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Nonalcoholic fatty liver disease associated with metabolic syndrome: Influence of liver fibrosis stages on characteristics of very low-density lipoproteins

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ABSTRACT

Background: We evaluated possible changes in VLDLcharacteristics, and metabolic related factors, in MetS-associated NAFLD and accompanying liver fibrosis.

Methods: We studied 36 MetS patients with biopsy-proven NAFLD (MetS + NAFLD) and 24 MetS without ultrasound NAFLD evidence. Further, MetS + NAFLD was sub-divided according to fibrosis stage into, non-to-moderate (F0–F2, n = 27) and severe (F3–F4, n = 9) fibrosis. We measured: lipid profile, VLDL composition and size (size exclusion-HPLC), CETP and lipoprotein lipase (LPL) activities and adiponectin. Additionally, in MetS + NAFLD type IV collagen 7S domain was measured.

Results: MetS + NAFLD showed increased VLDL-mass, VLDL particle number, VLDL-triglyceride% and large VLDL-% ($p < 0.04$). CETP activity tended to increase in MetS + NAFLD ($p = 0.058$), while LPL activity was unchanged. Moreover, in MetS + NAFLD, adiponectin was decreased ($p < 0.001$), and negatively correlated with VLDL-mass and VLDL particle number ($p < 0.05$), independently of insulin-resistance. Within MetS + NAFLD group, despite greater insulin-resistance, patients with severe fibrosis showed lower plasma triglycerides, VLDL-mass, VLDL-triglyceride%, large VLDL-% and CETP activity ($p < 0.05$), while type IV collagen was increased ($p = 0.009$) and inversely correlated with large VLDL-% ($p = 0.045$).

Conclusions: In MetS, NAFLD is associated with larger and triglyceride over-enriched circulating VLDLs, of greater atherogenicity. However, when NAFLD progresses to severe fibrosis, circulating VLDL features apparently improved, probably due to early alterations in hepatic synthetic function.

1. Introduction

Metabolic syndrome (MetS) is a constellation of factors leading to higher risk of cardiovascular disease and type 2 diabetes. MetS is characterized by an atherogenic dyslipidemia consisting of elevated very low-density lipoprotein (VLDL) triglycerides, reduced HDL-cholesterol and the predominance of small dense LDL particles [1].

The VLDL constitutes a heterogeneous family of sub-fractions varying in composition and size [2]. Circulating VLDL is the resultant of its hepatic production, catabolism -mainly by lipoprotein lipase (LPL)- and the remodeling activity of cholesteryl lecithin acyl transferase (CETP) [3,4]. A clue factor for atherogenic dyslipidemia in MetS is the hepatic over-production of VLDL, which is principally driven by increased free fatty acid (FFA) flux from adipose tissue to the liver [5]. Recently we demonstrated that the alterations in VLDL composition, occurring in MetS, are associated with endothelial dysfunction [6].

Nowadays, nonalcoholic fatty liver disease (NAFLD) is the leading cause of chronic liver disease in Western world, being considered the hepatic manifestation of MetS [7]. NAFLD comprises a spectrum of alterations varying from simple steatosis to nonalcoholic steatohepatitis and different degrees of liver fibrosis. Among these alterations, liver fibrosis is recognized to be the less reversible of liver changes occurring

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in NAFLD and it would be consequence of disease activity [8]. Liver biopsy constitutes the only diagnostic tool to confirm nonalcoholic steatohepatitis and is the gold standard for the assessment of hepatic fibrosis; however, it represents an invasive procedure for fibrosis evaluation [9]. Some components of the extracellular matrix, such as 7S fragment of type IV collagen, has been proposed as a potential non-invasive marker for liver fibrosis in NAFLD [10,11]; all these studies have been performed in Asian population.

An increasing bulk of evidence indicates that NAFLD is an independent risk factor for cardiovascular disease [12–14]; however, the mechanisms are not fully understood. It seems paradoxical that apparent opposite situations take place in MetS: increased VLDL secretion on the one hand and liver fat deposits on the other. In this context, it is important to take into account that, alterations on VLDL structure and composition attribute increased atherogenic capacity [15]. The VLDL features might be affected by NAFLD and even more by its progression to advanced fibrosis stages because of the hepatocyte function compromise. Adiels M et al. demonstrated, by means of an *in vivo* kinetic assay in type 2 diabetic men, that NAFLD was linked to over-production of VLDL presenting greater triglyceride content and presumably larger in size, however authors did not distinguish between NAFLD stages [16]. Discordant results have recently been reported in studies addressing the influence of NASH or liver cirrhosis on circulating VLDL sub-fractions [17,18], probably due to variety in reported populations and applied methodology. It would be of interest to evaluate VLDL composition and size, and associated factors, such as LPL and CETP, in non-diabetic MetS with associated NAFLD and liver fibrosis.

Adiponectin is an adipose tissue derived cytokine with atheroprotective and insulin-sensitizing actions. In a previous report we have demonstrated that the reduction in adiponectin concentrations in NAFLD was related to an increase in circulating VLDL particle number [17]. Adiponectin would be related to NAFLD progression to advance fibrosis stages, and adiponectin could have a role on VLDL composition and sub-fraction profile in fatty liver disease.

Our aim was to evaluate, in the spectrum comprising from fatty liver disease to hepatic fibrosis in MetS, possible changes in VLDL characteristics and metabolic related factors in order to understand how NAFLD contributes to the increase in cardiovascular risk.

2. Patients and methods

2.1. Subjects

Thirty-six patients with MetS –ATPIII criteria– [1] with biopsy-proven NAFLD were consecutively selected for the present study, among those patients attending to the Liver Unit from the National Hospital Professor Alejandro Posadas, Buenos Aires Province, Argentina. In parallel, other 24 patients with MetS without ultrasound evidences of fatty liver were also consecutively enrolled from the same service as a control group. To define the participants in the present study, the following exclusion criteria were considered: alcohol intake > 140 g/week, type 2 diabetes, recent history of acute illness, drugs which could modify lipid levels or induce fatty liver, seropositive hepatitis B or C, or any other health problem which could lead to liver steatosis.

