Basal and Postprandial Serum-Promoted Cholesterol Efflux in Normolipidemic Subjects: Importance of Fat Mass Distribution

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Excess of adipose tissue may affect the reverse cholesterol transport mediated by high-density lipoprotein (HDL). Impairments in this system may be one possible factor favoring atherosclerosis development in obesity. To investigate if gender and regional fat mass distribution independently influence reverse cholesterol transport (RCT), we studied in vitro the capacity of serum to promote the cell cholesterol efflux. Measurements were performed both in the fasting state and in the postprandial state, a setting known to stimulate cholesterol transport and altered in obesity. Thirteen obese women with an android phenotype, waist-to-hip ratio (WHR): 0.98 to 0.85 and 51 normal-weight subjects: 25 women and 26 men, with a similar WHR range: 0.96 to 0.67, were recruited. All the participants were normolipoproteinemic in the fasting state and were given an oral fat load. Blood samples were taken before giving the oral fat load and after every 2 hours. The measurements of the ability of serum to promote cholesterol efflux from cells were performed using ^{3}H-cholesterol labeled Fu5AH hepatoma cells in the fasting state 6 and 8 hours after the lipid rich meal. Incremental serum triglyceride (TG), area under the curve (iAUC) and AUC of retinyl palmitate (RP) for the obese women and nonobese subjects were similar. Basal cholesterol efflux was reduced in obese women compared with normal-weight women (26.75% ± 3.1% *v* 30.81% ± 4.2%, *P* = .004). However, the magnitude of cholesterol efflux promoted by whole serum increased similarly in all the groups. In the subjects with similar WHR, no gender difference was observed in the postprandial TG response and in the first step of RCT. Multivariate regression analyses indicated that plasma HDL-cholesterol (HDL-C) concentration is the best predictor of cholesterol efflux in the fasting state with an independent mild additive effect of WHR. Conversely, postprandial efflux appeared to be mostly related to the WHR with a mild additive effect of HDL-C. Our results indicate that alterations in the first step of RCT can occur in normolipidemic obese subjects and are tightly associated with the abdominal distribution of fat mass. Android obesity in women brings them to the level of men with respect to RCT.

AFFLUENT POPULATIONS, prone to overeating and overweight, are particularly exposed to atherosclerosis. Obesity is a frequent correlate of accession to affluence and an independent risk factor for coronary heart disease, even in the absence of dyslipidemia or clinically impaired glucose tolerance.[1] The causal relationship between obesity and atherosclerosis remains, however, unclear. One of the main mechanisms preventing the development of atherosclerosis is now understood to be the reverse cholesterol transport (RCT),[2,3] which is mediated by high-density lipoprotein (HDL).[4,5] In vitro experiments have shown that it is highly dependent upon HDL concentrations in plasma[6-8] and upon their relative enrichment with phospholipids.[9,10] HDL are often qualitatively affected in obese subjects,[11,12] even when their concentrations are not diminished, and it has been suggested that adipose tissue might play a direct role in these alterations.[13] Abnormalities in RCT are therefore one possible cause favoring atherosclerous development in early stages of obesity.[14] Moreover, obesity may favor atherosclerosis through other mechanisms. Obese patients have been claimed to display delayed lipid clearance related to decreased lipoprotein lipase activity.[15,16] Disturbances in postprandial metabolism are likely to have a significant impact because of the atherogenic properties of remnant lipoproteins.[17,18] However, postprandial metabolism may also act in opposite fashion by stimulating RCT, presumably through an enrichment of HDL with residues of lipolysis and through the transfer of cholesterol to remnant chylomicron particles. Consequently, the presence of excess adipose tissue may, in principle, affect RCT in either or both of these 2 ways. For instance, a lack of stimulation of cholesterol efflux in the postprandial state was observed in type 2 diabetic patients who have abdominal fat distribution.[19] From a qualitative viewpoint, 2 different types of obesity have to be considered. While there is little doubt that android obesity is a risk factor,[20,21] gynoid obesity has not objectively proved to be one.

