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# Predominance of large VLDL particles in metabolic syndrome, detected by size exclusion liquid chromatography

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ABSTRACT

**Objective:** To study size heterogeneity of triglyceride rich lipoproteins (TRL) in metabolic syndrome (MS).

**Design and methods:** Thirty MS patients and 14 healthy subjects were included. In fasting serum we measured: lipid profile, free fatty acids (FFA) and adiponectin; TRL were isolated ($d<1.006$ g/mL) and analysis by size exclusion HPLC followed by UV detection was performed; each subfraction was expressed as percentage of total TRL.

**Results:** MS patients, even those with normal triglycerides, presented higher proportion of very large VLDL (90 nm diameter) and large VLDL (60 nm) and slightly lower of typical VLDL (37 nm) ($p<0.04$); increased FFA ($p=0.04$) and lower adiponectin ($p=0.001$). FFA correlated with large VLDL% ($r=0.58$; $p=0.003$), independently of insulin-resistance and waist. Furthermore, the lower the adiponectin, the greater the predominance of large VLDL ($r=-0.40$; $p=0.04$).

**Conclusion:** MS was associated with large VLDL, described as more atherogenic beyond triglyceride levels. Size exclusion HPLC would represent a useful tool for assessing subfractions' lipoprotein profile.



## Introduction

Metabolic syndrome (MS) is a common disorder that consists of a cluster of several clinical and metabolic factors accompanied by an increased risk for cardiovascular disease and type 2 diabetes. The atherogenic dyslipidemia associated to this syndrome is characterized by elevated fasting triglyceride (TG) and low high-density lipoprotein cholesterol levels, predominance of small dense LDL subfraction with higher atherogenic capacity, and postprandial accumulation of TG rich lipoproteins (TRL) [1].

Lipoproteins constitute heterogeneous particle families based on their density, particle size and electrophoretic mobility. High performance liquid chromatography (HPLC) with size exclusion columns is at the moment an alternative accurate tool for determining lipoprotein size and would also represent a method for classifying lipoprotein subfractions [2]. Therefore, size exclusion HPLC could be applied to isolated TRL in order to analyze the lipoprotein fraction profile.

Triglyceride rich lipoproteins are not easy to define; they can be identified as a family of lipoproteins composed by chylomicrons, very low density lipoprotein (VLDL) and their remnants comprising a heterogeneous group of particles of different origin, size and composition [3]. In insulin resistant states, there is a hepatic overproduction of VLDL, in addition, TRL catabolism would be reduced, and remnant lipoproteins are consequently accumulated, being present in plasma even in fasting periods [4]. Beyond plasma lipid concentrations, increased TRL are recognized to have an important role in the atherogenic process [5,6], that is why their identification would be relevant.

Moreover, MS is closely associated with visceral fat accumulation contributing to a pro inflammatory state, characterized by a reduced adiponectin among other cytokine alterations. It is known that there is an inverse correlation between adiponectin and TG plasma levels [7]. However, whether adiponectin has a relation with TRL size heterogeneity has not been completely elucidated yet.

Our aim was to study TRL size in relation to metabolic and clinical parameters in patients with MS and healthy controls, applying a size exclusion HPLC method.

## Materials and methods

Thirty patients from both sexes (70% females and 30% males, mean age $39\pm12$ years) with diagnosis of metabolic syndrome (ATPIII) [1],

*Abbreviations:* MS, metabolic syndrome; TRL, triglyceride rich lipoprotein; HPLC, high performance liquid chromatography; VLDL, very low density lipoprotein; FFA, free fatty acids.

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were selected consecutively for the present study among subjects who attended at the outpatient clinic service of the University Hospital in Buenos Aires. In parallel, fourteen clinically and biochemically healthy subjects, maintaining similar proportion in gender and age (65% women and 35% males, mean age 34 ± 13 years, p = 0.1) were recruited among hospital employees as control group. Subjects were excluded when they presented diabetes mellitus, kidney disease, cardiovascular disease, neoplasia, hypothyroidism, or other endocrine diseases, and when receiving lipid-lowering drugs, hormonal or insulin-sensitizing treatments. Those who consumed more than 20 g alcohol/day were also excluded. Written informed consent was required of all the participants to be included in the study, which had the approval of the Ethics Committee of the Faculty of Pharmacy and Biochemistry, University of Buenos Aires.