Written informed consent was required of all the participants to be included in the study. Besides, the study had the approval of the Ethic Committees of the Hospital Nacional “Profesor Alejandro Posadas” and of the Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Argentina.

Weight and height of each participant were measured and body mass index (BMI) was calculated to evaluate obesity degree. Waist circumference –measured at the moment of MetS diagnosis– was taken midway between the lateral lower rib margin and the superior anterior iliac crest on a standing position, always by the same investigator.

2.2. Liver biopsy

Biopsy specimens were obtained under ultrasound guidance by the percutaneous route, using the Menghini method with a 16-gauge needle biopsy set (Hepafix ®, B. Braun Medical Ltd.). Our routine procedure includes performing 1 or 2 passes to assure samples at least 25 mm in length. Liver sections were routinely stained with hematoxylin and eosin, silver reticulin and Masson trichrome, and occasionally with Perls' Prussian blue and diastase-resistant periodic acid-Schiff. Liver biopsies were read by a single expert hepatopathologist, who estimated semi-quantitatively the NAFLD activity score and the fibrosis stage according to Kleiner et al. classification [8]. Briefly, specimens were examined for the grade of steatosis (grades 0–3), inflammation (grades 0–3) and ballooning (grades 0–2). NAFLD activity score was calculated as the sum of these three scores, ranging from 0 to 8. Moreover, fibrosis stage was also evaluated and graded as, stage F0: no liver fibrosis, stage F1: mild (F1A) or moderate (F1B) perisinusoidal fibrosis or portal/periportal-only fibrosis (F1C), stage F2: perisinusoidal and periportal fibrosis, stage F3: bridging fibrosis and stage F4: cirrhosis.

In order to evaluate whether the advance to liver fibrosis stages in NAFLD affects VLDL features and other metabolic parameters, patients with biopsy-proven NAFLD were sub-divided according to their fibrosis stage into those with non-to-moderate fibrosis (F0 to F2, n = 27) and those with severe fibrosis (F3 and F4, n = 9) [20].

2.3. Samples

After 12-h overnight fast blood samples were collected. Serum was kept at 4 °C for evaluation of liver enzymes, albumin, glucose, lipid and lipoprotein profile within 48 h, or stored at − 70 °C for further determination of insulin, FFA, CETP activity, adiponectin, type IV collagen 7s domain and for the isolation of VLDL by ultracentrifugation. Another aliquot of blood was collected on sodium citrate for the measurement of prothrombin activity in plasma.

For the determination of lipoprotein lipase activity, heparin (60 UI/kg body weight) was administered intravenously in those participants who accepted the administration. Ten minutes after, blood was drawn from the contralateral arm and the postheparin plasma was kept at − 70 °C until its processing.

2.4. Biochemical determinations

Total cholesterol, triglycerides, glucose, albumin and liver enzymes -aspartate aminotransferase (AST) and alanine aminotranferase (ALT) as markers of hepatic damage- were measured in serum, using commercial kits (Roche Diagnostics) in a Cobas c501 autoanalyzer, intra-assay CV < 1.9%, inter-assay CV < 2.4%, averaging CV values of these parameters. HDL and LDL cholesterol were determined by standardized homogeneous assays (Roche), CV intra-assay < 2.0% and inter-assay CV < 2.6%. Apo A-I and apo B were determined by immunoturbidimetry (Roche Diagnostics, Mannheim, Germany), intra-assay CV < 1.9% and inter-assay < 2.5%, for both parameters. FFA levels were determined by a spectrophotometric method (Randox; intra-assay CV < 2.6% and inter-assay CV < 3.9%. Insulin was measured with Immulite/Immulite 1000 Insulin (Siemens). In order to estimate insulin-resistance, the HOMA-IR index was calculated [21]. Prothrombin activity was determined in plasma by a standardized method.

2.5. Lipoprotein measurements

2.5.1. VLDL isolation and analysis

The VLDL was isolated by sequential preparative ultracentrifugation [22], density (d) < 1.006 g/mL, in a Beckman XL-90 using a fixed-angle rotor type 90 Ti. The 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, triglycerides and apo B, using the methods previously described, phospholipids were assessed by measuring the phosphorous in the dry residue after lipid extraction and proteins by the Lowry method. Data was expressed as the percentage of each component, and their sum as VLDL total mass per plasma decilitre.

2.5.2. VLDL sub-fractions by high performance liquid chromatography (HPLC)

As previously described, VLDL fraction was then subjected to size exclusion chromatography by HPLC [23]. In brief, 20 μL of isolated VLDL, corresponding to a full loading loop, 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, at controlled temperature (25 °C). Flow rate was 0.5 mL/min and the column eluate was monitored at 280 nm [24]. 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 (Magsphere Inc.). Efficiency and resolution was controlled throughout runs with the injection of 20 μL of a mix of the standards at every ten runs, finding no significant variations in retention time of standards (< 2.5%) all over the analysis.

In chromatograms different peaks can be detected (Fig. 1): a peak with a diameter of 94.98 ± 2.31 nm, which was compatible with chylomicron remnants and very large VLDL; a peak with an average diameter of 66.25 ± 4.89 nm, compatible with large VLDL; a majority peak with a diameter of 37.25 ± 0.09 nm, corresponding to typical VLDL sub-fraction, and finally smaller peaks at longer retention times (from 24 to 32 min) and sizes about 35 to 30 nm, identified as VLDL remnants [25]. Results were expressed as the percentage of each peak area in respect to total chromatogram area, using the ChromQuest 4.1 integration program.

2.6. CETP activity

Cholesteryl ester transfer protein activity was determined in serum samples as described previously [26]. Briefly, it consists in the evaluation of the ability of serum to promote the transfer of tritiatedcholesteryl esters from a tracer amount of biosynthetically labeled HDL3 (3H–CE–HDL3) (NENLife Science Products) towards serum apo B-containing lipoproteins was evaluated. Results were expressed as the percentage of 3H–cholesteryl esters transferred from HDL3 to apo B-containing lipoproteins, per mL, per h. Measurements were all carried out in duplicate within the same assay. The intra-assay CV was 4.9%.

