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PII: S1933-2874(15)00097-5

DOI: [10.1016/j.jacl.2015.03.010](https://doi.org/10.1016/j.jacl.2015.03.010)

Reference: JACL 747

To appear in: *Journal of Clinical Lipidology*

Received Date: 31 December 2014

Revised Date: 22 March 2015

Accepted Date: 30 March 2015

Please cite this article as: Sonmez A, Nikolic D, Dogru T, Ercin CN, Genc H, Cesur M, Tapan S, Karslioğlu Y, Montalto G, Banach M, Toth PP, Bagci S, Rizzo M, Low- and High-Density Lipoprotein Subclasses in Subjects with Non-Alcoholic Fatty Liver Disease, *Journal of Clinical Lipidology* (2015), doi: 10.1016/j.jacl.2015.03.010.

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Low- and High-Density Lipoprotein Subclasses in Subjects with Non-Alcoholic Fatty Liver Disease

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Abstract

Background: Non-alcoholic fatty liver disease (NAFLD) is associated with increased cardio-metabolic risk. Although dyslipidemia represents a key factor in this disease, its impact on serum levels of distinct lipoprotein subfractions is largely unknown.

Objective: To assess the full low-density lipoprotein (LDL) and high-density lipoprotein (HDL) profiles in patients with NAFLD.

Materials & Methods: Seven LDL and 10 HDL subfractions were assessed by gel electrophoresis (Lipoprint, Quantimetrix Corporation, USA) in men with biopsy proven NAFLD (simple steatosis (SS) (n=17, age: 34±7 years) and non-alcoholic steatohepatitis (NASH) (n=24, age: 32±6 years). Exclusion criteria included robust alcohol consumption, hepatitis B or C virus, body mass index (BMI) ≥40 kg/m², diabetes mellitus and hypertension.

Results: Compared to SS, NASH patients had similar BMI, HOMA-IR index and plasma lipids, with increased levels of both AST and ALT. NASH subjects had lower levels of larger LDL1 (10±4 vs. 13±4%, p=0.010) and increased smaller LDL3 and LDL4 particles (9±5 vs. 5±5%, p=0.017 and 3±3 vs. 1±2%, p=0.012, respectively). No changes were found in the HDL subclass profile. By multiple regression analysis we found that NASH was associated only with increased levels of LDL3 (p=0.0470).

Conclusion: The increased levels of small, dense LDL3 and LDL4 in NASH may help to at least partly explain the increased risk for atherosclerosis and cardiovascular diseases in these patients.

Key words: nonalcoholic fatty liver disease, non-alcoholic steatohepatitis, simple steatosis, lipids, lipoproteins

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is an asymptomatic disease defined by the presence of simple steatosis (SS) without hepatocyte ballooning, whereas the presence of steatosis and inflammation with ballooning, with or without fibrosis, is characteristic of non-alcoholic steatohepatitis (NASH).¹ The prevalence of NAFLD, which include both SS and NASH, is estimated to be about 30%, while NASH affects 3–5% of the population in Western countries.²⁻⁴ Such hepatic alterations are associated with augmented risk for cardiovascular disease (CVD), and this may occur independent of the presence of the metabolic syndrome.⁵⁻⁸

A number of studies have shown that several risk markers, including C-reactive protein (CRP), oxidized low-density lipoprotein (LDL), interleukin (IL)-6 and plasminogen activator inhibitor-1 (PAI-1) are associated with NAFLD and that they are involved in the progression from pure steatosis to steatohepatitis.^{9, 10} Changes in adipokine levels are associated with the development of insulin resistance (IR), fatty liver, and the progression of fatty liver to NASH.¹¹ It has been suggested that decreased levels of adiponectin and increased levels of tumor necrosis factor-alpha (TNF- α) are associated with the development of NAFLD and NASH, independently of IR and other metabolic factors.¹² In addition, there is a growing interest in new biomarkers for CVD in patients with NAFLD, such as fetuin A and asymmetric dimethylarginine (ADMA).¹³⁻¹⁵ The alterations of lipid metabolism in NAFLD have been reported.¹⁶ About 80% of subjects with NAFLD have dyslipidemia: LDL particles are increased with a predominance of small, dense particles that are known to be involved in atherogenesis and CVD.^{17, 18} Recently Siddiqui et al.¹⁹ have shown that NAFLD patients may have increased levels of pro-atherogenic lipoprotein subclasses, as well as increased lipid synthesis driving dyslipidemia in pre-cirrhotic NAFLD and impaired high-density lipoprotein (HDL) maturation in NAFLD.