The present work was designed to explore if alterations in cholesterol efflux, both in the fasting and postprandial state, occur in obese females without changes in plasma lipoprotein concentration and to assess the respective influence of fat mass distribution and overweight.

MATERIALS AND METHODS

Patients

Sixty-four subjects (26 men and 38 women) were included in this study. All women were premenopausal, and none were under hormone replacement therapy or contraceptive medication. All subjects displayed normal glucose tolerance (fasting glycemia < 6.1 mmol/L <

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7.8 mmol/L 2 hours after an oral glucose tolerance test) and fasting lipid levels (triglyceride [TG] < 1.07 mmol/L, HDL-cholesterol [HDL-C] > 0.90 mmol/L for men, >1.03 mmol/L for women and low-density lipoprotein-cholesterol [LDL-C] < 4.13 mmol/L). Fifty-one normal-weight subjects (26 men and 25 women) were selected according to the following criteria: body mass index (BMI) 18 to 25 kg/m^2. Among the women, 13 were obese as defined by a BMI ≥ 30 kg/m^2.[22] It was verified that body weight had remained stable (± 2%) over the last 3 months. None of the patients were morbidly obese (maximum BMI ≤ 37 kg/m^2). Abdominal (android) fat distribution was defined by a waist-to-hip ratio (WHR) 0.85 for women and 0.95 for men.

A 7-day food dietary record was used to determine usual food intake and to ensure homogeneity; a weight maintenance diet was prescribed during 7 days prior to the test. The diet contained 50% carbohydrate, 33% fat (polyinsatured to saturated ratio: P/S = 0.80) and 17% protein. Three days prior to the test, the subjects were requested to refrain from strenuous exercise and to abstain totally from drinking alcohol. The local Ethical Committee of the Nancy University Hospital approved the project, and all subjects gave their informed consent.

Oral Fat Load

On the evening preceding the test, the subjects were given a standardized 700-kcal meal (31% fat, 19% protein, and 50% carbohydrate) at 8:00 PM. After overnight fasting, they ingested the fat load in less than 15 minutes at 8:00 AM. They were requested to refrain from eating or drinking and to remain essentially in a supine position until the end of the experiment at 4:00 PM.

The oral fat load (Laboratories Pierre Fabre Santé, Castres, France) has been previously described in detail[23] and accounted for a total of 890 kcal (85% lipid, 13% carbohydrate, and 2% protein) with a P/S ratio of 0.43. It contained 88 mg cholesterol, and vitamin A (100,000 UI aqueous solution) was added to label intestinally derived particles.

Biochemical Determinations

Blood samples were drawn on EDTA and on a dry tube from a venous catheter before and several times (2, 4, 6, and 8 hours) after the fat load. Plasma was used fresh for preparation of chylomicrons and biochemical determinations. Serum was frozen until the cholesterol efflux assays.

Lipid Determinations

Chylomicrons were removed by ultracentrifugation (30 minutes at 78,000 × *g*, 15°C) of 5.0 mL of serum layered under 7.0 mL of a salt solution (density = 1.006 g/mL) in a SW 41 Ti rotor, Gagny, France). HDL-C, HDL_2-C, and HDL_3-C were measured after precipitation of very–low-density lipoprotein (VLDL) and LDL from chylomicron-free plasma using the dextran sulfate procedure.[24] Cholesterol and TG were measured by enzymatic assays (Boehringer Mannheim, Meylan France). Retinyl palmitate (RP) was assayed in total serum by reverse-phase high-performance liquid chromatography.[25]

Evaluation of Insulin Resistance

Plasma glucose was determined enzymatically (BioMérieux PAP250, Marcy-l'Etoile-France). Total plasma insulin concentration was determined by immune-enzymatic assay (Insulin IMX; Abbott Laboratoires, Tokyo, Japan). Cross-reactivity with proinsulin was below 0.05%. Insulin sensitivity was assessed from blood samples collected 30, 20, and 10 minutes prior to the fat load on the basis of the Homeostasis Model Assessment (HOMA) validated by Matthews et al[26]: (insulin mUI/L × plasma glucose mmol/L)/22.5.