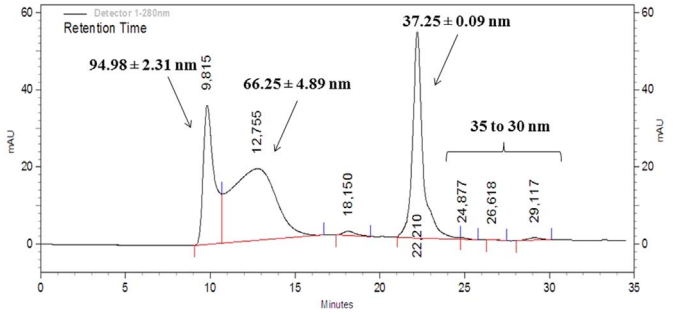

Fig. 1. Typical chromatogram, obtained after size exclusion chromatography of isolated VLDL fraction (randomly selected case).
From isolated VLDL fraction we detected, a peak with a diameter of 94.98 ± 2.31 nm, compatible with chylomicron remnants and very large VLDL; a peak with a diameter of 66.25 ± 4.89 nm, compatible with large VLDL; a majority peak with a diameter of 37.25 ± 0.09 nm, corresponding to typical VLDL, and smaller peaks at longer retention times (from 24 to 32 min) and sizes about 35 to 30 nm, identified as VLDL remnants.

2.7. Lipoprotein lipase activity

The activity of LPL was determined in PHP according to Nilsson-Ehle method [27]. Briefly, PPH was incubated with a mix of [3H]-labeled triolein (Amersham TRA191;) and unlabeled triolein (Sigma T-7140), in the presence of 10% v/v of human serum as source of apoC-II in 0.2 mol/L buffer Tris–HCl pH 8.0 with 0.3 mol/l NaCl. This reaction mixture was incubated in saline solution 1:10 to determine the total lipolytic activity, and simultaneously in saline solution 1:5 and 1 mol/L NaCl (as inhibitor of LPL) for 30 min at 37 °C. After incubation, the reaction was stopped and the released fatty acids were extracted. The [3H] oleic acid was quantified by counting with a Liquid Scintillation Analyzer (Packard TRI-CARB 2100; Packard Instruments). LPL activity of each individual was calculated from the difference between total activity and the activity measured in presence of 1 mol/L NaCl.

2.8. Adiponectin

Sera levels of adiponectin were determined by monoclonal antibody based enzyme-linked immunosorbent assay (ELISA) (R & D Systems). Adiponectin concentrations were calculated from a standard curve with implementation of recombinant standard protein of known concentration. Results were expressed as μg/mL.

2.9. Determination of type IV collagen 7s domain

In a group (n = 26) of randomly selected patients with biopsy-proven NAFLD (n = 19, F0–F2 and n = 7, F3–F4) type IV collagen 7s domain was determined, as noninvasive marker of liver fibrosis in NAFLD.

Sera levels of type IV collagen 7s domain were determined by radioimmunoassay (RIA) (Diaiatron Co.). Concentrations of type IV collagen 7s domain were calculated from a standard curve with implementation of standard protein of known concentration. Results were expressed as ng/dL.

2.10. Statistical analysis

Data is presented as mean ± SD or median (range) according to normal or skewed distribution. p Values < 0.05 were considered as significant. 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 variables, was used to determine correlations between parameters. Correlations were adjusted by those covariates considered appropriate. All analyses were performed using SPSS 21.0 software and Fig.s were designed with Graph Pad Prism 5 version 5.03 for Windows.

3. Results

Table 1 shows clinical and biochemical characteristics of the studied patients. No significant differences in gender proportion was observed between groups (Fisher's Exact Test, p = 0.573). MetS patients with NAFLD were older than MetS patients without NAFLD (p = 0.005). Subsequently, all differences between groups were adjusted by age as a co-variable. As all included subjects presented MetS, no differences in BMI and waist circumference were observed between groups (p = NS). Glucose levels were slightly increased in MetS patients with NAFLD (p < 0.049), while no differences in insulin and HOMA-IR were observed between groups (p = NS). As expected, MetS patients with NAFLD exhibited increased ALT values (p < 0.05), even after adjusting by age as co-variable (F = 6.1; p = 0.022). Notably, only 15 out of the 36 NAFLD patients presented an elevation in ALT, being in any case over 3 times the upper normal value.

Regarding lipid-lipoprotein profile (Table 1) MetS patients with NAFLD showed an increase in plasma triglyceride (p = 0.039) and FFA

Table 1
Clinical and biochemical characteristics and lipid and lipoprotein profile of studied subjects: Metabolic syndrome (MetS) patients (ATPIII) without non-alcoholic fatty liver disease (NAFLD) and MetS patients with biopsy-proven NAFLD.

	MetS without NAFLD	MetS with NAFLD
N, (Gender, male/female)	24, (6/18)	36, (12/24)
Age (y)	39 ± 16	51 ± 10*
BMI (kg/m^2)	35.2 ± 5.3	33.7 ± 5.8
Waist circumference (cm)	103.3 ± 8.2	107.1 ± 8.8
Glucose (mg/dL)	95 ± 18	115 ± 19*
Insulin (μU/mL)	10.6 (3.3–23.5)	8.2 (3.1–34.4)
HOMA-IR	2.58 (0.80–4.93)	2.20 (0.79–10.63)
ALT (U/L)	27 ± 24	60 ± 45*
AST (U/L)	25 ± 15	44 ± 26
Total cholesterol (mg/dL)	207 ± 42	206 ± 35
Triglycerides (mg/dL)	142 ± 49	180 ± 70*
HDL-cholesterol (mg/dL)	41 ± 8	43 ± 9
LDL-cholesterol (mg/dL)	150 ± 47	142 ± 34
Apo A-I (mg/dL)	166 ± 38	147 ± 31
Apo B (mg/dL)	105 ± 30	98 ± 21
Free fatty acids (mmol/L)	0.48 ± 0.19	0.82 ± 0.31*

