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Atherogenic alterations in hypertriglyceridemic patients would not depend on insulin resistance

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ABSTRACT

Background and aims: Several studies have been carried out to characterize the different alterations associated with hypertriglyceridemia (HTG) and to identify this dyslipemia as an independent risk factor for cardiovascular disease (CVD). HTG is frequently, but not always, associated with insulin resistance (IR). The present study was aimed to evaluate if the alterations observed in biomarkers of CVD were similar in HTG states independently of IR.
Methods: HTG was defined as triglycerides ≥1.69 mmol/l and IR as HOMA-IR ≥3.1. HTG-IR patients (n = 15) were compared with HTG subjects without IR (WIR) (n = 15) and with normotriglyceridemic (NTG)-WIR individuals (n = 30).
Results: Both HTG groups shared the increment in VLDL-C and non-HDL-C, HDL enrichment in triglycerides and depletion in phospholipids, the decrease in adiponectin concentration, and the increase in CETP activity. HDL-C and VCAM-1 levels were altered only in HTG-IR patients in comparison with the other groups, while oxidized LDL was only higher in HTG-IR than the control group. Multiple regression analysis identified triglycerides as the independent predictor of HDL-C, CETP activity and oxidized LDL levels.
Conclusion: The increase in triglycerides is the major determinant factor of the atherogenic modifications observed, while IR would be an amplifier factor.

1. Introduction

Many epidemiological studies have analyzed the association between triglyceride (TG) levels and the risk of cardiovascular disease (CVD) in different populations [1]. Interestingly, the National Cholesterol Education Program—Adult Treatment Panel III (NCEP-ATPIII) and a metaanalysis based on 29 prospective studies [2,3] concluded that there was insufficient evidence to consider increased TGs as an independent coronary risk factor in Western populations. Nonetheless, some studies such as the Prospective Cardiovascular Munster Study (PROCAM) and the Copenhagen Male Study [4–6] showed an increased risk of CVD with increasing TG levels independently of both, low density lipoprotein-cholesterol (LDL-C) and high density lipoprotein-cholesterol (HDL-C) levels. Thus, whether hypertriglyceridemia represents an independent risk factor or not still remains unclear [7].

Increased concentration of TGs is a common finding in general population and its prevalence in adult males rounds 20–35% [8,9]. It has been established that TG plasma concentrations are highly heritable, suggesting that TGs are, at least in part, genetically determined, though they also depend on the interaction with multiple environmental factors. In most cases, hypertriglyceridemia (HTG) is associated with obesity [10], metabolic syndrome (MS) [11], and type 2 diabetes [12], every condition related to insulin resistance (IR) and directly associated with increased morbidity and mortality due to CVD. This raises an important issue as to whether the association of hypertriglyceridemia with CVD or other forms of atherosclerosis is a direct effect of the triglyceride-rich lipoproteins themselves or the company they keep, such as IR [7].

It is well known that patients with hypertriglyceridemia usually present qualitative alterations in lipoprotein composition and, in particular, increased levels of small and dense LDL (sdLDL) particles [13]. It has been recognized that sdLDL are more susceptible to undergo oxidative modifications, thus generating oxidized LDL (oxLDL) [14]. In the intima of blood vessels, oxLDL could promote an immune response from vascular endothelial cells which is characterized by the expression of cell adhesion molecules such as the intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and E-selectin, which home inflammatory cells into the arteries [15]. In previous studies, both soluble and leukocyte cell adhesion molecules were found to be significantly increased in HTG patients [16,17]. In addition, hypertriglyceridemia was also shown to be associated with alterations in the

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antiatherogenic pathway known as reverse cholesterol transport [18] and an increase in the procoagulant state [19].

As most studies in HTG patients were carried out without considering the potential presence of IR, it is difficult to assign the abnormalities observed either to the increment in TG levels *per se* or to the IR state. The aim of the present study was to evaluate if the alterations observed in biomarkers of CVD were alike in HTG patients independently of the presence of IR.

