Arteriosclerosis, Thrombosis, and Vascular Biology

American
Heart
Association®

Role of SN1 Lipases on Plasma Lipids in Metabolic Syndrome and Obesity

Verónica Miksztowicz, Laura Schreier, Mary McCoy, Diego Lucero, Eduardo Fassio, Jeffrey Billheimer, Daniel J. Rader and Gabriela Berg

The online version of this article, along with updated information and services, is located on the
World Wide Web at:
http://atvb.ahajournals.org/content/34/3/669

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2014/01/23/ATVBAHA.113.303027.DC1.html

Role of SN1 Lipases on Plasma Lipids in Metabolic Syndrome and Obesity

Verónica Miksztowicz, Laura Schreier, Mary McCoy, Diego Lucero, Eduardo Fassio,
Jeffrey Billheimer, Daniel J. Rader, Gabriela Berg

Objective—To assess the phospholipase activity of endothelial (EL) and hepatic lipase (HL) in postheparin plasma of subjects with metabolic syndrome (MS)/obesity and their relationship with atherogenic and antiatherogenic lipoproteins. Additionally, to evaluate lipoprotein lipase (LPL) and HL activity as triglyceride (TG)-hydrolyses to complete the analyses of SN1 lipolytic enzymes in the same patient.

Approach and Results—Plasma EL, HL, and LPL activities were evaluated in 59 patients with MS and 36 controls. A trend toward higher EL activity was observed in MS. EL activity was increased in obese compared with normal weight group (*P*=0.009) and was negatively associated with high-density lipoprotein–cholesterol (*P*=0.014 and *P*=0.005) and apolipoprotein A-I (*P*=0.045 and *P*=0.001) in control and MS group, respectively. HL activity, as TG-hydrolase, was increased in MS (*P*=0.025) as well as in obese group (*P*=0.017); directly correlated with low-density lipoprotein–cholesterol (*P*=0.005) and apolipoprotein B (*P*=0.003) and negatively with high-density lipoprotein–cholesterol (*P*=0.021) in control group. LPL was decreased in MS (*P*<0.001) as well as in overweight and obese compared with normal weight group (*P*=0.015 and *P*=0.004, respectively); inversely correlated %TG-very low-density lipoproteins (*P*=0.04) and TG/apolipoprotein B index (*P*=0.013) in control group. These associations were not found in MS.

Conclusions—We describe for the first time EL and HL activity as phospholipases in MS/obesity, being both responsible for high-density lipoprotein catabolism. Our results elucidate part of the remaining controversies about SN1 lipases activity in MS and different grades of obesity. The impact of insulin resistance on the activity of the 3 enzymes determines the lipoprotein alterations observed in these states. (***Arterioscler Thromb Vasc Biol***. 2014;34:669-675.)

Key Words: hepatic lipase, human ■ LIPG protein, human ■ lipoprotein lipase ■ metabolic syndrome X
■ obesity ■ phospholipases

Lipoprotein lipase (LPL), hepatic lipase (HL), and endothelial lipase (EL) constitute a family of lipases involved in lipoprotein metabolism. These enzymes share a similar sequence structure at the genetic and protein level, thus indicating a common ancestral origin. However, they are expressed in different tissues and act on different lipoprotein substrates, indicating that they may have evolved to specific roles.[1] The 3 proteins are heparin-binding lipases anchored to the endothelial surface and mediate the hydrolysis of triglycerides (TG) and phospholipids (PL) at the SN1 position within circulating lipoproteins. Lipid hydrolysis results in structural changes within the lipoprotein species, which affects their removal from the plasma and releases fatty acids to be taken up by tissues. Although the 3 lipases have both TG and PL lipase activity, LPL is predominantly a TG-lipase, EL principally hydrolyses PL, and HL has an intermediate TG and PL lipase activity.[2] LPL is responsible for the hydrolysis of chylomicrons and very low-density lipoproteins (VLDLs). Lower LPL activity has been

associated with severe hypertriglyceridemia accompanied by low levels of high-density lipoprotein–cholesterol (HDL-C).[3,4] HL is involved in HDL metabolism as well as being responsible for the metabolism of apolipoprotein B–containing lipoproteins (especially intermediate-density lipoproteins [IDLs] and large low-density lipoproteins [LDLs]).[5] Higher concentrations and activity of HL, as TG hydrolase, have been associated with increased levels of small dense LDL (sdLDL)[6–8] and lower levels of HDL.[7,8] EL is primarily involved in HDL metabolism, and higher concentrations have been correlated with lower HDL levels.[9,10] Moreover, recently it has been shown that EL activity is responsible for the low HDL-C levels in patients who undergo hemodialysis.[11]

Alterations in the levels of plasma lipoproteins (high LDL-C and low HDL-C) are hallmarks of cardiovascular disease. Several comorbidities including diabetes mellitus, obesity, and metabolic syndrome (MS) have been shown to increase the risk of cardiovascular disease,[12] in part because of

Nonstandard Abbreviations and Acronyms	
BMI	body mass index
EL	endothelial lipase
FFA	free fatty acids
HDL-C	high-density lipoprotein–cholesterol
HL	hepatic lipase
HOMA-IR	homeostasis model assessment for insulin resistance index
LDLs	low-density lipoproteins
LPL	lipoprotein lipase
MS	metabolic syndrome
NW	normal weight
OB	obese
OW	overweight
PL	phospholipids
TG	triglyceride
VLDLs	very low-density lipoproteins

Table 1. Clinical and Biochemical Characteristics of Control and MS Groups

	Control (n=36)	MS (n=59)	P Value
Age, y	35±14	48±11	0.001
Sex (W/M)	20/16	43/16	0.083
BMI, Kg/m^2	23.5±2.7	34.2±5.9	0.001
Waist circumference, cm	81.5±11.6	105.3±10.1	0.001
TG, mmol/l	1.1 (0.4–2.9)	2.1 (1.0–5.6)	0.001
Total-C, mmol/l	4.6 (3.3–7.6)	5.3 (3.6–8.1)	0.042
LDL-C, mmol/l	3.0±1.1	3.7±0.9	0.001
HDL-C, mmol/l	1.5±0.4	1.1±0.2	0.001
apoA-I, g/l	1.7±0.4	1.4±0.3	0.001
apoB-100, g/l	0.9±0.3	1.1±0.3	0.002

Data are expressed as mean±SD or median (range) for skewed distributed data. apoA-I indicates apolipoprotein A-I; apoB, apolipoprotein B; BMI, body mass index; HDL-C, high-density lipoprotein–cholesterol; LDL-C, low-density lipoprotein–cholesterol; M, men; MS, metabolic syndrome; Total-C, total cholesterol; and W, women.

the changes in lipoprotein profile, including increased levels of TG rich lipoproteins, low levels of HDL-C, and increased levels of sdLDL. This profile, characteristic of insulin resistance (IR) states, is mainly attributed to abdominal obesity; however, it must be considered that the increase in body mass index does not always reflect IR. Additionally, there is evidence that weight change and IR individually can affect the atherogenic plasma lipid profile.[13,14] The behavior of LPL and HL (as TG hydrolases) has been widely studied in IR states and obesity[15–18]; however, to our knowledge, EL and HL activities, as phospholipases, have never been evaluated in these situations. The altered lipoprotein profile observed in patients with IR and during obesity could be in part a consequence of differing lipolysis of lipoproteins in these states. Furthermore, there are still controversies about the SN1 lipases activity in different grades of obesity.[19–22]

Our aim was to assess the phospholipase activity of EL and HL in postheparin plasma of subjects with MS/obesity and their relationship with atherogenic and antiatherogenic lipoprotein levels. Additionally, we evaluated LPL and HL activity as TG-hydrolyses to ascertain the individual roles of the 3 plasma lipolytic enzymes in the same patient and the consequent lipoprotein profile.

