16] – is important if proper dietary advice is to be given to them, especially considering that the aspect of cholesterol intake is generally neglected in clinical practice.

The aim of this study, based on a randomized double blind sequential cross-over design, was to evaluate the effects of dietary cholesterol supplementation (800 mg/day) on plasma lipids and concentration and composition of lipoprotein subclasses in a group of normolipidaemic, male IDDM patients compared to matched healthy control subjects.

Subjects and methods

Subjects. Ten male subjects diagnosed with IDDM according to World Health Organization (WHO) criteria [17], in stable metabolic control (HbA_{1c} 7.3 ± 0.9 %) (mean ± SD) and with normal plasma lipid levels (total cholesterol 4.66 ± 0.81 mmol/l, triglycerides 0.81 ± 0.18 mmol/l), and 11 male control subjects, matched to IDDM patients for age, BMI and plasma lipids, were included in the study after giving their informed consent. The protocol was approved by the ethical committee of the Federico II University Medical School, Naples, Italy.

Patients had no diabetic complications in the clinical examination, but 5 had microalbuminuria defined as albumin excretion rate over 20 μg/min and less than 70 μg/min. None of the patients was on any medication other than insulin. The clinical and biochemical characteristics of the patients and control subjects before entering the study are shown in Table 1.

Table 1. Clinical and biochemical characteristics of IDDM patients and control subjects at baseline

	IDDM patients $n = 10$	Control subjects $n = 11$
Age years	35 ± 10	37 ± 12
BMI (kg/m^2)	24 ± 2	25 ± 2
Duration of diabetes (years)	12 ± 6	–
HbA_{1c} (%)	7.3 ± 0.9	–
Blood glucose (mmol/l)	7.7 ± 0.8	–
Total cholesterol (mmol/l)	4.7 ± 0.8	4.8 ± 0.6
Triglycerides (mmol/l)	0.81 ± 0.18	0.89 ± 0.24
HDL cholesterol (mmol/l)	1.25 ± 0.29	1.13 ± 0.20
LDL cholesterol (mmol/l)	2.99 ± 0.83	3.28 ± 0.49
ApoE phenotype		
apoE2/2	$n = 1$	$n = 1$
apoE3/2	$n = 2$	$n = 1$
apoE3/3	$n = 6$	$n = 7$
apoE4/3	$n = 1$	$n = 2$

Data are mean ± SD

Experimental design. The study was based on a double blind sequential cross-over design and consisted of two study periods of 3 weeks each. Patients and control subjects were stabilized, during a run-in period of 3 weeks, on a weight-maintaining diet containing 50 % carbohydrates, 30 % fat, (12 % saturated fat), 20 % proteins, 200 mg/day cholesterol and 30 g/day dietary fibre. Patients followed their usual insulin therapy (three injections of regular insulin before meals with intermediate insulin before dinner), which remained unchanged throughout the two experimental periods. After the run-in period, subjects were randomly assigned to either cholesterol supplementation or placebo intake and after 3 weeks they were crossed to the other treatment.

Cholesterol supplementation (800 mg/day) and placebo were given as two liquid supplements, one containing egg yolk and the other containing a cholesterol-free fat mixture. The egg yolk supplement provided about 800 mg/day of cholesterol. The cholesterol free fat mixture was similar to the egg yolk supplements in terms of colour, energy, protein (16.7 vs 16.4 g per 100 g of product) and fat content (25.5 vs 26.1 g per 100 g of product, of which saturated fat 10 and 9 g, respectively). Frozen portions of egg yolk and placebo (kindly supplied by SME Research, Caserta, Italy) were given to the patients at the beginning of the study, with the instructions to consume them either fried or scrambled.

Compliance to the diet was checked by 7-day dietary records filled in by subjects at the end of the run-in period and the two experimental periods. At the end of the two periods the patients – who were already familiar with home blood glucose monitoring system – performed a daily blood glucose profile with samples taken in the morning after an overnight fast, before and 2 h after lunch and dinner. At the end of the experimental periods, blood samples were collected after a 12-h fast for the measurement of HbA_{1c}, plasma lipids and lipoproteins.