Cholesterol Efflux

Measurements of the ability of serum to promote cholesterol efflux from cells were performed essentially as described by Rothblat and Phillips[6] using Fu5AH cells grown in Eagles' minimum essential medium (MEM) supplemented with 5% bovine calf serum (Boehringer) and added with penicillin-streptomycin. For experiments, cells were seeded in 6-well plates at a density of 45,000 to 50,000 cells/well and grown in MEM with serum for 2 days. Radiolabeled cholesterol (1, 2- ^{3}H cholesterol, TRK 330, Amersham, Orsay, France) was added to the cells: a tracer amount was first mixed into 25% calf serum in MEM and then diluted with MEM to a 5% final serum concentration. The cells were grown in the presence of radiolabel for 2 more days to obtain confluent monolayers and to allow for radiolabeled cholesterol to distribute into all cellular pools. The labeling medium was finally replaced after 18 to 20 hours with MEM containing 0.5% bovine serum albumin to further ensure that cholesterol pools were equilibrated. The capacity of serum to promote efflux from the labeled cells was assayed in triplicate by adding individual samples of the patients' serum to the serum-free medium to a final concentration of 5% and by quantifying the amount of radiolabeled cellular cholesterol released during 4 hours. At the end of the efflux period, the medium was collected into ice-chilled tubes and centrifuged in the cold for 5 minutes at 2,000 rpm to remove cells. Labeled cholesterol release was then measured in an aliquot of the medium by standard liquid scintillation counting. Cells were washed with phosphate-buffered saline (PBS). Lipids were extracted by overnight incubation in isopropanol at room temperature, and radioactivity was quantified in an aliquot of the extract. Fractional efflux was calculated by dividing the radioactivity released into the medium by the total label in each well (medium+cells) and was expressed as the percent of total label released in 4 hours. Reproducibility was verified by inclusion of a standard pool of human serum in each efflux experiment. The interassay and intra-assay coefficients of variation were 6.5%, and 5.0%, respectively. Measurement of efflux at 0, 2, 4, 6, and 8 hours in a subset of 10 subjects showed that the maximal efflux was observed 6 to 8 hours after the lipid rich-meal (P = .004 *v* time 0 hour). Consequently, experimental measurements were performed at 3 times only: time 0, 6, and 8 hours.

Statistics

StatView 4.5 software (Abacus Concepts, StatView V; Brain Power, Calabasas, CA) was used for all calculations. Values are given as means ±SD in the text and tables, as means ±SE in figures. Repeated-measures analysis of variance (ANOVA) were performed to assess the effect of postprandial times on postprandial TG and RP concentrations and on percent of cholesterol efflux. When ANOVA was significant, means were compared between groups by Student's unpaired *t* test. Areas under the curves (AUC) were calculated using the trapezoid rule after subtracting the basal value (time 0) when incremental areas (iAUC) were desired.[27] Correlation coefficients were calculated assuming linear relationships between variables. The level of significance was $P \leq .05$.

RESULTS

Clinical Parameters and Lipid Concentrations

Clinical profiles and serum concentrations of lipids in the obese and nonobese subjects are shown in Table 1. Both glycemia and insulin were significantly higher in obese women than in the other groups. In addition to elevated BMI, obese women were characterized by a significant increase of their WHR and in HOMA values ($P < .0001$).