Materials and Methods

Materials and Methods are available in the online-only Supplement.

Results

Characteristics of the Study Population

The clinical and biochemical characteristics of MS and Control group are shown in Table 1. In the MS group, 43 patients were women and 16 men; whereas in the control group, 20 were women and 16 were men. Patients with MS were older (*P*<0.001) and presented higher body mass index (BMI; *P*<0.001) and waist circumference (*P*<0.001) than controls.

Regarding lipid and lipoprotein profile, the MS group presented higher TG (*P*<0.001), total cholesterol (*P*=0.042), LDL-C (*P*<0.001), and apolipoprotein B-100 (*P*=0.002), and lower HDL-C and apolipoprotein A-I levels (*P*<0.001).

With reference to IR and inflammatory state, as expected, values of glucose, insulin, homeostasis model assessment for insulin resistance index (HOMA-IR), TG/HDL-C, free fatty acids (FFA), and high-sensitivity C-reactive protein were higher (*P*<0.001), and adiponectin was lower in patients with MS compared with controls (*P*<0.001; Table 2).

Furthermore, in both groups, VLDL, IDL, and sdLDL were isolated and characterized. As shown in Table 3, in patients with MS, a prevalence of larger VLDL was observed, enriched in TG, as well as an increase of remnants and sdLDL.

The baseline characteristics of the subjects divided according to obesity degree are shown in Table 4.

Phospholipase Activities

EL Activity

EL activity was evaluated in control and MS group. A trend toward higher EL activity was observed in MS; however, it did not reach significance: 0.92 (0.09–1.93) versus 1.11 (0.15–3.06) µmol FFA/mL post heparin plasma per hour (PHP.h), *P*=0.097 (Figure 1A). There was no difference in EL activity between men and women: 1.25 (0.29–3.06) versus 1.0 (0.09–2.53) µmol FFA/mL PHP.h, *P*=0.330.

EL activity was not associated with age (*r*=−0.167; *P*=0.147) nor with waist circumference (*r*=0.183; *P*=0.126). Given the direct association between EL activity and BMI in the whole population (*r*=0.291; *P*=0.01), we analyzed the behavior of the enzyme according to the obesity degree of the subjects. EL activity was significantly increased in obese (OB) group compared with normal weight (NW) group: 1.25 (0.15–3.06) versus 0.71 (0.09–1.93) µmol FFA/mL PHP.h, *P*=0.009 (Figure 1B). Although no correlations with age and sex were observed, we performed an ANCOVA analysis including both variables. Difference between OB and NW group persisted significantly (*F*=6.9, *P*=0.004 and *F*=4.8, *P*=0.01, respectively).

Additionally, in the control and MS groups, EL activity was negatively associated with HDL-C (*r*=−0.369, *P*=0.014 and *r*=−0.480, *P*=0.005, respectively) and apolipoprotein A-I

Table 2. Insulin Resistance and Inflammatory Markers in Control and MS Groups

	Control (n=36)	MS (n=59)	P Value
Glucose, mmol/l	4.8 (3.9–5.6)	5.4 (4.5–7.0)	0.001
Insulin, pmol/l	36.1 (13.9–118.0)	66.0 (13.9–479.2)	0.001
HOMA-IR	1.2 (0.3–3.6)	2.5 (0.5–20.6)	0.001
TG/HDL-C	1.7 (0.5–7.9)	4.4 (2.1–16.2)	0.001
FFA, mmol/l	0.5 (0.1–0.8)	0.6 (0.3–1.1)	0.011
Adiponectin, µg/mL	12.3 (4.8–28.3)	5.6 (1.9–20.6)	0.001
hs-CRP, mg/l	1.3 (0.1–11.7)	3.1(0.3–29.7)	0.001

Data are expressed as median (range) for skewed distributed data. HDL-C indicates high-density lipoprotein–cholesterol; HOMA-IR homeostasis model assessment for insulin resistance index; hs-CRP, high-sensitivity C-reactive protein; MS, metabolic syndrome; and TG, triglycerides.

($r=-0.311$, $P=0.045$ and $r=-0.559$, $P=0.001$, respectively), highlighting the role of EL on HDL catabolism. Similarly, in both groups, EL activity was positively correlated with insulin ($r=0.301$, $P=0.05$ and $r=0.390$, $P=0.027$, respectively) and HOMA-IR ($r=0.310$, $P=0.047$ and $r=0.413$, $P=0.019$, respectively). In contrast, EL activity negatively correlated with adiponectin ($r=-0.515$; $P=0.006$) only in control group.

Given that there was no difference in EL activity between the MS and control groups, but a positive association between EL activity and HOMA-IR was observed, individuals were divided according to HOMA-IR quartile. The quartiles were defined according to the following range: quartile 1, HOMA-IR≤1.02; quartile 2, 1.03<HOMA-IR≤1.76; quartile 3, 1.77<HOMA-IR≤3.30; and quartile 4, HOMA-IR≥ 3.31. It was observed that individuals with the highest grade of IR (quartile 4) showed a significant increase in EL activity with respect to individuals with the lowest grade of IR (quartile 1; $P<0.05$; Figure 1C).

HL Activity

When HL was evaluated as phospholipase, there was no difference between control and MS groups: 5.85 (1.99–14.0) versus 5.62 (0.66–16.58) µmol FFA/mL PHP.h, $P=0.750$, neither between NW, overweight (OW), and OB group: 5.33 (1.99–12.89) versus 5.20 (1.54–14.0) versus 5.87 (0.66–16.58) µmol

Table 3. VLDL, IDL, and sdLDL in Control and MS Patients

	Control (n=36)	MS (n=59)	P Value
Large VLDL, %	7.8 (1.0–21.9)	33.5 (1.2–72.9)	0.010
VLDL-TG, %	49±12	56±6	0.018
VLDL-C, %	13±3	14±3	ns
VLDL-pt, %	14±4	16±4	ns
VLDL-pl, %	18±4	14±3	0.002
TG/apoB	6.2±2.8	11.7±4.7	0.001
IDL-C, mmol/l	0.1±0.06	0.2±0.08	0.036
sd LDL, %	11.3±6.3	28.7±5.4	0.001

Data are expressed as mean±SD or median (range) for skewed distributed data. apoB indicates apolipoprotein B; C, cholesterol; IDL, intermediate density lipoproteins; MS, metabolic syndrome; pl, phospholipids; pt, protein; sdLDL, small and dense low-density lipoprotein; TG, triglycerides; and VLDL, very low-density lipoproteins.