Waist circumference, weight and height were measured and body mass index (BMI) calculated. After a 12-h overnight fast blood samples were drawn. Serum was kept at 4 °C within 48 h for the measurement of glucose, total cholesterol and TG levels using commercial enzymatic kits (Roche Diagnostics, Mannheim, Germany) in a Hitachi 917 autoanalyzer, CV intra-assay <1.9%, and CV inter-assay <2.4%, averaging CV values of these parameters. HDL and LDL cholesterol were determined by standardized selective precipitation methods [8,9], CV intra-assay <2.0% and CV inter-assay<3.0%. Another aliquot of serum was stored at −70 °C for further determination of insulin (Immulite/Immulite 1000 Insulin, Siemens, USA), free fatty acids (FFA) (Randox, UK) and adiponectin (ELISA, R&D Systems, USA). In order to estimate insulin-resistance, the homeostasis model assessment for insulin resistance index (HOMA-IR) was calculated [10]. All parameters were assessed under good quality controls.

Fresh sera were centrifuged 30 min at 15,000 rpm in order to discard the eventual presence of chylomicrons. From the infranatant, TRL were isolated by preparative ultracentrifugation at density d<1.006 g/mL 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 [11]. Purity of lipoprotein was tested by agarose gel electrophoresis. Furthermore, the possible contamination with serum albumin was also investigated by SDS-polyacrylamide gel electrophoresis followed by silver staining [12] finding that only a tiny band corresponding to albumin was present. Quantification assay using Albumin Tina-Quant (Roche Diagnostics, Mannheim, Germany) in a Hitachi 917 yielded only traces of albumin (<1 mg/dL).

The obtained pure TRL fraction was then subjected to size exclusion chromatography by HPLC, as was previously reported [13,14]. A column TSK-Gel Lipopropack XL, 7.8 mm ID × 30 cm (Tosoh, Japan) was used, 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. For the conversion of elution time in particle diameter, a standard curve was implemented, constructed with the logarithm of retention time *vs.* the logarithm of the diameter of size standard latex particles of 100 nm (Fluka, Sigma-Aldrich), 39 and 27 nm in diameter (Magsphere INC). From chromatograms we could recognize a peak at 9.95 ± 0.10 min with a diameter of 90 ± 3.0 nm, which was identified as fraction 1 A, another peak at 12.46 ± 0.48 min with an average diameter of 60.0 ± 3.6 nm, fraction 1 B, a majority peak at 22.35 ± 0.05 min and a diameter of 37.3 ± 0.08 nm identified as fraction 2 and finally smaller peaks were detected at longer retention times (from 24 to 32 min) and sizes about 35 to 30 nm.

Results are expressed as the percentage of each peak area respect to total TRL area, using the ChromQuest 4.1 integration program. From repeated injections (n = 11) of isolated TRL from a donor sample, the within-day CV% for the retention times was 1.1% and for peak area proportions, average of CV% was 5.3%. The between-day CV% was performed through 20 consecutive days of a fractional sample conserved at −20 °C, being 2.1% for retention times and an average of 6.0% for peaks area proportions.

Data is presented as mean ± SD or median (range) according to normal or skewed distribution, respectively. Differences between groups were tested using the unpaired Student's *t* test for normally distributed data and the Mann–Whitney *U*-Test for skewed data. Pearson or Spearman analysis, for parametric or non parametric

**Table 1**
Clinical and biochemical characteristics of studied subjects: metabolic syndrome patients (ATPIII) and healthy controls.

| | Healthy controls | Metabolic syndrome | p Value |
|---|---|---|---|
| Age (years) | 34.2 ± 12.7 | 39.1 ± 12.9 | 0.3610 |
| BMI (kg/m$^2$) | 22.1 ± 1.7 | 35.4 ± 6.1 | 0.0001 |
| Waist circumference (cm) | 73.5 ± 8.6 | 102.6 ± 7.2 | 0.0001 |
| Triglyceride (mmol/L) | 1.02 ± 0.41 | 2.25 ± 114 | 0.0124 |
| Total cholesterol (mmol/L) | 5.51 ± 0.86 | 5.28 ± 0.88 | 0.5000 |
| LDL-cholesterol (mmol/L) | 3.56 ± 1.01 | 3.69 ± 0.91 | 0.8317 |
| HDL-cholesterol (mmol/L) | 1.51 ± 0.36 | 0.94 ± 0.23 | 0.0010 |
| Insulin (μU/mL) | 5.6 (2.0–16.8) | 13.5 (3.3–66.5) | 0.0498 |
| Glucose (mmol/L) | 5.00 ± 0.28 | 5.55 ± 0.66 | 0.0385 |
| HOMA-IR | 1.21 (0.43–3.98) | 3.33 (0.80–18.20) | 0.0485 |