Methods

Separation of lipoproteins. After an overnight fast, blood samples were taken by vein puncture without stasis. Plasma was isolated by low-speed centrifugation (3000 rev/min) for 10 min and mixed with merthiolate, EDTA disodium salt (final concentration 0.05 %). Major lipoprotein classes (VLDL and LDL) were separated by sequential preparative ultracentrifu-

gation under standard conditions [18]. Plasma HDL cholesterol concentration was determined by the precipitation method [19].

VLDL density gradient ultracentrifugation. A density gradient ultracentrifugation procedure was used to subfractionate VLDL at the end of the two experimental periods [20]. Four ml of plasma adjusted to a density of 1.10 g/ml with solid NaBr (0.1268 g/ml) were transferred to Beckman Ultra Clear centrifuge tubes (14 × 95 mm) (Beckman Instruments, Palo alto, CA, USA) previously coated with a thin layer of polyvinyl alcohol [21]. Three ml of NaBr solution at densities of 1.065, 1.020 and 1.006 g/ml were carefully layered above the plasma samples. These salt solutions were allowed to gravity-feed down the side of wettable centrifuge tubes, using a 22 gauge needle. Three VLDL subfractions of decreasing particle size, arbitrarily designated as 1, 2 and 3, were isolated. Centrifugation was carried out in a Beckman SW 40 Ti rotor at 20 °C on an ultracentrifuge Centrikon T 2060 (Kontron Instruments, Zurich, Switzerland) with operating mode preselection keys set at "vertical on-off". After correction for acceleration and deceleration forces, three consecutive runs were made: [1] 106 min at 40000 rev/min (VLDL1, Svedberg flotation unit [Sf] > 400–175, [2] 80 min) at 40000 rev/min. (VLDL 2, Sf 175–100); [3] 18 h at 37000 rev/min. (VLDL3, Sf 100–20). After each run, each subfraction (1 ml) was carefully aspirated from the top of the tube, which was then refilled with 1 ml of *d* 1.006 g/ml solution before the next spin cycle.

LDL density gradient ultracentrifugation. The LDL subclasses were determined in 9 IDDM patients and 9 control subjects at the end of both experimental periods. LDL1, LDL2 and LDL3 were separated, by density gradient ultracentrifugation in a Beckman SW40 Ti swinging bucket rotor, from the LDL samples obtained by sequential ultracentrifugation using Beckman Ultraclear (14 × 95 mm) tubes. The method was based on the one previously described by Griffin et al. [22] with some modifications [23]. Briefly, the discontinuous NaBr gradient was prepared by layering NaBr solutions from bottom to top, as follows: 0.5 ml $d = 1.190$ g/ml, 2.3 ml sample solution (0.8 ml LDL obtained from sequential ultracentrifugation in 1.5 ml NaBr $d = 1.100$ g/ml), 1.5 ml $d = 1.060$ g/ml, 1.5 ml $d = 1.045$ g/ml, 2.0 ml $d = 1.034$ g/ml, 2.0 ml $d = 1.023$ g/ml, and 1.0 ml $d = 1.019$ g/ml. Tubes were centrifuged in a Beckman L8–70 ultracentrifuge at 40000 rev/min for 24 h at 23 °C and the rotor was allowed to stop without braking.

After centrifugation the tubes were emptied from the top using a Beckman Recovery System, Perfusor V infusion pump (B. Braun, Melsungen, Germany) and Maxidens solvent (Nyegaard & C.A/S, Oslo, Norway). Protein absorbance profiles and densities in the tubes were monitored with an absorbance meter (Pharmacia, Uppsala, Sweden) and with a DMA 46 Digital Density Meter (Anton Paar, Graz, Austria). Three fractions, LDL1 ($d = 1.024$ to 1.031 g/ml), LDL2 ($d = 1.031$ to 1.040 g/ml), and LDL3 ($d = 1.040$ to 1.051 g/ml) with a volume of 1.5 ml each were collected into a fraction collector.