LDL-C, as well as HDL-C concentrations, were similar in all groups of subjects. Fasting TG concentrations, albeit remaining

Table 1. Clinical Profiles and Concentrations of Serum Lipids in Obese and Nonobese Subjects

	Obese Women	Nonobese Women	Nonobese Men	*P*†	*P*‡
No.	13	25	26		
Age (yr)	39.0 ± 6.70	34.8 ± 9.12	30.0 ± 9.90	NS	0.005
BMI (kg/m^2)	33.9 ± 3.98	21.6 ± 2.44	22.0 ± 1.92	.0001	.0001
WHR	0.91 ± 0.04	0.77 ± 0.06	0.86 ± 0.05	.0001	.01
Insulin (mUI/L)	11.35 ± 4.20	5.41 ± 2.29	5.11 ± 1.83	.0001	.0001
Glycemia (mmol/L)	5.48 ± 0.60	4.76 ± 0.41	4.96 ± 0.49	.0001	.006
HOMA*	2.82 ± 1.23	1.16 ± 0.53	1.12 ± 0.44	.0001	.0001
TG (mmol/L)	0.93 ± 0.20	0.76 ± 0.32	0.71 ± 0.23	NS	.007
LDL-C (mmol/L)	3.14 ± 0.44	3.45 ± 0.63	2.82 ± 0.80	NS	NS
HDL-C (mmol/L)	1.08 ± 0.28	1.25 ± 0.21	1.24 ± 0.26	NS	NS
HDL_2-C (mmol/L)	0.33 ± 0.16	0.56 ± 0.11	0.46 ± 0.23	.0003	NS
HDL_3-C (mmol/L)	0.74 ± 0.14	0.69 ± 0.15	0.79 ± 0.15	NS	NS

Abbreviation: NS, not significant.

*HOMA: (insulin mUI/L × plasma glucose mmol/L)/22.5

†Statistical difference in obese women *v* nonobese women.

‡Statistical difference in obese women *v* nonobese men.

in the normal range, were moderately increased in obese women. After a standardized lipid-rich meal, postprandial TG increased similarly in all 3 groups (Fig 1A). After 8 hours, TG concentrations were slightly more elevated in obese women than in the other groups due to delayed kinetics. However, these differences was not sufficient to elicit any significant differences in iAUC under the TG curve (insert Fig 1A). Likewise, no significant differences were observed in AUC under the curves. Similarly, the profiles of postprandial RP concentrations were comparable in the 3 groups (Fig 1B).

Cholesterol Efflux

Figure 2 shows the percentage of efflux of (^{3}H) cholesterol from Fu5AH cultured cells induced by fasting and postprandial serum (time 6 and 8 hours after the lipid-rich meal) obtained from obese women, nonobese women, and men.

Fasting serum capacity of obese women to induce cholesterol efflux was reduced by comparison to that of nonobese women ($P = .004$), reaching values even lower than those of men ($P = .14$). After a lipid-rich meal, the serum-promoted efflux of cholesterol increased significantly in the 3 groups of subjects. However, the magnitude of increase with respect to fasting values was similar in all groups.

Correlations Between Cholesterol Efflux and Obesity or Lipid Parameters

No significant relationship was apparent between basal efflux and LDL-C or TG concentrations (respectively, $r =$