Data is expressed as mean ± SD or median (range).
BMI: Body mass index, HOMA-IR: Homeostasis model assessment for insulin resistance index; AST: aspartate aminotransferase; ALT: alanine aminotranferase.
* $p < 0.05$ in comparison to MetS without NAFLD.

concentrations ($p = 0.001$) in comparison to MetS patients without fatty liver, while no differences were detected in total cholesterol, LDL-cholesterol, HDL-cholesterol or apolipoprotein concentrations. Noteworthy, FFA concentrations were positively associated with plasma triglyceride concentrations ($r = 0.34$; $p = 0.03$).

Isolated VLDL chemical composition and sub-fraction profile, obtained by size exclusion HPLC, are shown in Table 2. MetS patients with NAFLD, showed an increase in VLDL total mass ($p = 0.0001$), as well as in VLDL-apoB ($p = 0.0002$) in comparison to MetS patients without NAFLD, even after adjusting by age as a co-variable ($F = 32.6$; $p = 0.0001$ and $F = 60.1$; $p = 0.0002$, respectively). Moreover, VLDL from MetS patients with NAFLD presented triglyceride over-enrichment when compared to VLDL isolated from MetS patients without fatty liver ($p = 0.04$), adjusted by age: $F = 3.9$; $p < 0.05$. Additionally, MetS patients with NAFLD showed higher proportion of the peak of 66.25 ± 4.89 nm -compatible with large VLDL ($p = 0.0012$)- and a decrease in the proportion of peak 37.25 ± 0.09 nm, corresponding to typical VLDL ($p = 0.004$), adjusted by age: $F = 16.5$; $p = 0.0016$ and $F = 9.8$; $p = 0.004$, respectively. No differences were found in the proportion of the other sub-fractions between groups. Interestingly, the proportion of large VLDL correlated with FFA ($r = 0.35$; $p = 0.04$) and triglyceride content in VLDL ($r = 0.44$; $p = 0.009$). These correlations were still significant after adjusting by HOMA-IR as co-variable: $\beta = 0.36$; $p = 0.049$ and $\beta = 0.40$; $p = 0.02$, respectively.

Table 2
VLDL chemical composition and sub-fractions detected by size exclusion liquid chromatography in studied subjects: Metabolic syndrome (MetS) patients (ATPIII) without non-alcoholic fatty liver disease (NAFLD) and MetS patients with biopsy-proven NAFLD.

	MetS without NAFLD	MetS with NAFLD
VLDL chemical composition		
VLDL total mass (mg/dL)	64.6 ± 40.2	177.2 ± 78.9*
VLDL-apoB (mg/dL)	3.6 ± 2.7	10.1 ± 4.0*
Triglycerides (%)	49.4 ± 10.4	54.4 ± 5.8*
Cholesterol (%)	17.4 ± 4.3	13.2 ± 2.0
Phospholipids (%)	22.1 ± 8.7	15.7 ± 3.9
Proteins (%)	11.2 ± 5.1	17.1 ± 3.3
VLDL sub-fractions		
VL-VLDL and Chyl rem (%)	12.6 (0.6–30.6)	14.3 (4.6–43.7)
Large VLDL (%)	10.8 (1.2–57.4)	44.7 (15.2–72.9)*
Typical VLDL (%)	79.1 (27.2–98.2)	43.9 (16.5–74.9)*
VLDL remnants (%)	1.7 (0.7–6.3)	0.8 (0.1–2.2)

Data is expressed as mean ± SD or median (range). VL-VLDL: very large VLDL; Chyl rem: chylomicron remnants.
* $p < 0.05$ in comparison to MetS without NAFLD.

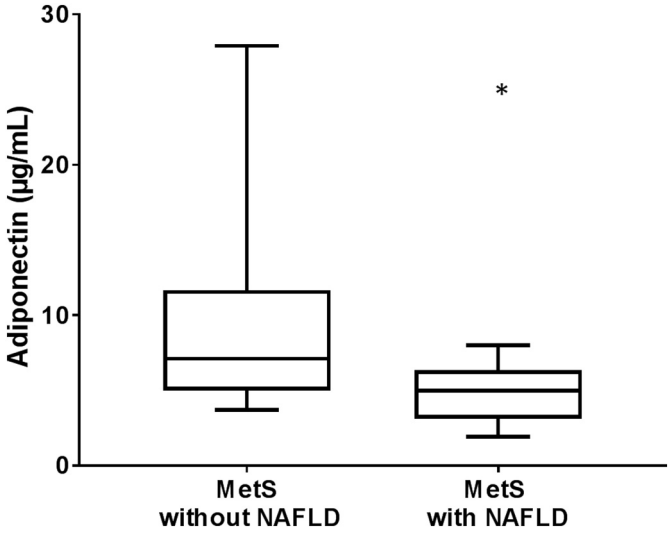

Fig. 2. Adiponectin levels in the studied subjects: Metabolic syndrome (MetS) patients (ATPIII) without non-alcoholic fatty liver disease (NAFLD) and MetS patients with biopsy-proven NAFLD. *p = 0.004, adjusted by age: F = 84.9; p = 0.005.

Activities of proteins related to lipoprotein metabolism were also evaluated in MetS patients with and without NAFLD. CETP activity showed a strong tendency to an increase in MetS patients with NAFLD in comparison to those without NAFLD (272.4 ± 80.3 *vs.* 237.9 ± 42.5% mL h; $p = 0.058$), whereas no differences were detected in LPL activity between groups [MetS with NAFLD, median (range): 0.83 (0.04–2.10) and MetS without NAFLD: 1.05 (0.69–2.02) μmol FFA/mL PHP h; $p = 0.336$].