HDL density gradient ultracentrifugation. Five high density lipoprotein subclasses were determined in eight IDDM patients and nine control subjects at the end of placebo and cholesterol intake periods. The density gradient ultracentrifugation method was based on the method described by Groot et al. [24], except that the apoB containing lipoproteins, VLDL, IDL, and LDL were removed from the serum as described previously [25]. The density of the infranatant was increased by adding 1.0 g of dry NaBr and this sample solvent was transferred to the bottom of Beckman Ultraclear 14 × 95 mm tubes. Thereafter, the discontinuous gradient above the sample was prepared by layering NaBr solution in the following order: 1.5 ml $d = 1.250$ g/ml, 6.7 ml d 1.220 g/ml and 2.0 ml distilled water. After centrifugation in a Beckman L8–70 ultracentrifuge with a SW40 Ti swinging bucket rotor (40000 rev/min, 18 h, 20 °C), the tubes were emptied from the top, as described for LDL, and HDL2 b, HDL2 a, HDL3 a, HDL3 b and HDL3 c subclasses were collected in a volume of 1.3 ml each.

Analytical measurements. Total cholesterol, triglyceride and phospholipids were assayed in serum, isolated lipoproteins and lipoprotein subclasses by enzymatic colorimetric methods using commercially available kits (Boehringer-Mannheim, Mannheim, Germany) adequately modified in order to get a high sensitivity also for low concentrations. Quality control of lipid analysis is regularly ensured by the WHO Prague Reference Centre. Proteins were measured in LDL and HDL subclasses by Lowry method [26]. The concentration of each LDL and HDL subclass was calculated by adding together the cholesterol, triglyceride, phospholipid and protein concentrations obtained by analytical measurements. HbA_{1c} was measured by a high pressure liquid chromatography [27]. Apo E phenotype of the subjects was determined by immunoblot assay [28].

Statistical analyses were performed with BMDP statistical software (University of California Press, 1995). Values are expressed as mean ± SD. Differences between placebo and cholesterol supplementation periods were calculated using Wilcoxon's signed rank test. Comparisons of changes in IDDM patient and control groups and of data between the groups were performed by Mann-Whitney non-parametric test.

Results

Dietary cholesterol and plasma lipids. The compliance to the diet, checked by 7-day food records, was good (on the average less than 10 % deviation from the prescribed diet for each patient) and virtually identical during the two experimental periods. All subjects consumed more than 90 % of the cholesterol-rich/placebo supplements. During the placebo and cholesterol intake periods the mean body weight of IDDM patients (68 ± 8 vs 68 ± 8 kg) and control subjects (72 ± 6 vs 72 ± 6 kg) remained the same. The glycaemic control of IDDM patients was similar at the end of the two experimental periods (average daily blood glucose of IDDM patients 7.5 ± 1.2 and 7.8 ± 1.9 mmol/l, respectively).

The concentration of total cholesterol increased significantly during the dietary cholesterol supplementation period compared to the placebo period in both groups. In IDDM patients total plasma cholesterol concentration increased from 4.53 ± 0.85 to 4.82 ± 0.98 mmol/l ($p < 0.05$) and in control subjects from 4.87 ± 0.80 to 5.33 ± 0.56 mmol/l ($p < 0.05$). No differences were observed in the concentration of serum triglycerides in either group between the placebo and cholesterol intake periods (IDDM patients: 0.77 ± 0.22 vs 0.79 ± 0.24 mmol/l, and control subjects

Table 2. Mean lipid concentrations of total VLDL and their subclasses in IDDM patients and control subjects during placebo and cholesterol intake periods