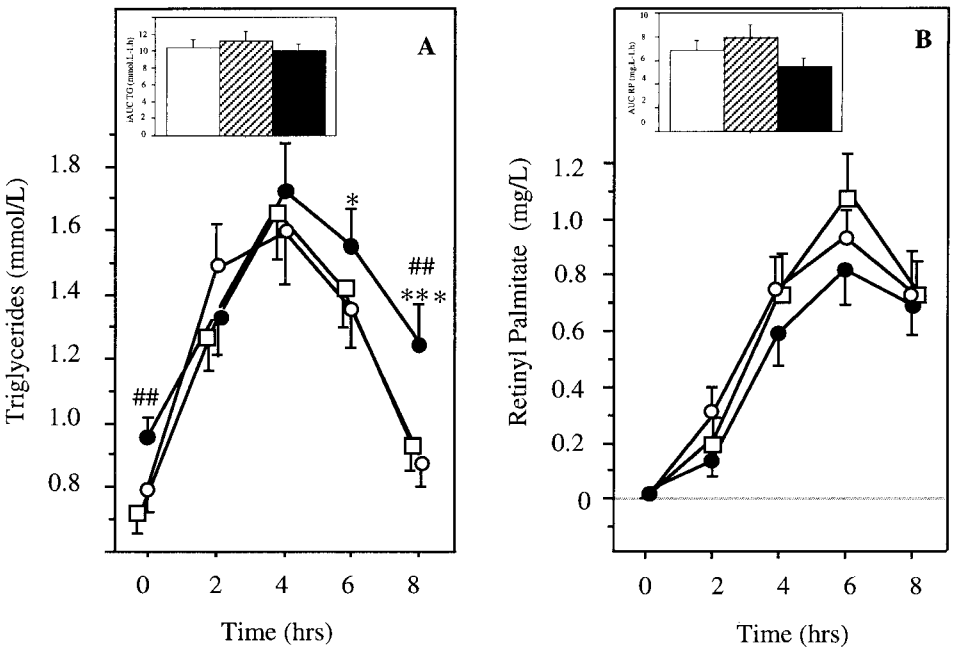

Fig 1. Plasma concentrations of TG and retinyl palmitate after ingestion of a lipid-rich meal, in nonobese women (○) or men (□) and obese women (●). The insets show the increments of the areas under the curves (iAUC, mmol/L. h-1) in nonobese women (○) or men (▨) and obese women (●). ## *P* < .01 *v* nonobese men. * *P* < .001 *v* nonobese women.**

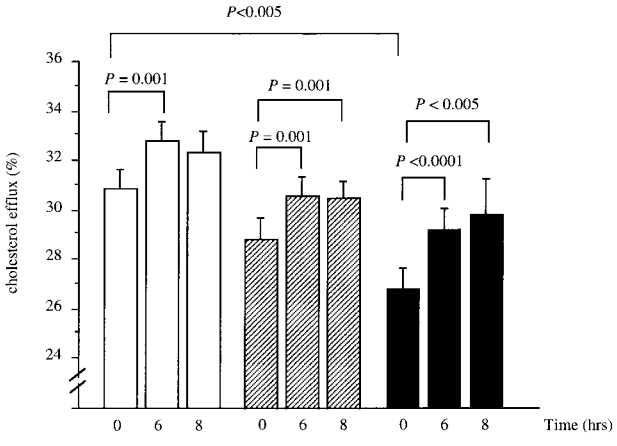

Fig 2. Fasting and postprandial serum-promoted efflux of ^{3}H-labeled cholesterol from Fu5AH cells in nonobese women (○) or men (▨) and obese women (●).

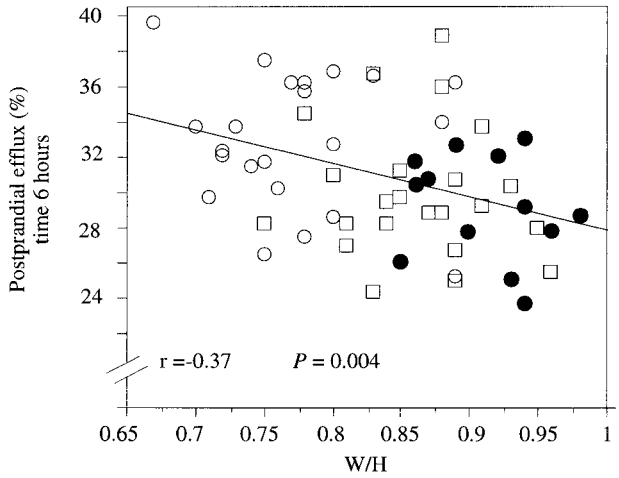

Fig 4. Correlations between serum-promoted efflux of ^{3}H-labeled cholesterol and the WHR in nonobese women (○) or men (□) and obese women (●) 6 hours after ingestion of a lipid-rich meal.