It has been suggested that NASH may contribute to a more atherogenic risk profile compared to SS.²⁰ However, it is still unclear whether such changes may occur in subjects with SS or only in those with NASH. Thus, the aim of this study is to evaluate the full spectrum of LDL and HDL subclasses in subjects with NAFLD and identify differences between subjects with SS and NASH. In addition, we analyzed the relationship between lipoprotein subfractions and markers of inflammation, IR, and endothelial dysfunction in patients with NAFLD.

MATERIAL AND METHODS

Study subjects

A total of 41 biopsy proven men with NAFLD were enrolled in this study. Subsequent histological diagnoses were SS (n=17, age: 34±7 years) and NASH (n=24, age: 32±6 years). Part of the data of these subjects was used in previous published studies.^{14, 15, 21} We used the following exclusion criteria: robust alcohol consumption (>140 g/wk), positive testing for hepatitis B or C virus (using the HBsAg Antigen and the Anti-HCV antibody serological tests), body mass index (BMI) ≥ 40 kg/m², diabetes mellitus, or hypertension. All subjects were recruited from the Gulhane Medical School. Written informed consent was obtained from each subject. This study was performed in accordance with the Good Clinical Practice guidelines, the Helsinki Declaration and it was approved by the Ethics Committee of the Gulhane Medical School. The study protocol included a clinical examination, biochemical analyses, and measurements of serum concentrations of 21 distinct lipoprotein fractions.

Liver histology

Routinely processed and analyzed liver biopsy specimens were read by the same liver pathologist who was unaware of the patient's clinical and laboratory data. The main histologic features commonly described in NAFLD, including steatosis, inflammation (portal and lobular), hepatocyte ballooning, and fibrosis, were scored by using the semi quantitative classification of Kleiner et al.²² Features of steatosis, lobular inflammation, and hepatocyte ballooning were combined in a score ranging from 0 to 8, the NAFLD activity score (NAS). Cases with NAS of 5 or more are diagnostic of NASH, cases with NAS of 2 or less are diagnostic of SS, and cases with scores in between are defined as borderline NASH.

Biochemical analyses

For biochemical analyses, all blood samples were collected from an antecubital vein, between 08:00 and 09:00 a.m. after an overnight fasting. The samples were centrifuged within 30 minutes of collection at 1500 g, aliquotted, and immediately frozen at -80 °C for analyses until examination. Aliquots were made of both serum and plasma. All samples were analyzed simultaneously in a blinded manner.

Fasting plasma glucose (FPG), total cholesterol (TC), triglycerides (TG), and HDL-cholesterol (HDL-C) levels were measured by the enzymatic colorimetric methods with Olympus AU2700 auto analyzer using reagents from Olympus Diagnostics (GmbH, Hamburg, Germany). LDL-cholesterol (LDL-C) was calculated by Friedewald's formula.²³

The serum basal insulin level was measured in duplicate by a chemiluminescence method using reagents from Roche Diagnostics (Mannheim, Germany). IR was calculated by modified homoeostasis model assessment of IR (HOMA-IR), with the following formula: $\text{HOMA-IR} = \text{fasting plasma insulin (IU/ml)} \times \text{fasting plasma glucose (mg/dl)} / 405$. HOMA-IR was originally

reported by Matthews et al.²⁴ and this index has been shown to be well correlated with the results of the euglycemic-hyperinsulinemic clamp method to determine IR.²⁵

A high-sensitivity CRP (hs-CRP) level was determined in serum by an immunoturbidimetric fixed rate method using an Olympus AU- 2700 autoanalyzer (Hamburg, Germany). Intra-assay coefficient of variation (CV) and inter-assay CV were 5.8% and 3.1%, respectively. The minimum detectable concentration for hs-CRP was 0.07 mg/L.

Plasma adiponectin levels were determined by an enzyme-linked immunosorbent assay (ELISA) (Total Human Adiponectin ELISA Kit, Lot no. TE1013; TECOmedical AG, Sissach, Switzerland). The minimum detectable concentration for adiponectin was 0.6 ng/ml. Intraassay CV ranged from 2.35% to 4.66%, while inter-assay CV ranged from 5.7% to 6.72%.