	IDDM patients ($n = 10$)	Control subjects ($n = 11$)
VLDL 1 (mg/dl)		
Placebo	14.9 ± 9.9	22.6 ± 16.1
Cholesterol	15.4 ± 8.4	21.9 ± 16.6
VLDL 2 (mg/dl)		
Placebo	14.7 ± 8.6	22.3 ± 14.8
Cholesterol	15.5 ± 8.5	20.9 ± 16.8
VLDL 3 (mg/dl)		
Placebo	33.1 ± 16.6	30.9 ± 13.9
Cholesterol	31.7 ± 17.9	28.7 ± 13.4
TOTAL VLDL (mg/dl)		
Placebo	65.0 ± 32.7	78.9 ± 42.7
Cholesterol	68.1 ± 32.9	71.7 ± 42.9

Data are mean ± SD

0.90 ± 0.31 vs 0.84 ± 0.31 mmol/l). The LDL cholesterol level increased in all patients but one with an average change of 12 % (from 2.95 ± 0.82 to 3.41 ± 1.03 mmol/l, $p < 0.01$). Similarly, in control subjects LDL cholesterol increased significantly after cholesterol supplementation. The increase was present in 7 of 11 subjects but the average change was only 7 % (from 3.28 ± 0.49 to 3.59 ± 0.64, $p < 0.05$). In IDDM patients plasma HDL cholesterol concentration did not change (1.13 ± 0.23 vs 1.16 ± 0.25 mmol/l), while in control subjects it tended to increased after cholesterol supplementation (from 1.14 ± 0.26 to 1.23 ± 0.27 mmol/l, $p = 0.06$). The different effect of dietary cholesterol on LDL and HDL cholesterol concentrations in the two groups of subjects resulted in a significant 9 % increase in the ratio of LDL/HDL cholesterol only in IDDM patients (from 2.75 ± 0.82 to 3.02 ± 0.98, $p < 0.05$) and in absolutely no change in the control group (from 2.92 ± 0.63 to 2.92 ± 0.51).

Dietary cholesterol and VLDL subclasses. No change in the lipid concentrations of total VLDL and their subfractions was observed during the two experimental periods in either group (Table 2). The percentage composition of VLDL subclasses (VLDL1, VLDL2, VLDL3) also remained unchanged at the end of the two periods of treatment for both groups (data not shown) and, moreover, no difference was observed between IDDM patients and control subjects during the placebo period (Table 2).

Effects of cholesterol intake on LDL subclasses. During the placebo period the concentrations of LDL subclasses were similar in IDDM patients and control subjects (Table 3). However, the percentage content

Table 3. Mean concentrations and compositions of different LDL subclasses in IDDM patients and control subjects during placebo and cholesterol intake periods

Total concentration	mg/dl	Cholesterol (%)	Triglycerides (%)	Phospholipids (%)	Protein (%)
IDDM patients (n = 9)					
LDL 1					
Placebo	55.8 ± 23.8	39.1 ± 1.9^{x}	9.4 ± 1.9	27.9 ± 0.5^{y}	23.6 ± 1.2^{x}
Cholesterol	67.5 ± 24.9^{a}	39.6 ± 2.2^{y}	9.0 ± 2.0	27.7 ± 0.6^{y}	23.7 ± 1.8
LDL 2					
Placebo	123.5 ± 56.9	40.7 ± 1.2	6.7 ± 1.4	27.7 ± 0.5	24.9 ± 0.7
Cholesterol	148.7 ± 56.3^{c}	40.6 ± 1.6^{x}	6.8 ± 1.4	27.7 ± 0.6^{x}	23.9 ± 1.8
LDL 3					
Placebo	68.9 ± 25.9	38.5 ± 1.4^{x}	7.4 ± 1.6	26.8 ± 0.6^{z}	27.3 ± 1.0^{x}
Cholesterol	76.5 ± 29.3	38.3 ± 3.7^{x}	7.0 ± 1.3^{x}	26.4 ± 0.4^{z}	28.3 ± 1.1^{x}
Total LDL					
Placebo	248.4 ± 97.9	39.3 ± 1.2	7.9 ± 1.4	27.5 ± 0.5^{y}	25.3 ± 0.7^{x}
Cholesterol	293.2 ± 107.6^{c}	39.2 ± 2.1^{y}	7.5 ± 1.7	27.9 ± 0.5^{z}	25.4 ± 1.5
Control subjects (n = 9)					
LDL 1					
Placebo	64.4 ± 18.7	41.0 ± 1.9	10.6 ± 1.8	26.0 ± 0.7	22.4 ± 0.5
Cholesterol	70.4 ± 18.5	42.3 ± 1.6^{a}	8.8 ± 1.3^{b}	26.2 ± 1.0	22.7 ± 1.4
LDL 2					
Placebo	133.4 ± 20.0	40.7 ± 2.0	6.9 ± 1.1	27.5 ± 3.1	24.9 ± 1.6
Cholesterol	151.2 ± 22.1	42.0 ± 1.0	5.7 ± 1.0^{a}	26.7 ± 0.9	25.6 ± 0.8
LDL 3					
Placebo	73.8 ± 23.2	40.2 ± 1.2	8.7 ± 1.6	25.2 ± 1.0	25.9 ± 1.5
Cholesterol	79.2 ± 25.2	40.4 ± 1.6	8.4 ± 1.6	24.9 ± 0.9	26.3 ± 0.7
Total LDL					
Placebo	271.6 ± 31.7	40.7 ± 1.6	8.8 ± 0.9	26.2 ± 1.1	24.3 ± 0.9
Cholesterol	300.8 ± 43.9	41.7 ± 1.3^{a}	7.4 ± 0.7^{a}	26.0 ± 0.9	24.9 ± 0.9