−.191, $P = .24$; $r = .135$, $P = .32$). In the fasting state, cholesterol efflux correlated with total HDL-C concentration ($r = .421$, $P < .005$) (Fig 3). This correlation remained valid with HDL_2-C ($r = .433$, $P < .002$), but not with HDL_3-C (not shown). The WHR, a criterion of body fat distribution, exhibited a strong correlation in the postprandial state ($r = -.37$, $P = .004$) (Fig 4). The correlation was even stronger when women were considered alone ($r = -.444$, $P = .006$). Similar, but milder, correlation was observed in the fasting state ($r = -.236$, $P = .06$). Only a trend toward a negative correlation between basal efflux and BMI was found ($r = -.282$, $P = .03$). Cholesterol efflux was found to correlate with the level of insulin resistance, as estimated from HOMA only in men, during the postprandial state (time [t] 6 hours: $r = -.439$, $P = .04$ and t 8 hours: $r = -.518$, $P = .01$).

Table 2 shows the stepwise regression analysis in the total population of the relationship between basal efflux (time = 0 hour) or postprandial efflux and independent variables. Similar results were obtained for the 6- and the 8-hour time points. For sake of clarity, only the results obtained at 6 hours are shown. The basal efflux was mostly correlated with HDL-C concentrations, which predicted 17.7% of the variation in the capacity of serum to promote cholesterol efflux. In addition, WHR led to a model accounting for 23.3% of the variability in basal efflux. No independent additional effect of sex, BMI, and HOMA was observed. Conversely, postprandial efflux was correlated mostly with WHR with an additive mild effect HDL-C; again no independent additional effect of sex, BMI, or HOMA was observed.

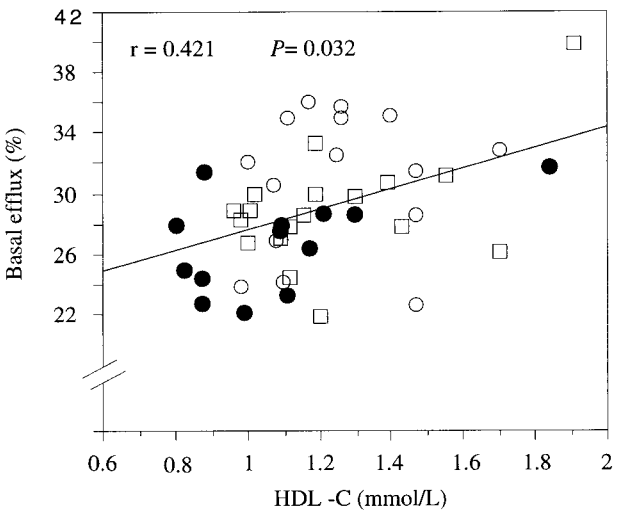

Fig 3. Correlation between basal efflux of ^{3}H-labeled cholesterol and plasma concentration of HDL-C in nonobese women (○) or men (□) and obese women (○).

DISCUSSION

RCT mediated by HDL, is now understood to be a major factor of protection against atherosclerosis. In agreement, the first step, the free cholesterol efflux measured in vitro, was found significantly lower in individuals with a history of cardiovascular ischemic disease.[28] However, no prospective data

Table 2. Stepwise Regression Analysis of the Relationship Between Basal Efflux (0 hour) and Postprandial Efflux (6 hours) and Independent Variables

Dependent Variable	Independent Variables	R^2%	*P* Value
Basal efflux	HDL-C	17.7	.004
	W/H	23.3	.05
	BMI	-	NS
	HOMA*	-	NS
Postprandial efflux (6 hours)	W/H	19	.003
	HDL-C	25.3	.05
	BMI	-	NS
	HOMA*	-	NS

Abbreviation: NS, not significant.