Table 4. Baseline Characteristics of the Subjects According to Obesity Degree

	NW (n=27)	OW (n=20)	OB (n=48)
BMI, Kg/m²	23.0 (16.7–24.9)	26.7 (25.2–29.1)*	34.5 (30.0–54.7)*†
Age, y	36±15	45±14‡	46±11*
Sex (W/M)	18/9	11/9	34/14
Waist circumference, cm	78±11	95±7*	107±10*†
TG, mmol/l	1.0 (0.4–2.9)	1.8 (0.5–4.6)*	2.1 (1.0–5.6)*
Total-C, mmol/l	4.7 (3.3–7.6)	6.2 (3.4–7.9)*	5.2 (3.6–8.1)
LDL-C, mmol/l	2.8±1.1	4.1±1.2*	3.5±0.8*
HDL-C, mmol/l	1.6±0.4	1.2±0.4*	1.0±0.2*
apoA-I, g/l	1.8±0.4	1.6±0.3	1.4±0.2*
apoB-100, g/l	0.9±0.3	1.1±0.3‡	1.0±0.3‡
Glucose, mmol/l	4.78 (3.9–5.4)	5.3 (4.4–6.6)*	5.5 (4.4–7.0)*
Insulin, pmol/l	32.5 (13.9–66.7)	43.0 (13.9–147.2)*	76.4 (27.1–479.2)†
HOMA-IR	0.9 (0.3–2.0)	1.4 (0.5–15.6)‡	2.7 (0.9–20.6) *†
TG/HDL-C	1.5 (0.7–7.9)	3.6 (0.5–15.7)	4.4 (2.1–16.2)*
FFA, mmol/l	0.5 (0.1–0.8)	0.6 (0.1–1.1)	0.6 (0.3–1.1)
Adiponectin, µg/mL	12.6 (7.0–28.2)	7.3 (1.9–20.6)*	5.6 (1.9–16.4)*
hs-CRP, mg/l	1.3 (0.1–11.7)	2.3 (0.2–6.2)	3.4 (0.3–29.7)‡

Data are expressed as mean±SD or median (range) for skewed distributed data. apoA-I indicates apolipoprotein A-I; apoB, apolipoprotein B; BMI, body mass index; FFA, free fatty acids; HDL-C, high-density lipoprotein–cholesterol; HOMA-IR, homeostasis model assessment for insulin resistance index; hs-CRP, high-sensitivity C-reactive protein; LDL-C, low-density lipoprotein–cholesterol; M, men; NW, normal weight; OB, obese; OW, overweight; TG, triglycerides; Total-C, total cholesterol; and W, women. vs NW ‡$P<0.05$ *$P<0.01$; vs OW †$P<0.01$.

FFA/mL PHP.h, $P=0.912$. In turn, in the whole population, HL as phospholipase was increased in men compared with women: 7.31 (1.61–16.58) versus 4.38 (0.66–16.17) µmol FFA/mL PHP.h, $P<0.001$.

Although no difference in HL activity was found between groups, regarding lipoprotein profile, in the control group, HL activity was negatively correlated with HDL-C ($r=-0.639$; $P=0.001$) and apolipoprotein A-I levels ($r=-0.623$; $P=0.001$) whereas in the MS group only a tendency with HDL-C was observed ($r=-0.281$; $P=0.062$). An inverse association with adiponectin was observed only in the control group ($r=-0.441$; $P=0.021$).

Effect of EL and HL as Phospholipase on HDL

Given that EL and HL as phospholipase were associated with HDL-C, the impact of both enzymes activities on HDL-C was analyzed through a multivariate regression analysis to distinguish the contribution of each one. In the MS group, HDL-C decrease remained mainly associated with EL activity ($\beta=-0.35$; $P=0.01$).

TG-Lipase Activities

HL Activity

As expected, HL activity, as TG-hydrolase, was increased in the MS group compared with the control group: 14.53±6.33 versus 11.26±4.92 µmol FFA/mL PHP.h, $P=0.025$ (Figure 2A). Similarly to its activity as phospholipase men presented
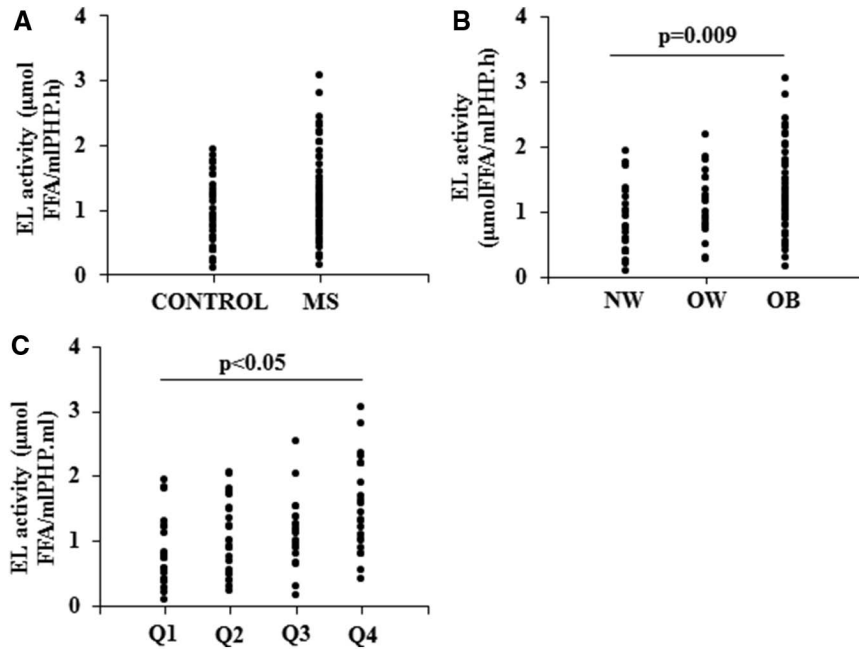
Figure 1. Endothelial lipase activity (EL) in (**A**) control and metabolic syndrome (MS) group; (**B**) different obesity grade: normal weight (NW), overweight (OW) and obese (OB); and (**C**) different homeostasis model assessment for insulin resistance index (HOMA-IR) quartile (Q): Q1, HOMA-IR≤1.02; Q2, 1.03<HOMA-IR≤1.76; Q3, 1.77<HOMA-IR≤3.30 and Q4, HOMA-IR≥ 3.31. FFA indicates free fatty acids.

higher values of HL than women (16.7±5.8 µmol FFA/mL PHP.h versus 11.8±5.5 µmol FFA/mL PHP.h, $P<0.001$). With reference to obesity degree, HL activity as TG-hydrolase was significantly increased in the OB group compared with the NW group: 15.0±6.3 versus 10.8±4.8 µmol FFA/mL PHP.h, $P=0.017$ (Figure 2B). HL activity was not associated with age ($r=0.031$; $P=0.802$). With reference to lipids and lipoproteins profile, in the control group, HL activity was directly correlated with LDL-C ($r=0.526$; $P=0.005$), apolipoprotein B ($r=0.560$; $P=0.003$), and negatively correlated with HDL-C ($r=-0.442$; $P=0.021$). These correlations were not found in the MS group in whom HL activity showed a weak inverse correlation with IDL-C ($r=-0.365$; $P=0.040$). With respect to IR markers, HL activity was positively associated with insulin ($r=0.378$; $P=0.011$) and HOMA-IR ($r=0.323$; $P=0.032$) in MS group.