Data are mean ± SD. ^{a} $p < 0.05$, ^{b} $p < 0.01$ and ^{c} $p < 0.001$ for differences between periods of placebo and cholesterol intake. ^{x} $p < 0.05$, ^{y} $p < 0.01$ and ^{z} $p < 0.001$ for differences between groups during placebo or cholesterol intake period

Table 4. Mean concentrations and compositions of HDL subclasses in IDDM patients and control subjects during placebo and cholesterol intake periods

Total concentration	mg/dl	Cholesterol (%)	Triglycerides (%)	Phospholipids (%)	Protein (%)
IDDM patients (n = 8)					
HDL2b					
Placebo	22.2 ± 18.6	20.5 ± 4.2	5.4 ± 2.0^{y}	28.4 ± 3.8	45.7 ± 7.1
Cholesterol	21.4 ± 17.5	20.9 ± 4.5	5.6 ± 2.4^{x}	29.3 ± 3.3	44.2 ± 6.7
HDL2a					
Placebo	25.4 ± 18.2	19.4 ± 3.2	5.0 ± 1.7^{x}	29.2 ± 2.8	46.4 ± 2.5
Cholesterol	25.5 ± 19.7	19.4 ± 3.7	4.6 ± 1.9^{x}	29.8 ± 2.4	46.2 ± 1.7
HDL3a					
Placebo	36.6 ± 22.7	17.8 ± 2.6^{x}	3.9 ± 1.4^{x}	26.7 ± 4.1	51.6 ± 2.2
Cholesterol	35.7 ± 19.8	17.6 ± 2.9	3.8 ± 1.4^{x}	27.3 ± 4.2	51.3 ± 1.9
HDL3b					
Placebo	49.2 ± 19.3	16.6 ± 3.0	3.3 ± 1.1^{x}	22.1 ± 5.5	58.0 ± 2.8
Cholesterol	49.3 ± 20.2	16.1 ± 3.2	3.0 ± 1.0^{x}	22.8 ± 5.5	58.1 ± 2.5
HDL3c					
Placebo	50.2 ± 16.3^{x}	13.6 ± 2.6	2.5 ± 0.8^{y}	18.1 ± 4.6	65.8 ± 3.5
Cholesterol	49.9 ± 17.6^{x}	12.9 ± 3.1	2.4 ± 0.7^{y}	17.9 ± 4.9	66.8 ± 4.1
Total HDL					
Placebo	183.5 ± 87.3	17.6 ± 3.0	4.1 ± 1.4^{y}	24.9 ± 3.6	53.4 ± 2.2
Cholesterol	181.9 ± 87.7	17.4 ± 3.5	3.8 ± 1.4^{x}	25.4 ± 3.5	53.4 ± 1.9
Control subjects (n = 9)					
HDL2b					
Placebo	15.2 ± 8.2	20.8 ± 1.2	11.0 ± 3.8	27.0 ± 3.4	41.2 ± 3.5
Cholesterol	17.9 ± 8.6	23.6 ± 3.8^{a}	8.9 ± 2.8^{b}	27.8 ± 2.9	39.7 ± 3.9
HDL2a					
Placebo	20.9 ± 11.7	17.6 ± 1.0	8.2 ± 3.3	28.2 ± 2.3	46.0 ± 2.1
Cholesterol	23.6 ± 11.2^{a}	19.1 ± 2.2^{a}	7.6 ± 2.6	28.1 ± 1.6	45.2 ± 2.5
HDL3a					
Placebo	38.8 ± 12.0	15.3 ± 1.2	6.5 ± 2.3	26.8 ± 1.6	51.4 ± 1.5
Cholesterol	38.9 ± 11.2	17.1 ± 1.9^{a}	6.0 ± 1.9	27.0 ± 2.7	49.9 ± 5.2
HDL3b					
Placebo	54.8 ± 9.8	13.7 ± 1.1	4.6 ± 1.1	23.9 ± 1.9	57.8 ± 2.0
Cholesterol	53.6 ± 8.3	14.2 ± 0.8	4.3 ± 1.0	23.2 ± 1.1	58.3 ± 1.9
HDL3c					
Placebo	67.0 ± 11.4	11.7 ± 0.9	3.9 ± 0.9	19.7 ± 1.5	64.7 ± 1.9
Cholesterol	68.4 ± 10.3	11.8 ± 0.8	3.6 ± 0.7	18.6 ± 1.3	66.0 ± 2.2
Total HDL					
Placebo	190.8 ± 45.0	15.6 ± 0.9	7.3 ± 2.3	25.0 ± 1.9	52.1 ± 2.0
Cholesterol	196.3 ± 40.7	17.2 ± 1.4^{a}	6.0 ± 1.8^{b}	25.0 ± 1.7	51.8 ± 2.5