Data is mean ± SD or median (range) for parameters with skewed distributed. BMI: Body mass index; HOMA-IR: Homeostasis model assessment for insulin resistance index.

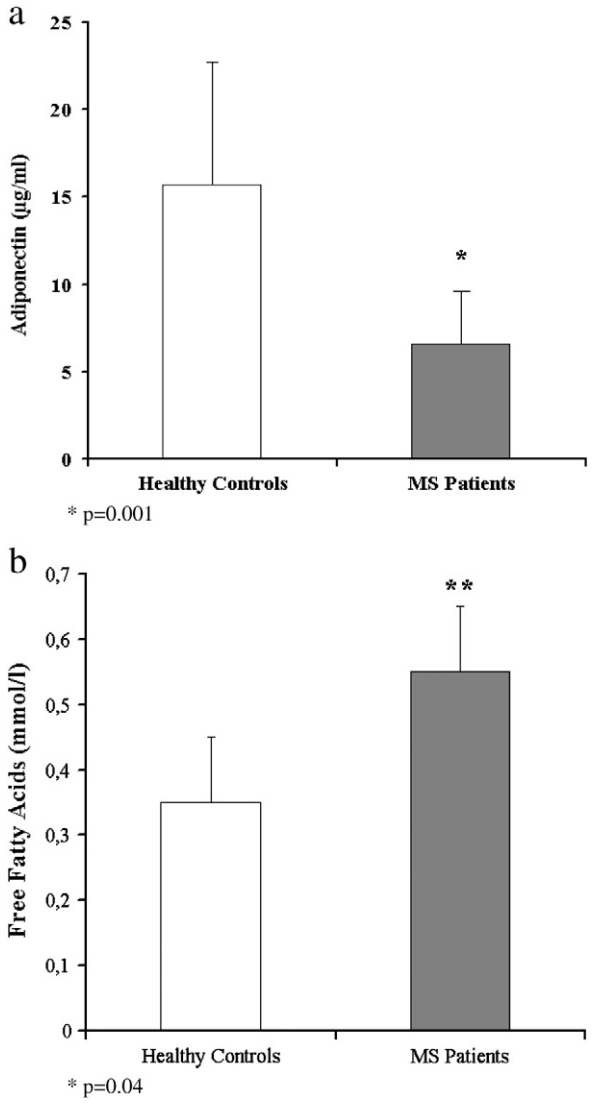


**Fig. 1.** Bar graph shows the adiponectin levels (panel a) and free fatty acids concentration (panel b) in metabolic syndrome (MS) patients (grey bar) and healthy controls (white bar).*p = 0.001.**p = 0.04.

variables, was used to determine correlations between parameters. A multivariate stepwise regression model was developed in order to assess associations between parameters. All analyses were performed using SPSS 17.0 software. P values $<0.05$ were considered significant.

## Results

Table 1 shows clinical and biochemical characteristics of the subjects studied. As expected, patients with MS presented decreased HDL cholesterol, increased TG and glucose levels, as well as higher waist circumference, body mass index and HOMA-IR than healthy controls ($p<0.02$).

Fig. 1 demonstrates that adiponectin was significantly reduced ($p=0.001$) and FFA levels were increased ($p=0.04$) in MS patients in comparison to healthy controls.

When isolated TRL was analyzed by HPLC size exclusion chromatography (Fig. 2), patients with MS presented higher proportion of peaks 1 A: median (range): 18.6% (0.6–36.9) *vs.* 6.2% (0.4–24.0) $p=0.039$ and 1 B: 23.0% (2.1–57.4) *vs.* 9.6% (1.0–26.5) $p=0.045$. These peaks correspond to larger TRL particles. Subsequently, a relative diminishment of peak 2 was observed, 62.5% (27.2–98.2) *vs.* 90.3% (72.2–98.9) $p=0.020$, this major peak would correspond to typical VLDL. Finally, no differences were observed in the proportion of the peaks with longer retention time, whose areas were practically negligible.