Given that HL activity was higher in men than in women, we performed an ANCOVA analysis including sex as an independent variable. Differences in HL activity remained significant between the MS group and controls ($F=8.9$, $P=0.004$) and among obesity degree groups ($F=6.4$, $P=0.003$). Although HL activity was not associated with age, we also included this variable in the ANCOVA analysis. Differences in HL activity persisted significantly between the MS group and controls ($F=5.2$, $P=0.02$) and among obesity degree groups ($F=4.1$, $P=0.02$).

It is important to point out that HL as TG-hydrolase directly correlated with HL as phospholipase: $r= 0.79$, $P<0.001$.
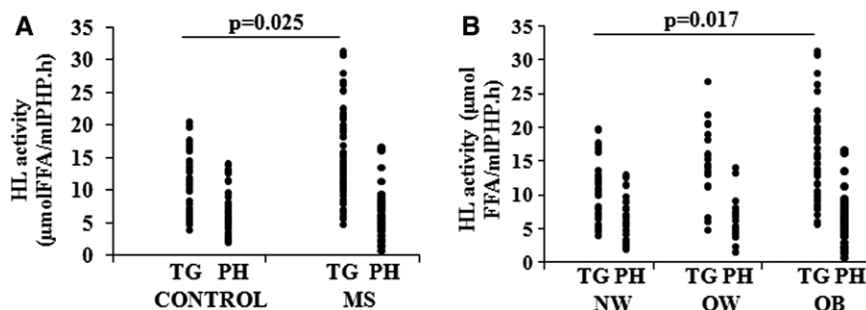
LPL Activity
Patients with MS presented lower LPL activity than controls: 0.75 (0.04–2.10) versus 1.38 (0.56–2.58) µmol FFA/mL PHP.h, $P<0.001$. In the whole population, there was no difference in LPL activity between men and women: 0.99 (0.26–1.55) versus 1.15 (0.04–2.58) µmol FFA/mL PHP.h, $P=0.160$, and it was not associated with age ($r=-0.171$; $P=0.173$).

When obesity degree was considered, LPL activity was significantly decreased in OW group: 0.81 (0.19–1.34) µmol FFA/mL PHP.h and OB group: 0.75 (0.04–2.10) µmol FFA/mL PHP.h compared with NW group: 1.54 (0.56–2.58) µmol FFA/mL PHP.h, $P=0.015$ and $P=0.004$, respectively (Figure 3).

Although no correlations with age and sex were observed, we performed an ANCOVA analysis including age and sex as independent variables. Differences in LPL activity remained significant between the MS and control groups ($F=10.7$, $P=0.002$ and $F=12.1$, $P=0.001$, respectively) and among obesity degree groups ($F=5.9$, $P=0.004$ and $F=6.6$, $P=0.002$, respectively).

The expected relationship between LPL activity and %TG-VLDL ($r=-0.636$, $P=0.04$) and TG/apolipoprotein B index ($r=-0.783$, $P=0.013$) was indeed found in the control

Figure 2. Hepatic lipase (HL) activity in (**A**) control and metabolic syndrome (MS) group and (**B**) HL activity in different obesity grades: normal weight (NW), overweight (OW), and obese (OB). FFA indicates free fatty acids.

group. However, in this group, only weak associations between LPL activity and insulin ($r=-0.462$, $P=0.04$) and HOMA-IR index ($r=-0.468$, $P=0.037$) were found.

Discussion

In the present study, we evaluated the role of EL, HL, and LPL activity in the lipoprotein abnormalities associated with MS and obesity. It is important to highlight that this is the first time that EL and HL as phospholipases were evaluated in MS and obesity. Moreover, to our knowledge, this study is the first that reports all 3 lipoprotein lipases activity in the same population.

It is well known that obesity and associated IR are main contributors to cardiovascular disease.[23] These relationships are directly linked with lipids and lipoproteins alterations which include elevated TG and lower HDL-C.

In this study, we showed for the first time that EL activity is increased in individuals with higher obesity degree and is associated with lower HDL-C and apolipoprotein A-I levels. Previous studies have shown that overexpression of EL in mice results in a dramatic decrease in HDL-C and apolipoprotein A-I levels, leading to the production of smaller HDL particles.[10] Furthermore, several studies in human plasma demonstrated higher expression of EL in MS[24] and obesity[25] associated with a significant decrease of HDL-C levels. In this study, we evaluated EL activity, and no significant difference between controls and patients with MS was found. However, there was a trend toward increase in EL activity in MS, and the lack of significance may reflect the inherent high assay variability. In this respect, a direct EL activity assay where HL is removed by immunoprecipition would be useful. Further studies including larger number of patients would possibly allow us to obtain significant differences between groups. When the subjects were analyzed according to their obesity degree, those with the highest obesity grade presented the highest EL activity. Our results support the previous findings of Badellino et al[26] who showed that plasma EL concentration is positively correlated with markers of adiposity, such as BMI and waist circumference in healthy individuals. Part of the association observed between EL and obesity could be attributed to the impact of the IR state, corroborated by the correlation between EL activity and the HOMA index in individuals with MS and controls. This finding is held up by the increased EL activity observed at the highest HOMA quartile. Additionally, it is important to highlight that, in contrast to LPL and HL, EL activity was associated with lower HDL-C and apolipoprotein A-I levels in individuals with MS

and controls. Although IR would be the best predictor of EL behavior, this study is the first to report an inverse association between EL activity and adiponectin in individuals without MS. It should be noted that adiponectin was also associated with HL phospholipase activity, suggesting a possible role of this cytokine in phospholipase activity regulation. Until now, it has only been described an inverse association between EL concentration and adiponectin levels in healthy individuals.[26] However, in vitro studies showed that tumor necrosis factor-α may stimulate EL secretion; thus, adiponectin would affect EL indirectly by inhibiting tumor necrosis factor-α.[26] Further studies are necessary to elucidate this finding.