2. Materials and methods

2.1. Subjects

A group of male HTG patients with IR (HTG-IR) (TG≥1.69 mmol/l and HOMA-IR≥3.1) (n=15) were compared with HTG individuals without IR (HTG-WIR) (TG≥1.69 mmol/l and HOMA-IR<2.6) (n=15) and with normotriglyceridemic controls without IR (NTG-WIR) (TG<1.69 mmol/l and HOMA-IR<2.6) (n=30). HTG was diagnosed according to NCEP-ATPIII definition [2]. IR was defined as HOMA-IR≥3.1. This cut-off point was the value that best characterized patients with MS in a male cohort from the south region of Argentina and also the highest HOMA-IR proposed for IR assessment in Argentinean general population [20,21]. In addition, to exclude IR, HOMA-IR values lower than 2.6, which was another cut-off point proposed for Argentinean population [21], were considered. All the subjects included in the present study were adult males recruited during a period of 10 months from the Institute of Cardiology and Cardiovascular Surgery of the Favaloro Foundation, Buenos Aires, Argentina. Subjects were excluded from the study if they matched any of the following criteria: 1) diabetes or other endocrine disorders, 2) hepatic or renal pathologies; 3) chronic inflammatory diseases such as rheumatoid arthritis or celiac disease, 4) excessive tobacco (>10 cigarettes/day) or ethanol (>30 g/day) consumption, and 5) therapy with drugs that could affect lipid or carbohydrate metabolism or with antioxidants. Informed consent was obtained from all participants and the protocol was approved by the Ethical Committees from Faculty of Pharmacy and Biochemistry, University of Buenos Aires.

2.2. Study protocol and samples

After a 12 hour overnight fast, venous blood was drawn from the antecubital vein. Aliquots were collected either in clean and $EDTANa_2$-containing tubes. Samples were centrifuged at 1500×*g*, for 15 min, at 4 °C, and serum was stored at 4 °C and used within 24 h for glucose, lipid profile and lipoprotein isolation by ultracentrifugation. Serum aliquots were also stored at −70 °C for determination of insulin, adiponectin, oxLDL, CETP, ICAM-1, VCAM-1 and E-selectin.

2.3. Analytical procedures

Glucose, TGs and total cholesterol were quantified by standardized methods (Roche Diagnostics, Mannheim, Germany) in a Hitachi 727 autoanalyzer. Low-density lipoprotein cholesterol (LDL-C) and high-density lipoprotein cholesterol (HDL-C) concentrations were determined by selective precipitation methods. Very low density lipoprotein-cholesterol (VLDL-C) was calculated as the difference between the cholesterol contained in the supernatants obtained for LDL and HDL measurements. In the isolated HDL fraction, TGs and cholesterol were measured by the previously mentioned methods, phospholipids by the Bartlett method [22], and proteins by the Lowry method [23]. Lipoprotein mass was estimated as the summatory of all components (TGs, cholesterol, phospholipids and total proteins) and then the percentual composition was calculated. Apo B and apo A-I were evaluated by immunoturbidimetry (Roche Diagnostics Mannheim, Germany).

2.4. Lipoprotein isolation

HDL (d: 1.063–1.210 g/ml) was isolated by sequential preparative ultracentrifugation [24] in a XL-90 Beckman ultracentrifuge, with a type 90 Ti rotor. The run was performed at 105,000×*g*, for 18 h, at 4 °C. HDL purity was tested by agarose gel electrophoresis and albumin content tested by inmunoturbidimetry (Roche Diagnostics, Mannheim, Germany). Albumin content was in no case >2% of total protein.

2.5. CETP activity

CETP activity was determined in serum samples following the general procedure previously described with few modifications [25]. Briefly, the ability of serum to promote the transfer of tritiated cholesteryl esters from a tracer amount of biosynthetically labeled HDL_3 (^{3}H-CE-HDL_3) (NEN Life Science Products, Boston, MA) toward serum apo B-containing lipoproteins was evaluated. Samples were incubated with ^{3}H-CE-HDL_3 (50 μmol/l cholesterol) and 1.5 mmol/l iodoacetate for 3 h, at 37 °C. After incubations, lipoproteins were separated by selective precipitation method employing 0.44 mmol/l phosphotungstic acid in the presence of magnesium ions. Radioactivity was measured in the incubation mixture and in the supernatant containing the HDL fraction in a liquid scintillation analyzer (Packard 210TR; Packard Instruments, Meridian, CT). Results were expressed as percentage of ^{3}H-cholesteryl esters transferred from HDL_3 to apo B-containing lipoproteins, per ml, per hour. Measurements were all carried out in duplicate within the same assay. Within-run precision (CV) was 4.9%.