Plasma asymmetric dimethyl arginine (ADMA) levels were determined by ELISA (ADMA direct ELISA kit, Lot no. K7828; Immundiagnostic AG, Bensheim, Germany) (detection limit of ADMA assay = 0.05 μ mol/L).

Plasma fetuin-A levels were determined by ELISA (Human Fetuin-A ELISA Kit, Lot no. D685; Epitope Diagnostics, San Diego, CA, USA). The minimum detectable concentration for fetuin-A was 5 ng/ml. Intraassay CV ranged from 4.8% to 5.5%, while inter-assay CV ranged from 5.7% to 6.8%.

Plasma visfatin levels were determined by ELISA (Human Visfatin-C-Terminal ELISA Kit, Lot no. 600482 Phoenix Pharmaceuticals, Belmont, CA, USA). The minimum detectable concentration for visfatin was 2.25 ng/ml. Intraassay CV and inter-assay CV were 5 % and 12%, respectively.

Plasma TNF- α and IL-6 levels were determined by ELISA, (Human TNF- α High Sensitivity ELISA Lot no. BMS223HS; and Human IL-6 High Sensitivity ELISA Lot no.

BMS213HS, respectively, eBioscience, Vienna, Austria). The minimum detectable concentration for TNF- α and IL-6 were 0.13 pg/mL and 0.03 pg/mL, respectively. The calculated overall intra-assay CV for TNF- α and IL-6 were 8.5% and 4.9%, while the calculated overall inter-assay CV for TNF- α and IL-6 and were 9.8% and 6.0%, respectively. Measurements were carried out using ELISA plate reader Bio-Tek Synergy HT [Biotek Instruments Inc., Winooski, VT, USA].

Lipoprotein subclass analysis

We assessed a total of 21 distinct lipoprotein subclasses including very low density lipoproteins (VLDL), 3 intermediate-density lipoprotein (IDL A, IDL B and IDL C), 7 LDL and 10 HDL subfractions. LDL subclasses were assessed by non-denaturing, linear polyacrylamide gel electrophoresis (Lipoprint, Quantimetrix Corporation, USA) as previously reported²⁶⁻²⁸, the only Food and Drug Administration cleared diagnostic tool for lipoprotein subfraction testing.²⁹ In short, the procedure was performed for 60 min with 3 mA for each gel tube. Each electrophoresis chamber involved two quality controls. The LDL bands in the sample are identified by their mobility using VLDL as the starting reference point and HDL as the leading reference point. The electrophoresed gels were scanned using a digital scanner and a computer. The relative area for each lipoprotein band is determined and multiplied by the total cholesterol concentration of the sample and expressed in mg/dl. LDL subclasses were distributed as seven bands (LDL1 to LDL7, respectively).²⁶⁻²⁸ LDL1 and -2 are defined as large LDL, while LDL3 to -7 as small LDL.²⁹

The Lipoprint system (Quantimetrix, Inc., Redondo Beach, CA) was also used to assess HDL profiles as previously described.³⁰ Briefly, HDL subfractions were separated using polyacrylamide gel electrophoresis and then identified using comparisons with LDL/VLDL as starting reference point and albumin (migrating the farthest) as leading reference point. In order

to determine the relative area of each lipoprotein subfraction, densitometry was performed. The relative area for each HDL subfraction band was multiplied by the total HDL-cholesterol concentration in the sample and expressed in mg/dl. Finally, 10 HDL subclasses were distinguished, grouped into three categories: large (HDL1-3), intermediate (HDL4-7) and small (HDL8-10)³⁰. A majority of studies indicate that elevations in small HDL subfractions (HDL8-10) are associated with IR and the metabolic syndrome.³¹

Finally, detailed assessment of the full (LDL+HDL) lipoprotein profile (a total of 21 distinct lipoprotein subclasses) was the primary outcome of interest of the present study.

Statistical Analysis

Statistical analyses were performed using SPSS software (V.17.0 for Windows, SPSS Inc., Chicago, USA). Non-parametric Mann-Whitney test was conducted. The Spearman rank correlation method was used to assess whether evaluated parameters were associated with LDL subclasses in both groups, while using multiple regression analysis we assessed if the presence of NASH was dependent on any other evaluated parameter.