Regarding HL, as expected, TG-activity was increased in MS group. HL activity seems to be regulated by several factors including age[27] and sex.[27,28] It has been reported that men have twice as high HL activity as women[29] and, in accordance, we have observed that men presented higher HL activity than women; however, the higher HL activity in the MS group was independent of sex and age. Even more, HL activity was increased in the OB group; these results suggest that the severe states of obesity would be implicated in the regulation of the enzyme, as described in other studies.[21] The specific mechanism that links HL activity to hyperinsulinemia and IR remains unclear. It is known that patients with type 1 diabetes mellitus present low HL activity,[17] and that chronic hyperinsulinemic states show increased activity of the enzyme.[18] It was suggested that secondary factors might contribute to the regulation of HL in obesity and IR states[30]; in fact in our study, only in the MS group, a weak association between HL activity and IR markers was found. HL activity directly correlated with atherogenic lipoprotein profile only in the control group. When analyzing HL as phospholipase, no differences between groups, MS or obesity degree, were observed. HL phospholipase activity inversely correlated with HDL and apolipoprotein A-I levels in Control group. HL enzyme activities, as TG-lipase and phospholipase, were directly correlated within individuals. The differences in importance within a disease state may reflect their different in vivo substrates: VLDL and LDL for TG-lipase activity and HDL for phospholipase activity.

According to our results, HDL-C levels would be influenced by phospholipase activities of EL as well as HL, with EL mainly responsible for HDL catabolism. Both HL and EL variants have been shown to affect HDL-C.[31,32] Because often they are similarly regulated, it is important to note which is the predominant determinant of HDL-C. The present data along with our previous analysis of individuals undergoing
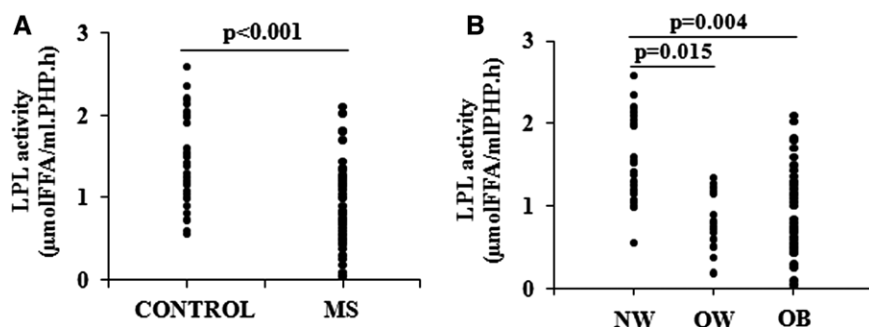
Figure 3. Lipoprotein lipase (LPL) activity in (**A**) control and metabolic syndrome (MS) group and (**B**) different obesity grades: normal weight (NW), overweight (OW), and obese (OB). FFA indicates free fatty acids.

Table 5.   LPL, HL, and EL behavior in Metabolic Syndrome and Obesity

	LPL	HL-TG	HL-PL	EL
MS	↓	↑	=	=/↑
Obesity	↓	↑	=	↑

EL indicates endothelial lipase; HL, hepatic lipase; LPL, lipoprotein lipase; MS, metabolic syndrome; PL, phospholipids; and TG, triglycerides.

hemodialysis[11] suggest that HDL-C decrease is mainly associated with EL activity. Our findings extend previous reports about factors that can modulate HDL-C levels, such as lecithin cholesterol acyl transferase, and ATP-binding cassette subfamily A member 1 expression, among others.[33]

With reference to LPL, this study shows a significant decrease in plasma activity in individuals with MS. Previous studies reported lower LPL mass in individuals with MS,[14] as well as negative correlations with BMI.[34] Our results of LPL activity in post heparin plasma are in agreement with the reported findings; we found a significant decrease from OW situations with a clear decrease in OB. Recent studies have shown that the expression and activity of LPL in post heparin plasma is lower in obese patients with diabetes mellitus with respect to obese individuals without diabetes mellitus and controls.[20] Moreover, a decrease in LPL gene expression in the visceral adipose tissue from morbidly obese individuals compared with obese and lean individuals has been reported.[35] In our study, we observed that LPL activity was inversely associated with surrogate markers of IR only in individuals without MS. With reference to the role of LPL in VLDL catabolism, our data revealed the expected inverse correlation with VLDL-TG content and size in the control group; however, these associations were not found in the patients with MS. These results are in accordance with previous studies, which suggest that other factors have more important regulatory roles in the removal of postprandial lipoproteins in IR states.[36,37]

With respect to high-sensitivity C-reactive protein, in this study, no significant association between this chronic inflammation marker and lipolytic enzymes activity was found. Different studies have shown controversial results according to the effect of C-reactive protein on the expression and activity of the enzymes.[26,30,38,39] In this study, the lack of association between high-sensitivity C-reactive protein and the lipolytic enzymes should not exclude more complex inflammatory mechanisms in the regulation of enzyme activity.

Finally, in this study, we describe for the first time the activity of the 3 main lipolytic enzymes, evaluated in the same population, highlighting the specific role of each one on the different lipoproteins metabolism. EL and HL as phospholipase are both responsible for HDL catabolism. Our results elucidate part of the remaining controversies about the SN1 lipases activity in different grades of obesity. The impact of IR and obesity on the 3 enzymes behavior (Table 5) determines the lipoproteins alterations in these pathological situations. Overall, lipolytic enzymes would be an interesting potential therapeutic target as a strategy to improve lipoprotein profile and reduce cardiovascular risk in IR and obese patients.