2.6. Insulin, adiponectin, oxidized LDL and soluble cell adhesion molecules

Insulin concentration was measured by microparticle enzyme immunoassay (MEIA) (ABBOTT, Japan). Results were expressed as mU/l. Homeostasis model assessment (HOMA-IR) was calculated by [glucose (mmol/l) · insulin (U/ml)]/22.5 and quantitative sensitivity check index (QUICKI) by 1/[ln glucose (mmol/l) + ln insulin (mU/l)]. Adiponectin levels were measured by monoclonal antibody based enzyme-linked inmunosorbent assay (ELISA) (R&D Systems, Minneapolis, MN). Results were expressed as ng/ml. Oxidized LDL (Mercodia AB, Uppsala, Sweden) levels were determined by ELISA. Results were expressed as U/l. Levels of VCAM-1, ICAM-1 and E-selectin were determined by monoclonal antibody-based enzyme-linked immunosorbent assay (ELISA) (R&D Systems). Results were expressed as ng/ml.

2.7. Data and statistical analysis

Minimum sample size was estimated employing power analysis for the main parameters studied in this work and taking into consideration that data would be analyzed by ANOVA test. For this estimation, given power and significance were 80% and 0.05, respectively. The G*Power 3.1.3 program was employed.

Data distribution was tested using the modified Shapiro–Wilks method. Parameters following Gaussian distribution were presented as the mean ± SD and ANOVA parametric test (Tukey test) was used to compare the different groups. The median (range) expression and the non-parametric test (Kruskal Wallis test) were employed for data that did not follow the Gaussian distribution. Differences were considered significant at $p<0.05$ in the bilateral situation.

Six different multiple regression analyses were performed considering in each case one of the following dependent variables: HDL-C, adiponectin, CETP, ICAM-1, VCAM-1 and oxLDL. In these models, age, waist circumference, TGs and HOMA-IR were included as independent variables.

Table 1
General characteristics from HTG-IR patients compared with HTG-WIR patients and NTG-WIR subjects.

	HTG-IR patients	HTG-WIR patients	NTG-WIR subjects	p<
N	15	15	30	–
Age (years)	46 ± 12	48 ± 14	41 ± 14	NS
BMI (kg/m^2)	29 (25–30)^a	27 (23–30)^a	25 (21–30)^b	0.005
Waist (cm)	100 ± 7^a	95 ± 9^{ab}	91 ± 8^b	0.01
Hypertension (%)	33	20	23	NS
Smokers (%)	25	20	13	NS
Glucose (mmol/l)	5.7 (4.9–10.7)^a	5.3 (4.5–6.4)^{ab}	5.1 (3.9–6.4)^b	0.05
Insulin (mU/l)	16.0 (14.1–30.0)^a	6.6 (4.6–10.3)^b	5.7 (2.6–9.6)^b	0.0001
HOMA-IR	4.2 (3.2–7.4)^a	1.7 (1.0–2.2)^b	1.3 (0.6–2.3)^c	0.0001
QUICKI	0.22 (0.20–0.24)^a	0.27 (0.24–0.32)^b	0.30 (0.25–0.40)^b	0.05

HTG, hypertriglyceridemic; NTG, normotriglyceridemic; IR, insulin resistance; WIR, without insulin resistance; BMI, body mass index; HOMA-IR, Homeostasis Model Assessment; QUICKI, Quantitative Sensitivity Check Index.; NS, non significant. Results were expressed as media ± S.D. or median (range), depending on parametric or non parametric distribution of the data, respectively. p value was obtained by ANOVA. Different letters show differences between groups.