Data are mean ± SD. ^{a} $p < 0.05$, ^{b} $p < 0.01$ and ^{c} $p < 0.001$ for differences between periods of placebo and cholesterol intake. ^{x} $p < 0.05$, ^{y} $p < 0.01$ and ^{z} $p < 0.001$ for differences between groups during placebo or cholesterol intake period

of cholesterol in LDL1 (39.1 %) and LDL3 (38.5 %) was significantly lower in IDDM patients than in control subjects (41.0 and 40.2 %, respectively, $p < 0.05$, Table 3). Conversely, the percentage content of phospholipid in LDL1 (27.9 %), LDL3 (26.8 %) and total LDL (27.5 %) was significantly higher in IDDM patients than in control subjects (26.0, 25.2 and 26.2 %, respectively, $p < 0.01$). In addition, the protein content in LDL1 (23.6 %), LDL3 (27.3 %) and total LDL (25.3 %) was significantly higher in IDDM patients than in control subjects during the placebo period (22.4, 25.9 and 24.3 %, respectively, $p < 0.05$).

During the cholesterol intake period the mean concentration of LDL1, LDL2 and LDL3 subclasses in IDDM patients showed a significant increase of 21.0 % ($p < 0.05$), 20.4 % ($p < 0.001$) and 11.1 % ($p < 0.05$), respectively, resulting in an 18.0 % increase in mean total LDL mass ($p < 0.001$, Table 3). However, the percentage distribution of different LDL subclasses between the cholesterol and the placebo periods did not vary. In contrast, in the control subjects the changes in the concentrations of LDL subclasses during cholesterol supplementation were less pronounced and not statistically significant compared to the placebo period, resulting in a 10.7 % increase in total LDL concentration (not statistically significant) (Table 3). Although the increments of all the LDL subclasses induced by cholesterol supplementation were clearly more pronounced in IDDM than in control subjects, none of the differences between the groups reached the conventional level of statistical signifi-

cance, probably as a consequence of the small sample size.