Adiponectin concentrations were decreased in MetS patients with NAFLD in comparison to those without NAFLD (Fig. 2) ($p = 0.004$), even after age adjustment ($p = 0.005$). Adiponectin concentrations negatively correlated with liver enzymes (ALT: $r = -0.38$; $p = 0.013$ and AST: $r = -0.39$; $p = 0.011$). Noteworthy, adiponectin also negatively correlated with FFA ($r = -0.31$; $p = 0.04$) and with VLDL total mass ($r = -0.30$; $p < 0.05$) and VLDL-apoB ($r = -0.43$; $p = 0.0003$).

3.1. Effect of liver fibrosis in NAFLD

In order to analyze the effect of NAFLD-associated fibrosis on VLDL features and related factors, MetS patients with NAFLD were sub-divided according to their fibrosis staging into those with non-to-moderate fibrosis (F0–F1–F2) and those with severe fibrosis (F3–F4). The characteristics of NAFLD patients, divided according to the histological presentation of liver fibrosis, can be appreciated in Table 3. Interestingly, there were no differences in gender proportion between both groups ($p > 0.90$); however, those patients with severe fibrosis showed higher waist circumference, insulin concentrations and HOMA-IR than those with non-to-moderate fibrosis ($p < 0.05$). In addition, NAFLD patients with severe fibrosis presented higher values of both liver enzymes, ALT ($p = 0.041$) and AST ($p = 0.024$). Interestingly, the group of NAFLD patients with severe fibrosis showed decreased concentrations of plasma triglyceride ($p = 0.043$) and apoA-I ($p = 0.035$) in comparison to NAFLD patients with non-to-moderate fibrosis, while no differences were found in the other lipid parameters.

Markers related to liver function were also evaluated in NAFLD patients. No differences were observed in albumin concentrations ($p = 0.537$), prothrombin activity ($p = 0.296$) or in total bilirubin concentrations ($p = 0.886$) between NAFLD patients with severe and

Table 3
Clinical and biochemical characteristics and lipid and lipoprotein profile of metabolic syndrome patients with biopsy-proven non-alcoholic fatty liver, classified according to histological analysis into those with non-to-moderate fibrosis (grades F0–F2) and those with severe fibrosis (grades F3 and F4).

	Non-to-moderate fibrosis	Severe fibrosis
N, (Gender, male/female)	27, (9/18)	9, (3/6)
Age (y)	51 ± 11	49 ± 8
BMI (kg/m^2)	33.1 ± 4.9	35.6 ± 8.2
Waist circumference (cm)	105.9 ± 8.5	110.9 ± 9.2*
Glucose (mg/dL)	113 ± 39	123 ± 41
Insulin (μU/mL)	7.1 (3.1–21.2)	15.2 (3.4–34.4)*
HOMA-IR	1.85 (0.79–6.60)	3.86 (0.90–10.60)*
ALT (U/L)	45 ± 22	104 ± 66*
AST (U/L)	35 ± 16	69 ± 33*
Total cholesterol (mg/dL)	212 ± 38	190 ± 20
Triglycerides (mg/dL)	195 ± 72	138 ± 39*
HDL-cholesterol (mg/dL)	44 ± 9	39 ± 7
LDL-cholesterol (mg/dL)	145 ± 38	135 ± 16
Apo A-I (mg/dL)	153 ± 31	127 ± 20*
Apo B (mg/dL)	100 ± 22	93 ± 13
Free fatty acids (mM)	0.84 ± 0.34	0.76 ± 0.24
Total bilirrubin (mg/dL)	0.68 ± 0.51	0.65 ± 0.30
Albumin (g/dL)	4.48 ± 0.35	4.34 ± 0.49
Prothrombin activity (%)	96 ± 14	90 ± 11

Data is expressed as mean ± SD or median (range).
BMI: Body mass index, HOMA-IR: Homeostasis model assessment for insulin resistance index; AST: aspartate aminotransferase; ALT: alanine aminotranferase.
* $p < 0.05$ in comparison to MetS with NAFLD and non-to-moderate fibrosis.

non-to-moderate fibrosis, suggesting that there was no evident impairment in liver function in NAFLD patients with severe fibrosis.

Furthermore, VLDL features were analyzed between these two NAFLD sub-groups (Table 4). Remarkably, the patients presenting severe fibrosis exhibited lesser VLDL total mass ($p = 0.0024$) and VLDL triglyceride content ($p = 0.0148$) than the patients with non-to-moderate liver fibrosis. In addition, the proportions of the peaks with larger size −94.98 ± 2.31 nm and 66.25 ± 4.89 nm– were decreased in NAFLD patients with associated severe fibrosis ($p < 0.01$).

Regarding the activities of proteins related to lipoprotein metabolism, patients with severe fibrosis presented lower CETP activity than the patients with non-to-moderate fibrosis (Fig. 3A; $p = 0.017$). On the other hand, again no differences in LPL activity were detected between fibrosis stages between sub-groups [non-to-moderate fibrosis, median (range): 0.75(0.04–2.10) and severe fibrosis: 0.84(0.54–1.44) μmol FFA/mL PHP h; $p = 0.554$].

A potential non-invasive marker of liver fibrosis, the type IV collagen 7s domain was measured in randomly selected MetS patients with biopsy-proven NAFLD (Fig. 3B). Type IV collagen 7s domain levels were increased in NAFLD patients with severe fibrosis in comparison to those with non-to-moderate fibrosis ($p = 0.009$). Importantly, clinical and chemical characteristics in the randomly selected patients did not differ from the whole NAFLD group ($p > 0.3516$). ROC curve analyzes showed an area under the curve of 0.81 (Fig. 4), and considering the highest sensitivity (71.5%) and specificity (83.3%) the cut off point for the detection of severe fibrosis (F3–F4) was 3.90 ng/dl. Interestingly, type IV collagen 7s domain positively correlated with liver enzymes (ALT; $p = 0.0002$ and AST; $p = 0.0007$, Fig. 5) and was negatively associated with the proportion of peak of 66.25 ± 4.89 nm, compatible with large VLDL detected by size exclusion liquid chromatography ($r = -0.53$; $p = 0.045$). These three correlations were still significant after adjusting by HOMA-IR as co-variable: $\beta = 0.50$; $p = 0.009$, $\beta = 0.65$; $p = 0.002$ and $\beta = -0.51$; $p < 0.05$ respectively.