References

1. Hasham SN, Pillarisetti S. Vascular lipases, inflammation and atherosclerosis. *Clin Chim Acta*. 2006;372:179–183.
2. McCoy MG, Sun GS, Marchadier D, Maugeais C, Glick JM, Rader DJ. Characterization of the lipolytic activity of endothelial lipase. *J Lipid Res*. 2002;43:921–929.
3. Wang H, Eckel RH. Lipoprotein lipase: from gene to obesity. *Am J Physiol Endocrinol Metab*. 2009;297:E271–E288.
4. Krentz AJ. Lipoprotein abnormalities and their consequences for patients with type 2 diabetes. *Diabetes Obes Metab*. 2003;5(Suppl 1):S19–S27.
5. Zambon A, Deeb SS, Bensadoun A, Foster KE, Brunzell JD. *In vivo* evidence of a role for hepatic lipase in human apoB-containing lipoprotein metabolism, independent of its lipolytic activity. *J Lipid Res*. 2000;41:2094–2099.
6. Berg GA, Siseles N, González AI, Ortiz OC, Tempone A, Wikinski RW. Higher values of hepatic lipase activity in postmenopause: relationship with atherogenic intermediate density and low density lipoproteins. *Menopause*. 2001;8:51–57.
7. Brunzell JD, Zambon A, Deeb SS. The effect of hepatic lipase on coronary artery disease in humans is influenced by the underlying lipoprotein phenotype. *Biochim Biophys Acta*. 2012;1821:365–372.
8. Miksztowicz V, Lucero D, Zago V, Cacciagiú L, Lopez G, Gonzalez Ballerga E, Sordá J, Fassio E, Schreier L, Berg G. Hepatic lipase activity is increased in non-alcoholic fatty liver disease beyond insulin resistance. *Diabetes Metab Res Rev*. 2012;28:535–541.
9. Jin W, Marchadier D, Rader DJ. Lipases and HDL metabolism. *Trends Endocrinol Metab*. 2002;13:174–178.
10. Maugeais C, Tietge UJ, Broedl UC, Marchadier D, Cain W, McCoy MG, Lund-Katz S, Glick JM, Rader DJ. Dose-dependent acceleration of high-density lipoprotein catabolism by endothelial lipase. *Circulation*. 2003;108:2121–2126.
11. Miksztowicz V, McCoy MG, Schreier L, Cacciagiú L, Elbert A, Gonzalez AI, Billheimer J, Eacho P, Rader DJ, Berg G. Endothelial lipase activity predicts high-density lipoprotein catabolism in hemodialysis: novel phospholipase assay in postheparin human plasma. *Arterioscler Thromb Vasc Biol*. 2012;32:3033–3040.
12. Reaven GM. Insulin resistance, the insulin resistance syndrome, and cardiovascular disease. *Panminerva Med*. 2005;47:201–210.
13. Mäntyselkä P, Kautiainen H, Saltevo J, Würtz P, Soininen P, Kangas AJ, Ala-Korpela M, Vanhala M. Weight change and lipoprotein particle concentration and particle size: a cohort study with 6.5-year follow-up. *Atherosclerosis*. 2012;223:239–243.
14. Wang J, Liu EY, Freudenreich O, Goff D, Henderson DC, Fan X. Phenotypic characteristics in metabolically obese but normal weight non-diabetic patients with schizophrenia. *Schizophr Res*. 2010;124:49–53.
15. Hanyu O, Miida T, Obayashi K, Ikarashi T, Soda S, Kaneko S, Hirayama S, Suzuki K, Nakamura Y, Yamatani K, Aizawa Y. Lipoprotein lipase (LPL) mass in preheparin serum reflects insulin sensitivity. *Atherosclerosis*. 2004;174:385–390.
16. Saiki A, Oyama T, Endo K, Ebisuno M, Ohira M, Koide N, Murano T, Miyashita Y, Shirai K. Preheparin serum lipoprotein lipase mass might be a biomarker of metabolic syndrome. *Diabetes Res Clin Pract*. 2007;76:93–101.
17. Caixàs A, Pérez A, Payés A, Otal C, Carreras G, Ordóñez-Llanos J, Reviriego J, Anderson JH, de Leiva A. Effects of a short-acting insulin analog (Insulin Lispro) versus regular insulin on lipid metabolism in insulin-dependent diabetes mellitus. *Metabolism*. 1998;47:371–376.
18. Syvänne M, Ahola M, Lahdenperä S, Kahri J, Kuusi T, Virtanen KS, Taskinen MR. High density lipoprotein subfractions in non-insulin-dependent diabetes mellitus and coronary artery disease. *J Lipid Res*. 1995;36:573–582.
19. Eckel RH, Yost TJ. Weight reduction increases adipose tissue lipoprotein lipase responsiveness in obese women. *J Clin Invest*. 1987;80:992–997.
20. Costabile G, Annuzzi G, Di Marino L, De Natale C, Giacco R, Bozzetto L, Cipriano P, Santangelo C, Masella R, Rivellese AA. Fasting and

post-prandial adipose tissue lipoprotein lipase and hormone-sensitive lipase in obesity and type 2 diabetes. *J Endocrinol Invest*. 2011;34:e110–e114.

21. Pardina E, Baena-Fustegueras JA, Catalán R, Galard R, Lecube A, Fort JM, Allende H, Vargas V, Peinado-Onsurbe J. Increased expression and activity of hepatic lipase in the liver of morbidly obese adult patients in relation to lipid content. *Obes Surg*. 2009;19:894–904.

22. Després JP, Ferland M, Moorjani S, Nadeau A, Tremblay A, Lupien PJ, Thériault G, Bouchard C. Role of hepatic-triglyceride lipase activity in the association between intra-abdominal fat and plasma HDL cholesterol in obese women. *Arteriosclerosis*. 1989;9:485–492.

23. Reaven GM. Insulin resistance: the link between obesity and cardiovascular disease. *Med Clin North Am*. 2011;95:875–892.

24. Badellino KO, Wolfe ML, Reilly MP, Rader DJ. Endothelial lipase concentrations are increased in metabolic syndrome and associated with coronary atherosclerosis. *PLoS Med*. 2006;3:e22.

25. Paradis ME, Badellino KO, Rader DJ, Tchernof A, Richard C, Luu-The V, Deshaies Y, Bergeron J, Archer WR, Couture P, Bergeron N, Lamarche B. Visceral adiposity and endothelial lipase. *J Clin Endocrinol Metab*. 2006;91:3538–3543.

26. Badellino KO, Wolfe ML, Reilly MP, Rader DJ. Endothelial lipase is increased *in vivo* by inflammation in humans. *Circulation*. 2008;117:678–685.

27. Huttunen JK, Ehnholm C, Kekki M, Nikkilä EA. Post-heparin plasma lipoprotein lipase and hepatic lipase in normal subjects and in patients with hypertriglyceridaemia: correlations to sex, age and various parameters of triglyceride metabolism. *Clin Sci Mol Med*. 1976;50:249–260.

28. Krauss RM, Levy RI, Fredrickson DS. Selective measurement of two lipase activities in postheparin plasma from normal subjects and patients with hyperlipoproteinemia. *J Clin Invest*. 1974;54:1107–1124.

29. Carr MC, Hokanson JE, Zambon A, Deeb SS, Barrett PH, Purnell JQ, Brunzell JD. The contribution of intraabdominal fat to gender differences in hepatic lipase activity and low/high density lipoprotein heterogeneity. *J Clin Endocrinol Metab*. 2001;86:2831–2837.

30. Schneider JG, von Eynatten M, Schiekofer S, Nawroth PP, Dugi KA. Low plasma adiponectin levels are associated with increased hepatic lipase activity in vivo. *Diabetes Care*. 2005;28:2181–2186.

31. Zambon A, Deeb SS, Hokanson JE, Brown BG, Brunzell JD. Common variants in the promoter of the hepatic lipase gene are associated with lower levels of hepatic lipase activity, buoyant LDL, and higher HDL2 cholesterol. *Arterioscler Thromb Vasc Biol*. 1998;18:1723–1729.

32. Brown RJ, Edmondson AC, Griffon N, Hill TB, Fuki IV, Badellino KO, Li M, Wolfe ML, Reilly MP, Rader DJ. A naturally occurring variant of endothelial lipase associated with elevated HDL exhibits impaired synthesis. *J Lipid Res*. 2009;50:1910–1916.

33. Vaziri ND. Causes of dysregulation of lipid metabolism in chronic renal failure. *Semin Dial*. 2009;22:644–651.

34. Kobayashi J, Saito K, Fukamachi I, Taira K, Takahashi K, Bujo H, Saito Y. Pre-heparin plasma lipoprotein lipase mass: correlation with intra-abdominal visceral fat accumulation. *Horm Metab Res*. 2001;33:412–416.