3. Results

Table 1 shows general characteristics from the 3 studied groups, which presented similar ages, percentage of hypertensive subjects and smokers. As it was expected, body mass index (BMI), waist circumference and glucose levels were higher in the HTG-IR group compared with NTG-WIR subjects. In agreement with the inclusion and classification criteria, insulin levels and HOMA-IR were significantly higher while QUICKI significantly lower in the HTG-IR group compared with WIR subjects. It is remarkable that even though HTG-WIR subjects showed significantly higher HOMA-IR than the NTG-WIR controls, there were no significant differences in glucose and insulin levels or in QUICKI between both groups. Adiponectin concentration was significantly lower in HTG patients independently of IR than NTG controls (Fig. 1).

On the other hand, the most atherogenic lipid and lipoprotein profile was observed in HTG-IR patients (Table 2). In fact, HDL-C concentration was significantly lower only in the HTG-IR subjects in comparison with both WIR groups. Moreover, both HTG groups exhibited the expected increase in TGs and non-HDL-C levels, due to an increment in VLDL fraction.

HDL chemical composition results are depicted in Table 3. HDL from HTG patients showed TG enrichment and decreased phospholipid content compared with NTG-WIR controls, while only particles from HTG-IR patients were partially depleted in cholesterol.

Both groups of HTG patients, regardless of the presence of IR, showed increased CETP activity with respect to NTG-WIR controls [HTG-IR = 281 (113–341), HTG-WIR = 270 (243–302) and NTG-WIR = 242 (138–297) %/ml.h; p<0.001]. Conversely, soluble cell adhesion molecules from vascular endothelium (VCAM-1, ICAM-1 and E-selectin) were higher only in the HTG-IR group, being only statistically significant for VCAM-1 (Fig. 2). Similarly, oxLDL levels were significantly higher in the HTG-IR patients compared to NTG-WIR controls (Fig. 3).

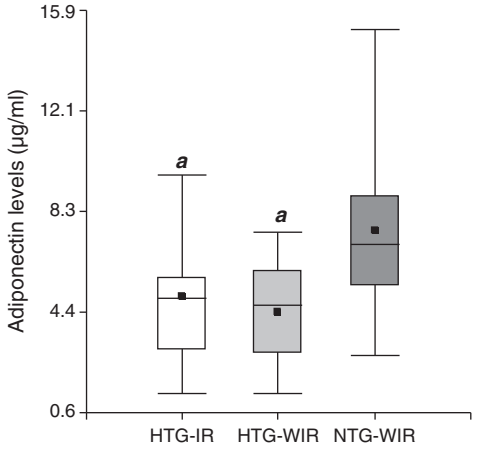

Fig. 1. Adiponectin plasma levels in HTG-IR patients (n = 15) compared with HTG-WIR patients (n = 15) and NTG-WIR controls (n = 30).NTG, normotriglyceridemic; HTG, hypertriglyceridemic; IR, insulin resistance; WIR, without insulin resistance. ^{a}p<0.05 *vs*. NTG-WIR.

TGs and HOMA-IR effects on HDL-C, adiponectin, CETP, ICAM-1, VCAM-1 and oxLDL were analyzed by multiple regression analyses. Remarkably, TG levels were the only independent predictor for the decrease in HDL-C and for the increase in CETP and oxLDL (ß = −0.034, $r^2 = 0.27$, p = 0.02; ß = 0.149, $r^2 = 0.24$, p<0.0001; and ß = 0.109, $r^2 = 0.22$, p = 0.001, respectively) in a model adjusted by age, waist circumference and HOMA-IR.

4. Discussion

The main finding of the present study was that most of the alterations described in lipid and lipoprotein profile, in lipid transfer proteins and in other biomarkers of CVD were associated with hypertriglyceridemia independently of the existence of an IR state.