Finally, no differences were found in adiponectin concentrations in relation to fibrosis grade [non-to-moderate fibrosis: 4.9 ± 1.5 *vs.* severe fibrosis: 4.3 ± 2.4 μg/mL; $p = 0.4431$]; however, adiponectin negatively correlated with type IV collagen 7s domain ($r = -0.54$; $p = 0.032$).

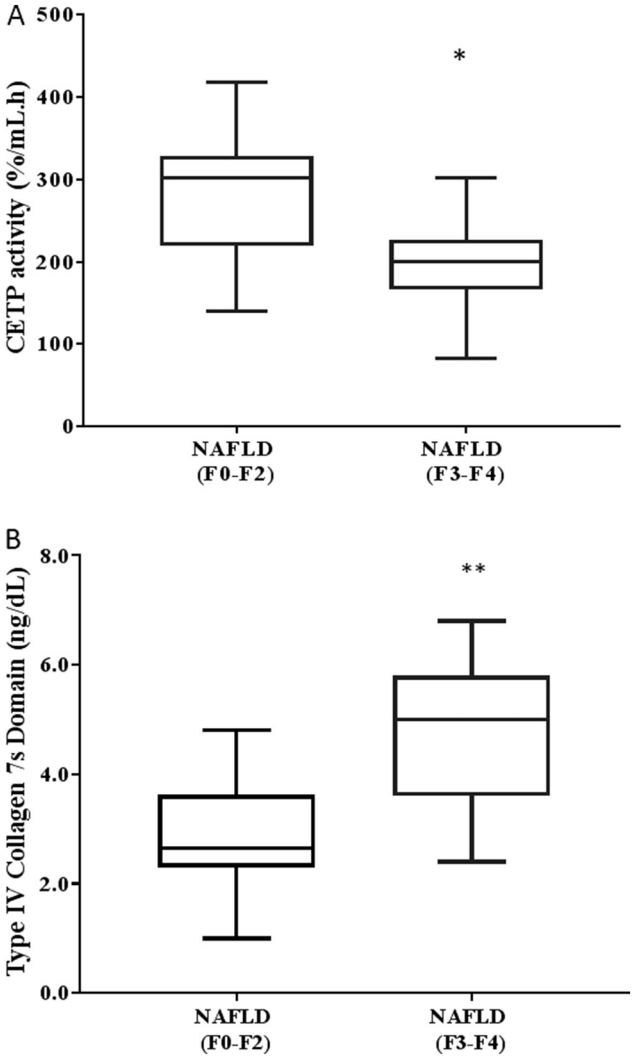

Fig. 3. CETP activity (A) and serum type IV collagen 7s domain (B) in metabolic syndrome patients with biopsy-proven non-alcoholic fatty liver (NAFLD), classified according to histological analysis into those with non-to-moderate fibrosis (grades F0–F2) and those with severe fibrosis (grades F3 and F4): *p = 0.017, **p = 0.009.

Table 4
VLDL chemical composition and sub-fractions detected by size exclusion liquid chromatography in metabolic syndrome patients with biopsy-proven non-alcoholic fatty liver, classified according to histological analysis into those with non-to-moderate fibrosis (grades F0–F2) and those with severe fibrosis (grades F3 and F4).

	Non-to-moderate fibrosis	Severe fibrosis
VLDL chemical composition		
VLDL total mass (mg/dL)	196.8 ± 80.6	118.4 ± 31.7*
VLDL-apoB (mg/dL)	10.5 ± 4.3	8.8 ± 2.5
Triglycerides (%)	55.7 ± 5.2	50.1 ± 5.7*
Cholesterol (%)	13.2 ± 1.9	13.4 ± 2.2
Phospholipids (%)	15.1 ± 3.1	17.5 ± 5.7
Proteins (%)	16.5 ± 3.2	19.0 ± 3.7
VLDL sub-fractions		
VL-VLDL and Chyl rem (%)	18.3 (7.1–43.7)	8.8 (4.6–10.4)*
Large VLDL (%)	48.6 (33.2–72.9)	29.6 (15.2–60.7)*
Typical VLDL (%)	43.9 (16.5–64.4)	53.4 (18.9–74.9)
VLDL remnants (%)	0.7 (0.4–2.2)	1.1 (0.1–1.3)

Data is expressed as mean ± SD or median (range). VL-VLDL: very large VLDL; Chyl rem: chylomicron remnants.
* $p < 0.05$ in comparison to MetS with NAFLD and non-to-moderate fibrosis.

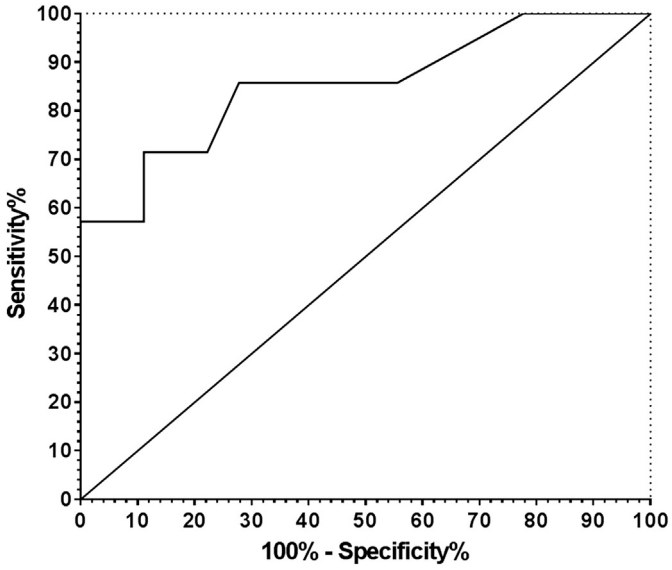

Fig. 4. Receiver operating characteristic (ROC) curve for serum type IV collagen 7s domain. Area under the curve = 0.81.