In order to appreciate whether the higher proportion of peaks 1 A and 1B is connected to the increase in plasma TG in MS group, TRL subfractions were analyzed after subdividing MS patients into two groups regarding their TG levels: $>1.71$ mmol/L ($n=18$) and $\leq 1.71$ mmol/L ($n=12$). No statistical differences were observed between TRL subfraction profiles in both MS sub-groups. Furthermore, differences between MS group with TG $\leq 1.71$ mmol/L and healthy controls, were still statistically significant (Table 2). In Fig. 3 it can be appreciated the dispersion of peaks 1A and 1B proportion in both MS sub-groups.

The increment of larger TRL showed interesting associations with other metabolic parameters. Both fractions, 1 A and 1 B, correlated positively with plasma triglyceride levels, $r=0.50$; $p=0.015$ and $r=0.56$; $p=0.0051$ respectively. Circulating FFA correlated with the proportion of peak 1 B ($r=0.58$; $p=0.003$), even after adjusting by HOMA-IR and waist girth ($F=5.9$; $p=0.027$). This correlation was not significant with peak 1 A ($r=0.03$; $p=0.92$), however the proportion of this peak correlated with waist circumference ($r=0.46$; $p=0.026$), also after adjusting by HOMA-IR ($F=3.9$; $p<0.05$). On the other hand, waist circumference presented a strong tendency to correlate with peak 1 B ($r=0.39$; $p=0.054$). As regards to adiponectin, it was inversely associated with peak 1 B proportion ($r=-0.40$; $p=0.04$), showing a tendency to correlate with peak 1 A ($r=-0.38$; $p=0.06$).

**Table 2**
Triglyceride rich lipoprotein (TRL) proportion in metabolic syndrome (MS) patients, divided according to their triglyceride (TG) levels ($>$ or $\leq 1.71$ mmol/L), and healthy controls.

| TRL fractions | MS patients | | Healthy controls |
|---|---|---|---|
| | TG $>1.71$ mmol/L | TG $\leq 1.71$ mmol/L | |
| Peak 1 A (%) | 21.3 (3.3–36.6) | 16.7 (0.6–36.9) | 6.2 (0.4–24.0)* |
| Peak 1 B (%) | 20.6 (4.9–50.2) | 14.8 (2.1–57.4) | 9.6 (1.0–26.5)* |
| Peak 2 (%) | 61.9 (27.2–98.2) | 77.6 (47.2–96.4) | 90.3 (72.2–98.9)** |
| Peak 3 (%) | 2.5 (0.7–15.2) | 2.3 (1.0–6.3) | 2.0 (0.1–6.2) |

No differences were observed between both MS groups. *$p<0.05$ in relation to both MS groups. **$p<0.020$ in comparison to MS patients with plasma TG levels $>1.71$ mmol/L.

## Discussion

In this study, we applied size exclusion HPLC to TRL fraction isolated from MS patients and controls, expressing the results as proportions of the subfractions obtained in each chromatogram. Results showed that in MS in comparison to controls there was a higher proportion of peaks corresponding to larger lipoprotein subfractions (peaks 1 A and 1 B), and less proportion of the major peak (peak 2) compatible with typical VLDL. This profile does not seem to be exclusively related to the presence of hypertriglyceridemia. It is also notable the observation that the lower the adiponectin levels, the greater the proportion of large TRL particles. Moreover, the increased proportion of these particles was directly related to abdominal obesity, expressed as waist circumference. Based on other significant correlations obtained, it can be