Both HTG groups evaluated in the present study, with and without IR, shared the increase in the anthropometric measures, BMI and waist circumference, the increment in VLDL-C and non-HDL-C levels, the alteration in HDL chemical composition, consistent of TG-enrichment and phospholipid depletion, the decrease in adiponectin concentration, and the increase in CETP activity. In addition, the present study highlights the fact that IR contributes to amplify CVD risk in HTG patients. In fact, HTG-IR patients exhibited lower HDL-C and higher oxLDL and VCAM-1 concentrations than controls, while HTG-WIR patients did not. Nonetheless, multiple regression analysis identified TG as the only independent predictor of HDL-C concentration, a relationship largely reported in the bibliography [26,27], of CETP activity and of oxLDL.

Up to our knowledge, this would be the first time that oxLDL levels are directly evaluated in plasma from non-diabetic HTG patients. It is noteworthy that the method employed in the present study evaluates LDL particles oxidized *in vivo*, which probably underwent minimal oxidation in the artery wall and then returned to plasma circulation [28]. Thus, these results confirm previous suggestions made based on the evaluation of LDL susceptibility to *in vitro* oxidation [29] and on the detection of negatively-charged LDL particles from HTG subjects [1]. One of the factors responsible for the increment in oxLDL levels could be the higher CETP activity observed in both HTG groups, which is known to render LDL TG-enriched. Then, hepatic lipase may act on this modified LDL with high affinity thus generating easily oxidable sdLDL particles [30,31].

In parallel, high CETP activity which promotes the exchange of TGs by cholesteryl esters between apo B-containing lipoproteins and HDL might be accountable for the enrichment in TGs of HDL particles from

Table 2
Lipid and lipoprotein profile from HTG-IR patients compared with HTG-WIR patients and NTG-WIR subjects.

	HTG-IR patients (n = 15)	HTG-WIR patients (n = 15)	NTG-WIR subjects (n = 30)	p<
TG (mmol/l)	2.84 (1.93–7.48)[a]	2.18 (1.70–8.59)[a]	1.18 (0.45–1.63)[b]	<0.001
TC (mmol/l)	6.5 ± 1.9	6.3 ± 1.2	5.7 ± 1.2	NS
VLDL-C (mmol/l)	1.09 (0.57–5.38)[a]	1.01 (0.49–3.28)[a]	0.46 (0.23–1.53)[b]	<0.001
LDL-C (mmol/l)	4.4 ± 1.0	4.4 ± 1.2	4.1 ± 1.2	NS
HDL-C (mmol/l)	0.90 ± 0.21[a]	1.03 ± 0.34[b]	1.27 ± 0.26[b]	<0.0005
Non-HDL-C (mmol/l)	5.2 (2.7–10.8)[a]	5.4 (3.1–7.1)[a]	4.6 (2.3–6.5)[b]	<0.05
Apo B (g/l)	1.12 ± 0.25	1.21 ± 0.30	1.05 ± 0.32	NS
Apo A-I (g/l)	1.27 (1.00–1.98)	1.20 (0.86–1.91)	1.29 (1.00–2.89)	NS

HTG, hypertriglyceridemic; NTG, normotriglyceridemic; IR, insulin resistance; WIR, without insulin resistance; TGs, triglycerides; TC, total cholesterol; VLDL, very low density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins; apo, apolipoprotein. NS, non significant. Results were expressed as media ± S.D. or median (range), depending on parametric or non parametric distribution of the data, respectively. p value was obtained by ANOVA. Different letters show differences between groups.

Table 3
HDL chemical composition from HTG-IR patients compared with HTG-WIR patients and NTG-WIR subjects.

	HTG-IR patients (n = 15)	HTG-WIR patients (n = 15)	NTG-WIR subjects (n = 30)	p<
TGs (%)	10.0 (4.3–19.3)[a]	9.2 (4.2–22.3)[a]	4.8 (2.6–10.7)[b]	<0.0005
Cholesterol (%)	14.3 ± 3.0[a]	17.6 ± 4.4[b]	17.4 ± 2.9[b]	<0.05
Phospholipids (%)	16.8 (11.0–28.2)[a]	18.0 (26.7–9.0)[a]	23.4 (29.8–9.3)[b]	<0.05
Proteins (%)	57 ± 6	56 ± 8	54 ± 5	NS

HTG, hypertriglyceridemics; NTG, normotriglyceridemics; IR, insulin resistance; WIR, without insulin resistance; HDL, high density lipoproteins; TGs, triglycerides; C, cholesterol; PL, phospholipids; TP, total proteins. NS, non significant. Results were expressed as media±S.D. or median (range), depending on parametric or non parametric distribution of the data, respectively. p value was obtained by ANOVA. Different letters show differences between groups.