4. Discussion

Our results essentially showed that in the presence of NAFLD associated to MetS large and triglyceride over-enriched VLDL particles are likely produced. It is known that this type of VLDL presents a greater atherogenic potential. Furthermore, it was observed that the presence of liver fibrosis in NAFLD was linked to a reduction in VLDL mass, VLDL triglyceride content and the proportion of large VLDL sub-fraction, as well as a decrease in CETP activity, as a consequence of an early impairment in liver function. This finding is considered paradoxical since patients with liver fibrosis presented higher degree of insulin-resistance.

NAFLD is commonly related to obesity and insulin-resistance and then to lipoprotein alterations, leading to increased cardiovascular risk [7]. Then, it is difficult to separate the specific effect of the presence of NAFLD on lipoprotein metabolism. In this context, our study design, including all MetS patients segregated only by the presence of NAFLD, represents a useful model to differentiate whether the observed alterations in VLDL can be attributed to liver steatosis, independently of insulin-resistance. Liver biopsy was implemented to confirm NAFLD and to assess the grade of fibrosis. The histological study is still considered the gold standard method for NAFLD confirmation [28].

On the other hand, despite no differences were observed in BMI, waist circumference and HOMA-IR between MetS patients, with and without NAFLD; FFA concentrations were increased in patients with NAFLD. Perhaps, if insulin sensitivity had been evaluated by means of a more precise method, such as euglycemic insulin clamp [29], NAFLD patients would have evidenced a greater insulin-resistant degree.

The main objective in our study was to evaluate VLDL in NAFLD associated to MetS at different grades. Patients with fatty liver presented higher circulating VLDL total mass and VLDL particle number, reflected by VLDL-apoB concentrations. In obese patients, Chan DC and coworkers demonstrated, by means of an *in vivo* kinetic assay, that increased liver fat deposits were associated with higher VLDL-apoB secretion rate [30]. According to our and other authors' results, it can be assumed that in insulin-resistant states, triglycerides are over-produced in the liver and would be over-secreted as VLDL to the plasma at the same time that triglycerides are deposited as lipid droplets inside hepatocytes [14,30,31].

Total VLDL isolated from MetS patients with NAFLD presented triglyceride over-enrichment. Alike, in type 2 diabetic patients, other authors demonstrated, by an *in vivo* kinetic study, that increased liver fat content was the determinant of greater secretion of triglyceride-rich VLDL particles, presumable larger in size [14]. In the present study, size sub-fractions were assessed by size exclusion chromatography analysis, showing that NAFLD presence in MetS, even previous to progression to type 2 diabetes, is associated to predominance of large VLDL subfractions. It is important to note that the excess of FFA concentrations in the insulin-resistant condition were positively associated with the greater proportion of large VLDL particles. It must be taken into account that, unlike typical VLDL, this type of VLDL has proved to exert endothelial dysfunction [32].

VLDL is mainly catabolized by LPL on endothelial surface of adipose tissue and skeletal muscle. Herein, no differences were found in LPL activity of MetS patients, with and without NAFLD. Other authors reported that the presence of NAFLD in MetS was associated to a slight reduction in LPL mass measured in pre-heparin plasma [33]. Controversies in the results could be due to different methodology to assess the enzyme, as protein mass on the one hand, and as the actual activity on the other. Thus, we suggest that based on the lipolytic activity of LPL, observed alterations in circulating VLDL would not be related to its catabolism.

In circulation, VLDL suffers from lipid exchange with other lipoproteins by CETP action. In accordance with our previous finding [34], herein patients with MetS and fatty liver showed a strong tendency to an increase in CETP activity in comparison to MetS without fatty liver. The CETP activity might promote a more dynamic cholesterol ester exchange by triglyceride on circulating VLDL. This suggests that in NAFLD, VLDL particles would be secreted even richer in triglycerides.

In line with previous reports [35,19], adiponectin concentrations were decreased in MetS patients with NAFLD. The negative correlation between adiponectin concentrations and FFA, VLDL total mass and VLDL particle number suggests that the reduction in adiponectin would be associated with the overproduction of altered VLDL in NAFLD. In fact, adiponectin regulates fatty acid oxidation in skeletal muscle [36], and thus its reduction would favor FFA transport to the liver. Moreover, adiponectin modulates VLDL assembly in the liver by direct action on hepatocytes [36].

Liver fibrosis is the strongest predictor of overall mortality in

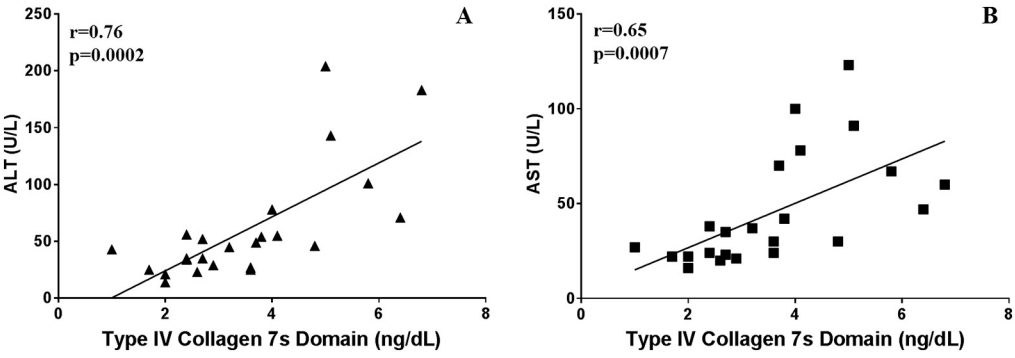

Fig. 5. Correlations between type IV collagen 7s domain and alanine aminotranferase (ALT) (A) and aspartate aminotransferase (AST) (B).