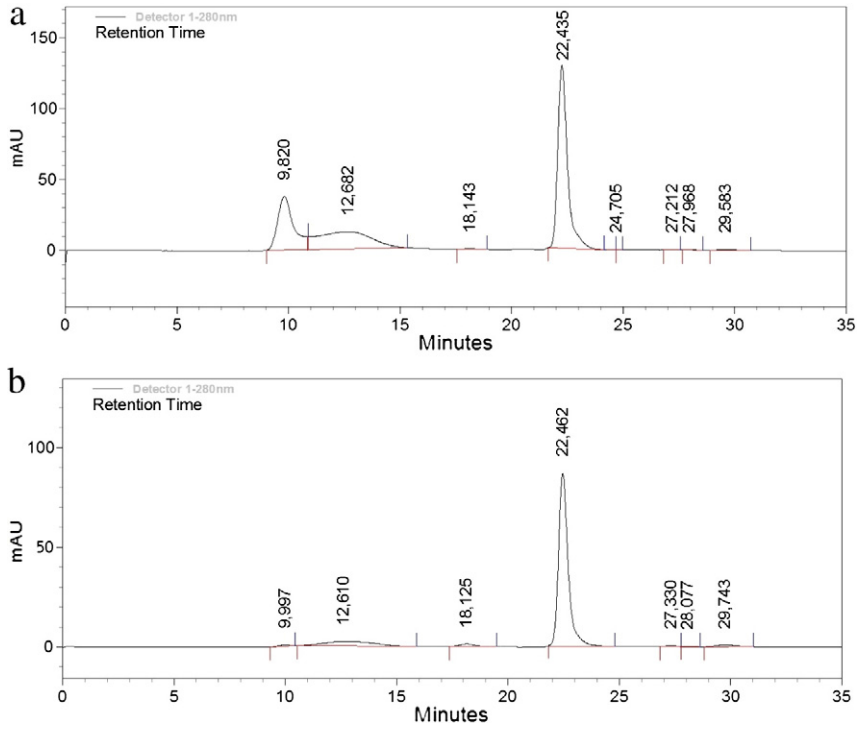


**Fig. 2.** Triglyceride-rich lipoprotein (TRL) profiles from a metabolic syndrome (MS) patient (panel a) and from a healthy control (panel b).

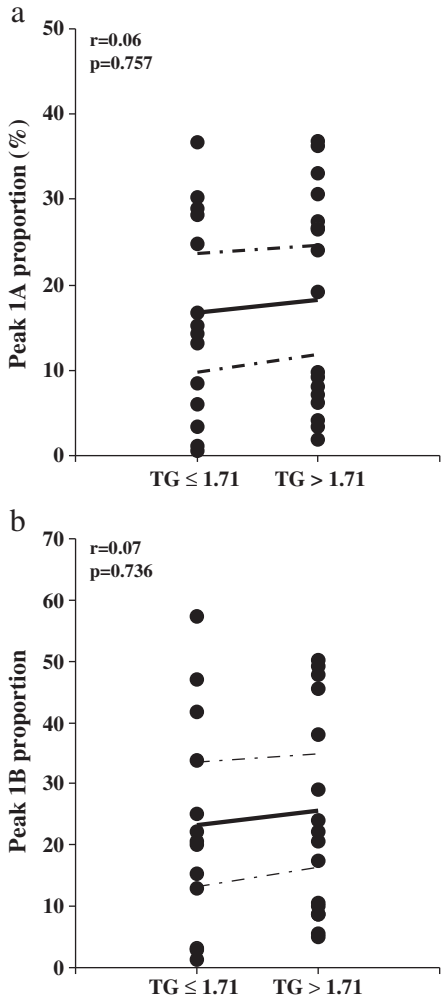


**Fig. 3.** Distribution of peak 1 A and 1 B proportion (panels A and B respectively) in metabolic syndrome (MS) sub-groups divided according to their triglyceride (TG) levels (> or ≤ 1.71 mmol/L).

suggested that the increase in circulating FFA induces the production of large VLDL particles (peak 1 B), independently of insulin resistance.