HTG patients, among other lipoprotein alterations. The modification of HDL core lipids in conjunction with the depletion in superficial phospholipids was found to have major negative impacts on HDL atheroprotective capacities [18,32]. Actually, in HTG-IR patients, a reduction in HDL anti-inflammatory capacity to inhibit the expression of cell adhesion molecules and the increment observed in oxLDL levels could act together to induce the endothelial dysfunction evidenced by the detected increase in VCAM-1 concentration.

In the present study, the use of HOMA-IR as indicator of insulin resistance and even the employ of a cut-off point not calculated from the studied population might be a source of bias. However, the choice of specific selection criteria designed to prevent overlapping between HTG-IR and HTG-WIR groups tends to improve subject categorization and to avoid misclassification. In particular, the HOMA-IR value <2.6 which characterized patients without IR was the lowest reported for Argentinean population [21], while the cut-off point to define presence of IR was the highest described in our country population [20]. Even if the number of subjects evaluated in the present study could seem quite low, the sample size required to detect significant differences between groups was previously estimated by adequate statistical methods.

5. Conclusions

Overall, the differences observed in most lipoprotein parameters and CVD biomarkers in both HTG groups in comparison with controls suggest that the increase in TG levels is the major determinant of the atherogenic modifications here described, while, in some cases, IR would be an amplifier factor. The higher oxLDL levels associated with HTG might be attributed to increased CETP activity, predominance of sdLDL particles and impaired HDL antioxidant capacity. To further confirm these findings, it would be necessary for other studies to add a model characterized by NTG and IR, a condition scarcely observed in humans. Moreover, a similar study carried out not only in fasting but also in postprandial state could further extend the results obtained.

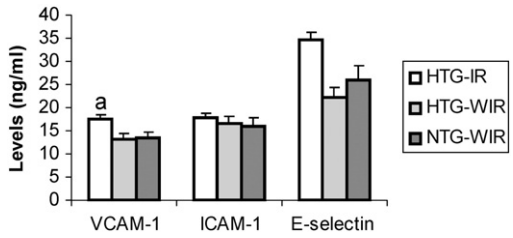

Fig. 2. Cell adhesion molecules' (VCAM-1, ICAM-1 and E-selectin) plasma levels from HTG-IR patients (n = 15) compared with HTG-WIR patients (n = 15) and NTG controls (n = 30).HTG, hypertrigliceridemic; NTG, normotriglyceridemic; IR, insulin resistance; WIR, without insulin resistance. [a]p<0.05 *vs.* HTG-WIR and NTG-WIR.

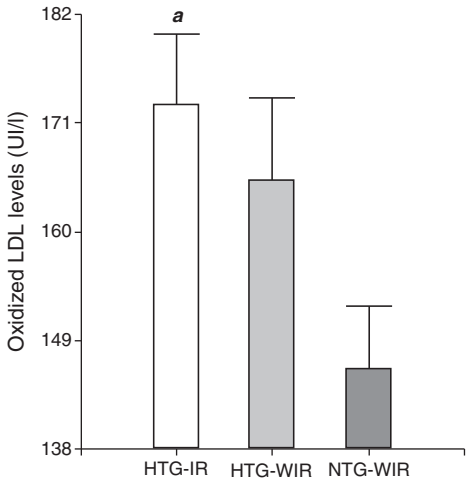

Fig. 3. OxLDL plasma levels from HTG-IR patients (n = 15) compared with HTG-WIR patients (n = 15) and NTG controls (n = 30).NTG, normotriglyceridemic; HTG, hypertriglyceridemic; IR, insulin resistance; WIR, without insulin resistance; LDL, low density lipoproteins. [a]p<0.05 *vs.* NTG-WIR.

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