NAFLD [37]. The progression of fibrosis is recognized to be the less reversible of liver changes occurring in NAFLD, leading to hepatic cirrhosis and liver failure [8]. We aimed to evaluate if the presence of severe liver fibrosis in NAFLD, affected metabolic factors and the composition and sub-fractions of circulating VLDL. To fulfill our aim, patients with biopsy proven hepatic steatosis were divided into those with non-to-moderate fibrosis (stages F0–2) and severe fibrosis (stages F3–4), as classified elsewhere [10,18]. It is interesting to note that patients with severe fibrosis showed reduced plasma triglyceride concentrations, even when they were more insulin-resistant. This result is in line with Jiang ZG and coworkers who described and inverse association of fasting triglyceride concentrations and non-invasive liver fibrosis indicators [38]; however, authors did not confirm hepatic fibrosis by histological analysis as was done herein. Overall, these results suggest that in NAFLD, a decrease in plasma triglycerides would constitute an early indicator of severe liver fibrosis, taking into account that no alterations were detected in traditional marker for cirrhosis and hepatic dysfunction.

In agreement with decreased plasma triglyceride concentrations in NAFLD patients with severe fibrosis, circulating VLDL total mass was also reduced, mainly due to its triglyceride content. Total VLDL chemical composition in the different stages of liver fibrosis associated to NAFLD has not been reported so far. In spite of an increasing insulin-resistance, in the NAFLD progression towards more severe fibrosis stages, an apparent improvement in circulating VLDL would occur as part of early alteration in hepatic synthetic function. Of note, in our cohort just 2 patients with severe fibrosis presented histological findings compatible with liver cirrhosis, but statistical significance of results did not change after data were analyzed excluding these 2 cases (data not shown).

There are few and controversial reports regarding VLDL sub-fractions related NAFLD progression to liver fibrosis. Some authors reported a decrease in circulating small VLDL particles but not in large VLDL, in diabetic and non-diabetic NAFLD patients with significant fibrosis [39]. However, Männistö VT et al. showed a greater reduction in larger VLDL sub-fractions in obese patients with advanced stages of liver fibrosis in comparison to NAFLD patients without fibrosis, compatible with our results [18]. It can be suggested that fibrosis progression in NAFLD is associated to a shift in VLDL to triglyceride poorer and smaller particles.

Circulating VLDL features are consequence of the action of different factors, such as LPL and CETP. When they were analyzed in patients with non-to-moderate and severe fibrosis, we have not observed variations in LPL, whereas CETP activity was decreased in severe fibrosis. Thus, it can be deduced that characteristics of VLDL in patients with severe fibrosis are consequence of the hepatic secretion, given that VLDL catabolism by LPL would not be impaired. To our knowledge LPL activity in NAFLD associated to severe fibrosis has not been addressed so far.

Due to invasiveness and the risk of complication related to liver biopsy, investigators have been proposing different strategies for estimation of fibrosis in NAFLD [40]. Serum type IV collagen 7s domain has been proposed as an independent marker of advanced fibrosis in NAFLD [10,20]. Serum type IV collagen 7s domain was increased in patients with severe fibrosis. In addition, of more interest is the negative correlation of type IV collagen 7s domain with the proportion of large VLDL, which has not been reported until now. This association reinforces the concept that progression of NAFLD to advanced liver fibrosis is related with a decrease in the secretion of larger VLDL particles.

Regarding adiponectin, in all NAFLD patients it was decreased, however, severe fibrosis group did not show lower levels than non-to-moderate fibrosis group. Probably, with a greater number of studied cases a significant difference could have been observed. However, adiponectin reduction was associated with the increase in type IV collagen 7s domain, suggesting a cyto-protective role of adiponectin on the liver.

Non-alcoholic fatty liver disease is characterized by lipid-lipoprotein abnormalities and increased cardiovascular risk, however safety of lipid-lowering therapies would be a matter of concern in patients with such chronic liver disease. Nonetheless, statins are effective in reducing lipid concentrations in patients with NAFLD without hepatotoxic effects [41,42]. Moreover, a preliminary study with statin intervention on patients with NAFLD suggests that statins would decrease liver fat content detected by ultrasound [43], but there is still lack of convincing histological support for the recommendation of statins in NAFLD treatment [44]. On the other hand, recently we observed that dietary supplementation with omega-3 poly-unsaturated fatty acids (PUFA) for 12 weeks to insulin-resistant rats, prevented alterations in VLDL as well as insulin-resistance; without changes in liver fat content [45]. Nevertheless, Lu W et al. in a meta-analysis, that summarized 10 different clinical trials, conclude that treatment with omega-3 PUFA might improve not only lipid and lipoprotein profile, but also could decreases liver fat content [46]. Thus, the use of lipid-lowering strategies in patients with NAFLD would be recommended to safely reduce associated high cardiovascular risk; however, whether statins and omega-3 PUFA improve liver histology still needs further studies.

The present study has some limitations. First, due to ethical concerns, liver biopsies were not available from MetS patients without NAFLD, included in the control group. Only MetS patients without fatty liver, according to abdominal ultrasonography, were included in this group. Furthermore, as was mentioned above, the cohort of patients included in this cross-sectional study is relatively small. Larger longitudinal studies, with the monitoring of NAFLD progression along time, would be needed to confirm the link between liver fibrosis developing in NAFLD and changes in VLDL composition and sub-fraction profile.

In summary, our results suggest that in MetS the presence of fatty liver without significant liver fibrosis is associated with circulating larger and triglyceride over-enriched VLDL particles, with greater atherogenic potential. However, when NAFLD progresses to severe fibrosis, circulating VLDL features apparently improve, probably as a consequence of early alterations in the hepatic synthetic function.

Our study contributes with results regarding a topic not quite studied so far, opening a scope to understand the mechanisms linked to lipoprotein metabolism in the context of fatty liver and its progression to advanced fibrosis stages.

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