RESULTS

As shown in Table-1, NASH subjects had similar BMI, HOMA-IR index and plasma lipid levels compared with those SS only. Statistical differences were found between the two groups for AST ($p=0.007$), ALT ($p=0.017$), hemoglobin ($p=0.010$), hematocrit ($p=0.021$) and platelets ($p=0.015$). Regarding LDL subclasses (see Table-2), NASH subjects had decreased presence of larger LDL1 (9.9 ± 4.1 vs. $13.3\pm 4.2\%$, $p=0.010$) and increased presence of smaller LDL3 and LDL4 particles (9.2 ± 5.1 vs. $5.3\pm 4.6\%$, $p=0.017$ and 3.0 ± 3.4 vs. $1.0\pm 2.4\%$, $p=0.012$,

respectively). This led to decreased LDL size ($p=0.026$). No significant between group differences were found for VLDL-C or any of the HDL-C subfractions. The largest IDL subfraction differed significantly between groups with higher levels found among patients with SS compared to those with NASH. *The NAS of 24 NASH patients enrolled was 6, while their average Fibrosis stage was 1.*

Table-1. Biochemical and lipids parameters of subjects included in the study

	SS (n=17)	NASH (n=24)	P value
Age (years)	34.47±7.32	32.04±6.05	0.301
BMI (kg/m ²)	29.14±2.42	27.72±1.99	0.128
Waist circumference (cm)	101.91±6.27	99.48±5.73	0.300
Dyslipidemia, n (%)	10 (58)	17 (70)	0.424
Smoking habit, n (%)	7 (41)	4 (16)	0.081
Insulin (mU/ml)	16.55±9.63	16.74±10.29	0.909
Fasting glucose (mmol/l)	5.07±0.68	5.24±0.42	0.295
HOMA index	3.73±2.23	3.92±2.52	0.842
Total Cholesterol (mmol/l)	4.68±0.99	5.02±1.38	0.250
Triglycerides (mmol/l)	1.91±1.41	2.22±1.31	0.195
HDL-cholesterol (mmol/l)	1.11±0.22	1.04±0.19	0.241
LDL-cholesterol (mmol/l)	2.84±0.83	2.93±1.08	0.479
AST (IU/L)	44.1±12.10	61.2±23.15	0.007
ALT (IU/L)	89.1±34.88	126.5±49.96	0.017
GGT (IU/L)	74.9±88.34	66.8±33.22	0.366
Urate (mmol/l)	6.3±0.94	6.7±1.26	0.376
Bilirubin (conjugated) (µmol/l)	0.19±0.11	0.24±0.23	0.375
Bilirubin (unconjugated) (µmol/l)	0.68±0.32	0.84±0.42	0.218
Hb (g/dl)	15.7±1.05	16.4±1.07	0.010
Hct (%)	45.3±2.91	47.23±3.30	0.021
WBC (mcL)	7265±1484	7017±1741	0.633
Plat (mcL)	259882±58002	218083±43617	0.015
Adiponectin (µg/mL)	4.83±1.55	3.91±1.43	0.066
ADMA (µmol/L)	0.51±0.293	0.59±0.378	0.751
Fetuin (µg/mL)	71.7±19.57	78.8±11.36	0.165
Visfatin (ng/ml)	13.5±1.45	13.98±2.09	0.481
TNF-α (pg/mL)	11.8±3.65	10.93±4.92	0.548
IL-6 (pg/mL)	0.38±0.20	0.57±0.34	0.052
hsCRP (mg/L)	2.92±2.84	2.53±3.10	0.362

ADMA: asymmetric dimethylarginine; ALT: alanine transaminase; AST: aspartate aminotransferase; BMI: body mass index; GGT: gamma-glutamyl transpeptidase; Hb: hemoglobin; Hct: hematocrit; HDL: high-density lipoprotein; HOMA: homeostatic model assessment; hsCRP: high-sensitivity C reactive protein; IL-6: interleukin-6; LDL: low-density lipoprotein; NAFLD: nonalcoholic fatty liver disease; NASH: nonalcoholic steatohepatitis; Plat: platelets; TNF-α: tumor necrosis factor α; WBC: white blood cell.