35. Clemente-Postigo M, Queipo-Ortuño MI, Fernandez-Garcia D, Gomez-Huelgas R, Tinahones FJ, Cardona F. Adipose tissue gene expression of factors related to lipid processing in obesity. *PLoS One*. 2011;6:e24783.

36. Goldberg IJ, Vanni-Reyes T, Ramakrishnan S, Holleran S, Ginsberg HN. Circulating lipoprotein profiles are modulated differently by lipoprotein lipase in obese humans. *J Cardiovasc Risk*. 2000;7:41–47.

37. Olivecrona T, Bengtsson-Olivecrona G. Lipoprotein lipase and hepatic lipase. *Curr Opin Lipidol*. 1993; 4: 187–196.

38. Maingrette F, Li L, Renier G. C-reactive protein enhances macrophage lipoprotein lipase expression. *J Lipid Res*. 2008;49:1926–1935.

39. Chen Z, Ma G, Zhang X, Wang J. Lipoprotein lipase and premature coronary artery disease. *Acta Cardiol*. 2009;64:379–383.

Significance

The novelty of this study is that this is the first time that endothelial and hepatic lipase activities as phospholipases have been evaluated in metabolic syndrome and obesity. Additionally, this is the first study in which all 3 SN1 lipases were evaluated in the same population. The altered lipoprotein profile observed in patients with insulin resistance and during obesity could be in part a consequence of differing lipolysis of lipoproteins in these states.

MATERIAL AND METHODS

Subjects

For the present study, 59 patients with Metabolic Syndrome (MS), according to Adult Treatment Panel III definition,[1] were consecutively selected at the Hepatology Unit of Prof. Alejandro Posadas National Hospital (Buenos Aires, Argentina). All the MS patients presented hepatic steatosis which was diagnosed by abdominal ultrasonography performed by a single operator and in all cases confirmed by a liver biopsy showing macro vesicular steatosis in at least 5% of hepatocytes. In parallel, 36 subjects recruited among hospital employee volunteers were selected as controls; all of them underwent hepatic ultrasonography in order to discard steatosis.

The following exclusion criteria were considered for both groups: alcohol intake >20 g/day, diabetes, cardiovascular disease (CVD), neoplasia, hypothyroidism, recent history of acute illness, renal disorders, seropositive hepatitis B or C. None of the subjects received corticosteroids, immunosuppressive agents or drugs known to influence lipid metabolism such as statins or fibrates.

The weight, height and blood pressure of each participant were measured, and body mass index (BMI) was calculated to evaluate obesity degree. Waist circumference was taken midway between the lateral lower rib margin and the superior anterior iliac crest in a standing position, always by the same investigator.

According to BMI, all the subjects were subdivided in: -Normal Weight (NW, $18.5 \leq BMI \leq 24.9$ kg/m^2), n= 27; Overweight (OW, 25 kg/m$^2 \leq BMI \leq 29.9$ kg/m^2), n= 20 and Obese (OB ≥ 30 kg/m^2), n=48.

Written informed consent was obtained from all the participants included in the study. The study had the approval of the Ethics Committees from the Faculty of Pharmacy and Biochemistry, University of Buenos Aires.

Samples

After a 12-h overnight fast, blood samples were drawn. Serum was kept at 4ºCwithin 48 h for the evaluation of glucose, lipids and lipoproteins or stored at -70 ºC for further determination of insulin, free fatty acids (FFA), adiponectin, high sensitivity C-reactive protein (hs-CRP) and VLDL, IDL and small and dense LDL isolation.

To measure LPL, HL and EL activities, heparin (60 IU/kg body weight) was administered intravenously. After 10 minutes, blood from the contralateral arm was collected in tubes on ice. Post-heparin plasma (PHP) was obtained by centrifugation at 1500 g at 4°C for 10 minutes and kept at −70°C.

Measurements

Total cholesterol, TG, and fasting glucose were measured using commercial enzymatic kits (Roche Diagnostics, Mannheim, Germany) in a Cobas C-501 autoanalyzer, coefficient of variation (CV) intra-assay<1.9%, CV inter-assay<2.4%, and averaging CV values of these parameters. HDL and LDL-cholesterol were determined by standardized selective precipitation methods, using phosphotungstic acid/MgCl$_2$ [2] and polyvinylsulfate [3] as precipitating reagents, respectively CV intra-assay<2.0%, and CV inter-assay<3.0%. Serum hs-CRP, apoA-I, and apoB-100 were determined by immunoturbidimetry (Roche Diagnostics, Mannheim, Germany), CV intra-assay<1.9%, and CV inter-assay<2.5% for the 3 parameters. FFA were determined by a spectrophotometric method (Randox, UK) CV intra-assay <2.6% and CV inter-assay <3.9% and insulin was measured with Immulite/Immulite 1000 Insulin (Siemens, USA), CV intra-assay<2.6%, and CV inter-assay<3.9%. To estimate IR, the homeostasis model assessment for insulin resistance (HOMA-IR) index was calculated as fasting

insulin (µU/mL)×fasting glucose (mmol/L)/22.5. TG/HDL-cholesterol index was also used as a surrogate marker of IR. Sera levels of adiponectin were determined by monoclonal antibody–based ELISA (R&D Systems, USA).

Lipoprotein measurements

VLDL and IDL isolation. VLDL [density (d) <1.006 g/ml] and IDL [d:1.006–1.019 g/ml] were isolated by sequential preparative ultracentrifugation,[4] in a Beckman XL-90 using a fixed-angle rotor type 90 Ti. Each run was performed at 105.000×g, for 18 h, at 14 °C. Purity of lipoprotein was tested by agarose gel electrophoresis. Isolated VLDL composition was characterized by the following parameters: cholesterol and TG, using the methods previously described, phospholipids by the Bartlett method[5] and proteins by the Lowry method.[6] Data was expressed as the percentage of each component. VLDL TG/protein (TG/PT) ratio was calculated as estimator of lipoprotein size. In order to assess IDL concentration, its cholesterol content was measured, as described before.

VLDL size by high performance liquid chromatography (HPLC). In brief, isolated VLDL were injected in a column TSK-Gel Lipopropack XL, 7.8 mm ID×30 cm(Tosoh, Japan) and runs were performed using as mobile phase: Tris acetate buffer 0.05 mol/l (pH 8) containing 0.3 mol/l sodium acetate,0.05% sodium azide and 0.005% Brij-35. Flow rate was 0.5 ml/min and the column eluate was monitored at 280 nm. [7] For the conversion of elution time in particle diameter, a standard curve was used, constructed with the logarithm of retention time and the logarithm of the diameter of standard diameter latex particles, 100 nm in diameter (Fluka, Sigma-Aldrich) and of 27 and 39 nm in diameter (MagsphereINC).

Small and dense LDL isolation. The small and dense LDL subfraction was measured by a precipitation commercial kit (sdLDL-C, Denka Seiken, Japan) using heparin sodium salt and $MgCl_2$. After precipitation LDL-cholesterol was measured by a direct and selective homogenous assay method (LDL-EX; Denka Seiken, Japan).[8] Results were calculated as sdLDL-chol mg/dl serum and expressed as percentage.