*HOMA values were normalized by logarithmic transformation.

showing that a defect in RCT may influence the atherogenesis are available. One way to indirectly approach this problem is to compare populations known to differ in their relative risk of cardiovascular heart disease (CHD), such as men and women, or lean and obese subjects and to ascertain whether alterations in HDL function can be observed independently of low HDL-C. To investigate as much as possible in this study the direct effects of adiposity on serum ability to promote cholesterol efflux, we have dealt with clinically normal lean and obese women devoid of metabolic or cardiovascular complications. To compare subjects with a similar range of WHR and lipid profile, the control group included normolipidemic nonobese men. All were normolipidemic without any impairment in the clearance of TG or chylomicron remnants as judged from the postprandial profiles of plasma TG and retinyl palmitate (Fig 1A and B). The data confirm that the basal fasting capacity of serum to promote cholesterol efflux from Fu5AH cells is significantly lower in obese women than in nonobese subjects. Sasahara et al[14] have also compared a smaller number of fasted obese and normal weight subjects for the capacity of their sera to promote efflux of cholesterol from fibroblasts. They have concluded, in agreement with our data, to a defect of efflux in obese subjects that they related to BMI by way of lower HDL concentrations. In agreement with their findings and with those of De la Llera Moya et al,[8] we found that basal efflux displayed a strong positive correlation with HDL-C. No other lipid parameters, whether in the basal or postprandial state, were correlated to cholesterol efflux. Besides HDL-C, efflux appeared to be related to obesity. Rather than BMI, WHR was the main additional factor influencing efflux. Multivariate analysis suggested that body fat distribution might have on RCT an additional effect independent of HDL-C concentrations, particularly in the postprandial state (Table 2). These findings obtained in normolipidemic subjects extend those from Couillard et al[29] who recently showed that visceral adipose tissue accumulation was tightly associated with the postprandial TG response after fat load independently of gender. In our group of normolipidemic subjects, differences in free cholesterol efflux were independent of gender, but were mostly modulated by WHR. WHR characterizes the degree of truncal adiposity and is often claimed to be more directly related to insulin resistance and cardiovascular risk than BMI.[29] This is particularly true in women for whom excessive BMI can be due either to gluteal (gynoid) obesity, which has little impact on insulin resistance and cardiovascular risk, or to truncal (android) obesity, which is a risk factor to the same extent as in men.[21] Thus, obesity does not only act on RCT by way of its accompanying often low concentrations of HDL. The effect may bear on the composition of HDL; accordingly, we found that large HDL_2 and not smaller HDL_3 particles were correlated with cholesterol efflux. Moreover, the phospholipid content in HDL particle, which is altered in the insulin-resistant state, could modify the increase in RCT seen in the postprandial state.[30] Adipose tissue is a source of lipid transfer proteins: cholesteryl ester transfer protein (CETP) and plasma phospholipid transfer protein (PLTP). A differential level of expression of both genes was reported according to the adipose tissue site.[31,32] These proteins are capable of altering the composition of HDL by their interaction either in the circulation or even in the vicinity of adipocytes.[33] Additionally, it can also be the source of some circulating factor(s) unrelated to HDL susceptible to influence RCT.

The process of RCT is not uniform during the course of the day. An increase in the capacity of serum to promote cholesterol efflux has been repeatedly reported in the postprandial state.[9,10] It was considered that this increase was due to an increment in HDL-PL transferred from chylomicron upon their lipolysis.[34,35] Our data confirm, in obese subjects, that when TG lipolysis is efficient, there is no alteration of the increase in the efflux capacity during the postprandial state (iAUC of efflux women: obese 12.8 ± 5.87 *v* nonobese 9.51 ± 13.44, $P = .7$ and *v* iAUC of efflux men: 10.62 ± 13.62, $P = .4$) even in obese subjects despite a slightly lower basal level. Decreased serum-promoted efflux has been described in CHD[28,36] and in diabetic[19,37] patients compared with normal controls. We have further observed in healthy subjects without basal or postprandial hyperlipidemia a wide range of efflux potential correlating independently with HDL concentrations and an index of fat distribution. This shows the measure of cholesterol efflux to be a sensitive and integrative index, which might show prognostic value. Our results also show that android obesity in women brings them to the level of men with respect to cholesterol efflux, a fact that has been difficult to ascertain on the basis of individual classical risk factors. The use of a continuous integrative surrogate variable, such as capacity of serum for cholesterol efflux, may prove useful in this respect.

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