Moreover, in IDDM patients the cholesterol intake did not affect the composition of either total LDL or LDL subclasses. In contrast, multiple changes in the composition of LDL subclasses were observed after cholesterol supplementation in control subjects (Table 3). In fact, in this group the percentage content of cholesterol increased significantly in LDL1 (from 41.0 to 42.3 %, $p < 0.05$), and a similar trend was observed in LDL2, the major LDL subclass. Overall, cholesterol supplementation induced an increase in the cholesterol content of total LDL (from 40.7 to 41.7 %, $p < 0.05$). On the contrary, the percentage content of Tg decreased in LDL1 (from 10.6 to 8.8 %, $p < 0.05$), LDL2 (from 6.9 to 5.7 %, $p < 0.05$) and in total LDL (from 8.8 to 7.4 %, $p < 0.05$) during the cholesterol supplementation as compared to the placebo period.

Effects of cholesterol intake on HDL subclasses. During the placebo period, the mean concentration of small HDL particles, HDL3 c, was 25.1 % lower ($p < 0.05$) and the mean concentration of the large HDL particles, HDL2 b, tended to be higher in IDDM patients than in control subjects (Table 4). In addition, the cholesterol content of the HDL3 a and HDL3 b subclasses was higher in IDDM patients than in control subjects (17.8 vs 15.3 % and 16.6 vs 13.7 %, respectively, $p < 0.05$). Conversely, the relative content of triglycerides in all HDL subclasses as well as in total HDL was significantly lower in IDDM patients compared to control subjects.

In IDDM patients cholesterol supplementation did not have any significant effect on the concentration of HDL subclasses or total HDL mass (Table 4). Neither did cholesterol intake in IDDM patients affect the composition of HDL subclasses (Table 4). In contrast, in control subjects cholesterol intake increased the mean concentration of HDL2 a by 12.2.% ($p < 0.05$) and this change was significantly different from that obtained in IDDM patients ($p < 0.05$). A similar, but non-significant increase in the mean concentration of HDL2 b was observed. In addition, always in control subjects the relative content of cholesterol in HDL2 b, HDL2 a and HDL3 a subclasses increased (from 20.8 to 23.6 %, from 17.6 to 19.1 % and from 15.3 to 17.1 %, respectively, $p < 0.05$) resulting in a significant increase in the relative content of cholesterol in total HDL (15.6 vs 17.2 % $p < 0.05$). On the other hand, the relative content of triglycerides in all HDL subclasses in the control subjects tended to decrease with the cholesterol supplementation compared to placebo. The change was significant for the HDL2 b subclass (11.0 vs 8.9 %, $p < 0.01$) and for total HDL (7.3 vs 6.0 %, $p < 0.01$, Table 4).

Discussion

Four major considerations emerge from this study. First, a dietary cholesterol supplementation of 800 mg/day (high, but easily reached in the diet of western countries) significantly increased plasma LDL cholesterol, both in IDDM patients (by 12 %) and control subjects (by 7 %). Second, the increase in LDL cholesterol concentration in IDDM patients was exclusively a consequence of the increased concentration of LDL particles, while in control subjects it was a consequence of a change in the composition of LDL particles (enriched with cholesterol) and only partially was it due to a higher concentration of LDL particles. Third, no changes in the density distribution of LDL were noticed in either group during cholesterol supplementation. Fourth, in IDDM patients dietary cholesterol did not induce any changes in either serum total HDL cholesterol level or in the concentration and composition of HDL subclasses. However, in control subjects, the cholesterol supplementation induced an increase in total serum HDL cholesterol levels in parallel with changes in the distribution of HDL subclasses (the concentration of HDL2 b increased by 15 %, NS, and that of HDL2 a by 12.2 %, $p < 0.05$) and in the composition of HDL particles (cholesterol content increasing from 15.8 to 17.4 %). Only for HDL2 a subclasses were the effects induced by dietary cholesterol significantly different in IDDM patients compared to the control group. Previous studies have reported an increase in HDL cholesterol [1, 7, 29] and apolipoprotein A-I (apo A-I) levels [30] in non-diabetic normolipidaemic and hyperlipidaemic individuals after dietary cholesterol intake. It has also been established that a diet rich in fat and cholesterol increases not only LDL cholesterol levels, but also plasma apoB levels in normal subjects [6, 31]. To the best of our knowledge no data is available on the effects of dietary cholesterol on either plasma lipoproteins or their subclasses in IDDM patients.