It is recognized that an accurate measurement of TRL as a whole has been difficult because in plasma they comprise a continuous spectrum of lipoproteins of different size, density and composition. Size exclusion HPLC is proposed as a useful tool for classifying lipoproteins subclasses in base on their size [2], because this technique presents a short experimental time, small sample requirement, high resolution and excellent reproducibility. Herein, HPLC also demonstrated to be an efficient method for the determination of TRL subfractions based on their differences in size, giving separate peaks for the TRL fractions that we have identified as: peak 1 A, that would be a group of TRL composed by very large VLDL, probably including some apoB-48 carrying lipoproteins, present even in fasting state in the MS; peak 1 B with a diameter around 60 nm which would match to large VLDL identified by Okazaki M et al. [15]; peak 2, of the greatest proportion and a size about 37 nm, that would correspond to typical VLDL; and at last very small peaks at low concentration that could represent some smaller VLDL fractions. Okazaki M et al. reported that TSK gel columns were suitable for discriminating among lipoprotein subclasses in whole serum using a dual online detection method [15]. In our study, the detection of lipoprotein subfractions in the column eluate was made by monitoring at 280 nm, given that we employed the isolated TRL fraction, avoiding interferences from other plasma proteins, in fact only traces of contaminant albumin were found in the isolated TRL fractions.

Size of lipoproteins constitutes one of their characteristics that determine the heterogeneity related to their atherogenicity. Herein, we found that patients with MS present not only a higher proportion of large VLDL particles, but also a probable increase in chylomicron remnants. Different reports carried out in patients with MS, have described that there is an increase in remnants-like particle cholesterol [4], measured by the immunoaffinity method developed by Nakajima K [16], as well as an increase in fasting apoB-48 as an indicator of chylomicron remnants presence [17]. Adiels M. et al., based on VLDL *in vivo* kinetic studies, discussed that in MS there is an over-secretion of TG enriched VLDL estimated of large size (called $VLDL_1$) [18]. Apparently, there are no previous reports as regards to VLDL subfraction detection by size exclusion HPLC in MS.

As expected, analyzing the studied groups together, an association between plasma TG levels and large VLDL subfractions was observed. However, when evaluating MS group subdivided according to the presence or not of hypertriglyceridemia, the predominance of large VLDL was also found in MS patients with normal TG levels, which in turn, was still higher in comparison to healthy controls. These results suggest that the alterations in TRL profile in MS are not exclusively dependent on TG levels.

The predominance of large VLDL in insulin-resistant state could be explained by a hepatic overproduction of TG that are assembled into a VLDL secreted overloaded in TG. It was described that these types of lipoproteins are precursors of the atherogenic small dense LDL particle [19]. Moreover, in previous studies we have observed that VLDL over enriched in TG is highly hydrolysed by lipoprotein lipase *in vitro* [20]. Thus, it can be expected that large VLDLs present in MS will be more efficiently lipolyzed, leading to the formation of smaller particles that are able to pass through the endothelial wall, enhancing TG and cholesterol ester accumulation in the intima. Our results contribute to confirm that in MS there is an accumulation of large VLDL and posprandial remnants lipoproteins, which are known to have high atherogenic potential.

Visceral fat accumulation also plays a role in this hallmark. The FFA flux from adipose tissue to the liver is related with the increase in intra-hepatic TG production [21]. In fact, we have found a positive correlation between the FFA levels and the proportion of fraction 1 B, equivalent to circulating large VLDL, independently of insulin resistance degree. Other authors support the relationship between FFA levels and the increase in the production of VLDL rich in TG and probably larger in size, studying type 2 diabetic men [22].

It was reported that waist circumference, as an indicator of abdominal obesity, is related to a greater concentration of chylomicrons and large VLDLs measured altogether by nuclear magnetic resonance [23]. This is in accordance with the significant direct correlation observed in this study between waist circumference and the proportions of the peaks 1 A and 1 B detected separately by HPLC, probably corresponding to chylomicron remnants and large VLDL, respectively.

Adiponectin, a fat-derived adipocytokine, has a role on the regulation of lipoprotein metabolism. It is notable that hipoadiponectinaemia, frequent in insulin resistant states, was associated with peak 1 B or large VLDL particles. This finding is in line with Weiss et al., who described that there was an opposite relationship between adiponectin levels and VLDL size, measured by nuclear magnetic resonance [24]. The action of adiponectin on TRL profile would be a consequence of increasing VLDL secretion over enriched in TG [25] or of reducing their catabolic rate [26], or probably a combination of both effects.

## Conclusion

Results have shown that MS presents an altered TRL subfraction profile, evidenced in isolated fasting TRL fraction by size exclusion liquid chromatography. The predominance of larger TRL particles in MS would contribute to the atherogenic risk beyond fasting plasma TG concentrations.

## Conflict of interest

There are no conflicts of interest to disclose.

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