Table-2. Lipoprotein profile of subjects included in the study

	SS (n=17)	NASH (n=24)	P value
LDL size (angstrom)	264.34±6.17	259.27±7.32	0.026
HDL size	26.21±8.33	26.38±9.64	0.999
VLDL (%)	17.64±3.02	17.08±2.84	0.302
IDL C (large) (%)	9.24±1.88	6.68±3.23	0.005
IDL B (medium) (%)	6.98±2.03	8.03±2.94	0.397
IDL A (small) (%)	4.95±1.48	4.13±1.13	0.078
LDL 1 (%)	13.26±4.22	9.87±4.11	0.010
LDL2 (%)	14.73±4.67	15.1±4.65	0.905
LDL3 (%)	5.29±4.59	9.16±5.05	0.017
LDL4 (%)	1.05±2.36	2.97±3.36	0.012
LDL5 (%)	0.12±0.51	0.30±0.99	0.504
LDL6 (%)	---	---	---
LDL7 (%)	0.11±0.44	---	---
HDL 1 (%)	11.79±18.23	9.95±5.57	0.109
HDL2 (%)	5.19±2.92	6.15±3.18	0.637
HDL3 (%)	5±2.26	5.13±2.12	0.813
HDL4 (%)	6.72±2.17	7.15±1.63	0.850
HDL5 (%)	7.6±2.66	8.66±1.03	0.479
HDL6 (%)	21.5±4.20	22.06±2.74	0.850
HDL7 (%)	11.01±2.00	9.83±1.58	0.179
HDL8 (%)	8.72±2.21	8.55±1.72	0.759
HDL9 (%)	6.64±2.92	6.63±1.51	0.409
HDL10 (%)	15.62±10.58	15.91±4.88	0.944

HDL: high-density lipoprotein; IDL: intermediate-density lipoproteins; LDL: low-density lipoprotein; VLDL: very low-density lipoprotein.

We also performed correlation analysis (data not shown), and we found in NAFLD subjects positive correlations between TNF- α and LDL4 ($r=.547$, $p<0.05$), as well as between ALT and LDL4 ($r=.533$, $p<0.05$), gamma-glutamyl transpeptidase (GGT) and LDL3 and LDL4 ($r=.746$, and $r=.497$, respectively; $p<0.05$ for both); while GGT also inversely correlated with LDL1 ($r=-.552$, $p<0.05$). In addition, as shown in Figure-1, LDL3 subclasses significantly correlated with triglycerides (positively) and with HDL-C (inversely) in both SS and NASH subjects. Finally, we performed multiple regression analysis (data not shown) to test whether

NASH was independently associated with any of the evaluated parameters; we have found an association only with the presence of smaller LDL3 ($p=0.047$).