HDL is a heterogeneous spectrum of particles differing in size, density, and apolipoprotein content [32]. HDL metabolism is complex and regulated by its apolipoprotein A-I and A-II synthesis and catabolism [33], lipoprotein and hepatic lipase activities [34], plasma cholesteryl ester transfer protein (CETP) activity [35], and plasma lecithin cholesteryl acyltransferase activity [36]. CETP has been reported to increase, either in terms of activity or mass, after cholesterol rich diets in both animals and men [37]. Martin et al. [37] found that the increase in HDL cholesterol in response to cholesterol feeding varies according to apoE genotypes. In particular, individuals with apoE3/2 and apoE3/3 genotypes showed no increase in HDL cholesterol levels but a significant increase in CETP, while in those with apoE4/3 genotype there was a significant increase in HDL but no

change in CETP. However, Marshall et al. [38] have shown that apoE polymorphism plays only a minor role in the association between dietary factors and serum lipids. In the present study the distribution of apoE phenotypes was similar in our IDDM patients in comparison to control subjects excluding the possibility that the different effects of cholesterol supplementation in the two groups were associated with apoE polymorphism. Moreover, the increase in the concentration of HDL2 a subclass, albeit small, occurred in all the control subjects, independently of their apoE phenotype. Our finding is in line with the results of Martin et al. [37] that dietary cholesterol (in amounts comparable to those used in the present study) primarily increases HDL2 cholesterol.

The lack of any effect of cholesterol supplementation on HDL in IDDM patients is a matter of concern. One explanation could be the presence of an overstimulation of CETP consequent to subcutaneous insulin therapy [39, 40], which could prevent the increase in concentration of HDL particles.

Dietary cholesterol did not induce any change in VLDL concentration or in the composition and percentage distribution of their subclasses in either group of subjects. One of the main effects of dietary cholesterol in animals is an increase in the concentration of small size beta VLDL [11, 12]. This was not observed in the present study probably because, among other causes, in animal studies the amount of cholesterol/kg body weight is much higher than what was used in this study. Unfortunately data in man are far too limited and also contradictory. Nestel et al. [41] have found an increase in the concentration of small VLDL, but the amount of dietary cholesterol used in that study was much higher (1700 mg/day). On the other hand, Miettinen et al. [42] have studied the composition of VLDL and IDL after cholesterol supplementation (the same amount used by us) in a free-living population but they did not find any significant change in VLDL composition.

In conclusion, our study clearly shows that dietary cholesterol supplementation (800 mg/day) does not induce any increase in the concentration of beta-VLDL (the smallest and most atherogenic VLDL particles) even in IDDM patients, who are already characterized by an abnormal high concentration of these particles. On the other hand, dietary cholesterol supplementation significantly increases the concentration of most atherogenic lipoproteins (LDL) in IDDM patients and these effects tend to be more homogeneous in IDDM patients than in control subjects. Moreover, it is important to underline that, contrary to what happened in the control subjects, cholesterol supplementation in IDDM patients was not accompanied by any positive effect on the antiatherogenic HDL2 a particles [43]. This induces a significant increase in the LDL/HDL cholesterol ratio, which is considered an index of potential lipoprotein atherogenicity, only in IDDM patients. Therefore, recognizing the high susceptibility of IDDM patients to atherosclerosis, special attention must be paid to the control of dietary cholesterol intake in the presence of this pathology, whereas this aspect is most often neglected in clinical practice, where the emphasis in dietary counselling is focused on the amount and quality of carbohydrates.

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