Lipase Activities

Lipoprotein Lipase and Hepatic Lipase as TG hydrolase. LPL and HL as TG hydrolase were determined in PHP by measuring the oleic acid produced by the enzyme-catalyzed hydrolysis of an emulsion containing [^{3}H]-triolein (Amersham TRA191; Amersham, Buckinghamshire, UK) according to Nilsson-Ehle method.[9] The assay mixture for LPL activity contained labelled and unlabelled Triolein (Sigma T-7140) (1.3 mmol/ml of glyceryltrioleate with a specific activity of 10 $\times 10^6$cpm/mmol), mixed with 0.11 mmol/ml of L-lysophosphatidylcholine (Sigma L-4129), 4% bovine serum albumin (Sigma A-6003), 10% v/v of human serum as source of apoC-II in 0.2 M buffer Tris–HCl pH 8.0 with NaCl 0.3 M. This mixture was incubated with PHP in saline solution 1:10 to determine the total lipolytic activity, and simultaneously with PHP in saline solution 1:5 and NaCl 1M (as inhibitor of LPL) for 30 minutes at 37°C. After incubation, the reaction was stopped and the released fatty acids were isolated by extraction with a carbonate-borate buffer, pH 10.5. The [^{3}H] oleic acid was quantified by counting with a Liquid Scintillation Analyzer (Packard TRI-CARB 2100; Packard Instruments, Meridian, CT). LPL activity of each individual was calculated from the difference between total activity and the activity measured in presence of NaCl 1M.

For HL, the assay mixture was prepared as previously described using 0.2 M buffer Tris–HCl pH 9.0 with NaCl 0.15 M and without human serum. In this case, the mixture was incubated with PHP in saline solution 1:10 and NaCl 1M (as inhibitor of the other lipases) for 30 min at 30ºC. After the incubation, the extraction and measurement of

free fatty acids was perform as described above. Results were expressed as μmolFFA released per milliliter of PHP per hour. CV intra-assay was 4% and CV inter-assay 9%. Because of the complexity of this assay, the CV is considered to be quite satisfactory.

Endothelial Lipase and Hepatic Lipase as Phospholipase. EL and HL as phospholipase were determined as previously described.[10] Briefly, total SN1-specific phospolipase activity was determined using (1-decanoylthio-1-deoxy-2-decanoyl-sn-glycero-3-phosphoryl) ethylene glycol (ThioPEG) as the substrate.[11] An emulsion of 4.09mmol/L ThioPEG (Avanti) in 100mmol/L HEPES, pH 8.3, and 7mmol/L Triton X-100 was prepared by sonication with a Bransom Sonifier. A 1:1 mol mixture of ThioPEG to 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) was prepared by adding the DTNB 271mmol/L (Sigma) solution to the ThioPEG emulsion, resulting in a chromogenic substrate solution. Total SN1 phospholipase activity was measured by adding 20 μL of a one-tenth dilution of PHP and 80 μL of chromogenic substrate to the wells of a 96-well plate and following the absorbance at 412 nm for 30 minutes in a Molecular Devices Spectra Max 250 microplate reader. To determine the HL phospholipase activity, the PHP dilutions were pre-incubated in ice for 15 minutes with 1mol/L NaCl to inhibit the EL activity. Then, the chromogenic substrate solution was added, and the plate was read at 412nm as described previously. Finally, EL activity was calculated as the difference between total phospholipase activity and hepatic phospholipase activity. The intra-assay CV for HL and EL were 3.1%, and 15.7%, respectively. The inter-assay CV for HL and EL were 5.3% and 28.8%, respectively. The EL CV is higher because it is the difference between total lipase and HL. All enzyme activities were calculated using the molar adsorption coefficient value for the nitro-5-thiobenzoate anion, the depth of solution, and the volume of the enzyme source. Results were expressed in μmol of FFA per milliliter of PHP per hour.

Statistical Analysis

Data are presented as mean±SD or median (range) according to normal or skewed distribution, respectively. Differences between Control and MS group were tested using the unpaired Student t test, χ2 test, or the Mann–Whitney U test, according to the data distribution. One-way analysis of variance was used to test differences among patients with different obesity degree. Previously, each variable was examined for normal distribution, and abnormally distributed variables were log transformed. Further evaluation was performed using Scheffé multiple comparison test. Pearson or Spearman analysis, for parametric or non-parametric variables, was used to determine correlations between parameters. To verify the difference of lipases activity between groups, we performed an analysis of covariance, controlling for necessary confounders such as age and gender. Stepwise and multiple linear regression analyses were used to indentify independent correlates of enzymes activities. Previously, each variable was examined for normal distribution, and abnormally distributed variables were log-transformed. The SPSS 19.0 software package (Chicago, IL) was used for statistical analysis.

REFERENCES

1. Grundy S, Cleeman J, Bairey Merz C, Brewer H, Clark L, Hunninghake D, Pasternak R, Smith S, Stone N. Implications of Recent Clinical Trials for the National Cholesterol Education Program Adult Treatment Panel III Guidelines. Circulation 2004;110:227-239.

2. Assmann G, Schriewer H, Schmitz G, Hägele EO. Quantification of high-density-lipoprotein cholesterol by precipitation with phosphotungstic acid/MgCl2. Clin Chem. 1983; 29: 2026-2030.

3. Assmann G, Jabs HU, Kohnert U, Nolte W, Schriewer H. LDL-cholesterol determination in blood serum following precipitation of LDL with polyvinylsulfate. Clin Chim Acta. 1984; 140:77-83.

4. Schumaker VN & Puppione DL. Sequential flotation ultracentrifugation. Methods Enzymol. 1986; 128: 155–70.

5. Bartlett G. Phosphorus assay in column chromatography. J Biol Chem. 1959; 234: 466–468.

6. Lowry O, Rosbrough N, Farr A, Randall R. Protein measurement with the Folin phenol reagent.J. Biol. Chem. 1951; 193:265-275.

7. Hara I, Okazaki M. High-performance liquid chromatography of serum lipoproteins. Methods Enzymol. 1986; 129:57–78.

8. Hirano T, Ito Y, Yoshino G. Measurement of small dense low-density lipoprotein particles. J Atheroscler Thromb. 2005; 12:67-72.

9. Nilsson-Ehle P, Ekman R. Rapid, simple and specific assay for lipoprotein lipase and hepatic lipase. Artery. 1977; 3:194 – 209.

10. Miksztowicz V, McCoy MG, Schreier L, Cacciagiú L, Elbert A, Gonzalez AI, Billheimer J, Eacho P, Rader DJ, Berg G. Endothelial lipase activity predicts high-density lipoprotein catabolism in hemodialysis: novel phospholipase assay in postheparin human plasma. Arterioscler Thromb Vasc Biol. 2012; 32: 3033-3040.

11. Eacho, PI, Foxworthy-Mason PS, Harper, RW, Lin, H, Lopez, JE and Richett, ME, Chromogenic Compound, United States Patent # 6,448,422 B1. (2002).