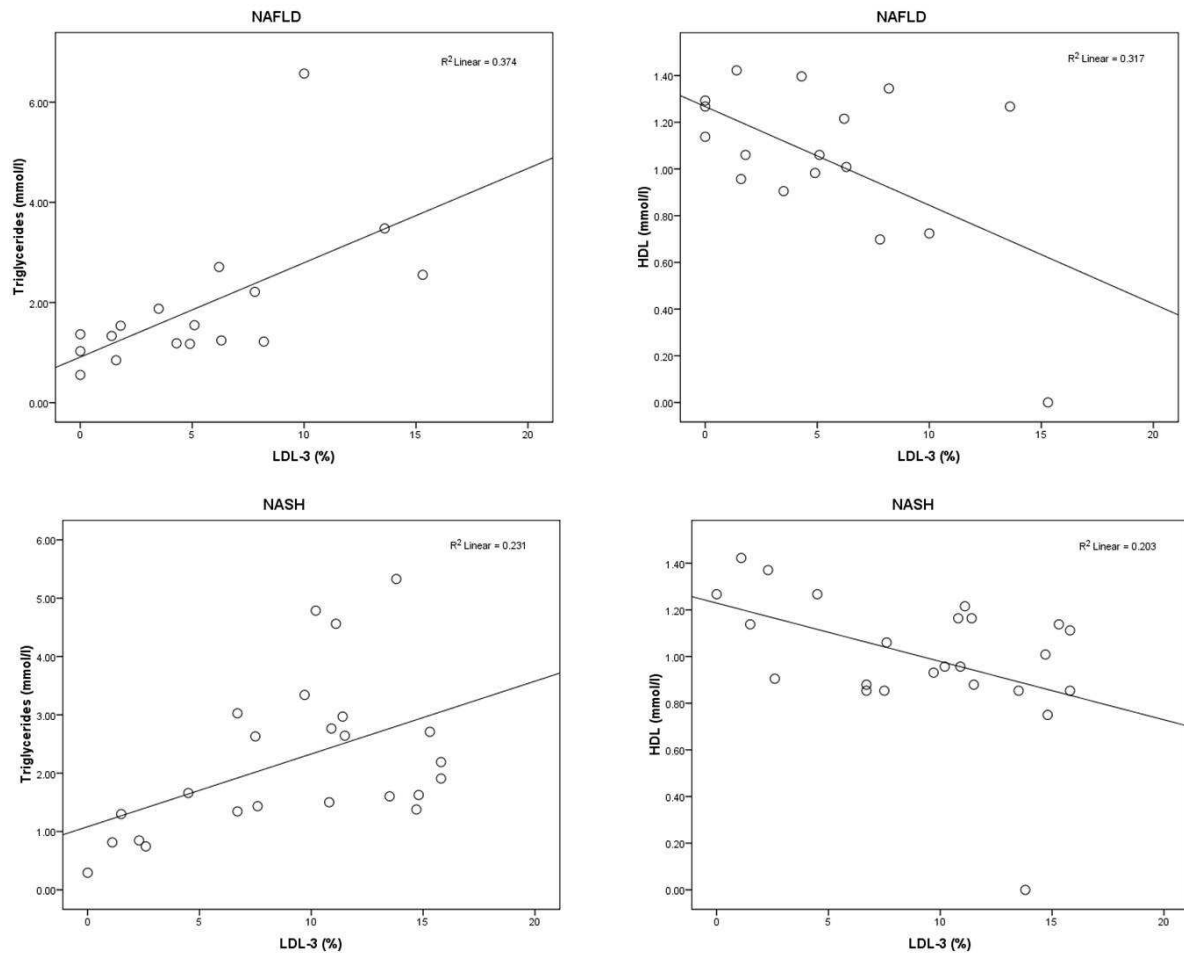


Figure-1. LDL3 subclasses vs. triglycerides and HDL-C in both SS and NASH groups

DISCUSSION

The present study suggests worsening of the LDL profile in NAFLD. Subjects with NASH, compared to those with SS, had increased small dense LDL (sdLDL) particles. Our findings are consistent with those from a recent case-control study, where SS and NASH were associated with increased pro-atherogenic lipoprotein subclasses compared to lean and obese controls as determined by nuclear magnetic resonance spectroscopy.¹⁹ Our study was more detailed as we evaluated changes in 21 distinct lipoprotein subfractions.

Results from the Multi-Ethnic Study of Atherosclerosis suggest that NAFLD is not associated with changes in TC or LDL-C, but is associated with higher TG, lower HDL-C level, higher LDL particle concentration, and smaller LDL particle size.³² We found that sdLDL significantly correlates with TG (positively) and with HDL-C (inversely) in both SS and NASH subjects.

Beyond lipoprotein concentrations, there is evidence supporting correlations between the liver enzymes ALT and GGT with liver fat content.³³ We found in the present study significant differences between AST and ALT in NASH compared to SS, while there were no differences for GGT and TG. However, in SS subjects we demonstrate a positive correlation between ALT, GGT and sdLDL particles. This is consistent with previous studies reporting a significant association between increased ALT and apolipoprotein B, LDL particles and the percentages of sdLDL.³⁴ Although there is evidence that subjects with NAFLD have higher levels of inflammatory markers such as IL-6, hsCRP, TNF- α and lower concentrations of adiponectin compared with controls^{9, 12, 35-37}, we did not find any significant differences for such parameters between SS and NASH subjects. This may be explained by the very young age of the cohort included in our study, as well as the early stage of NASH. The same reasons may explain no significant changes in the level of other measured adipokines (visfatin) as well as the level of

fetuin-A, a liver-derived peptide, which has been suggested to participate in both the pathogenesis of NAFLD and its metabolic complications.³⁸ Our finding is in accordance with a previous study¹³ which found no significant differences in levels of fetuin A between NASH subjects and those with pure steatosis. Of interest, correlation analysis indicated that changes in TNF- α were associated with changes in small LDL4 particles in subjects with SS. Finally, we found significantly increased platelets in subjects with NASH but not in those with SS. This is consistent with a recently published study.²⁰ Regarding the markers of endothelial dysfunction which have been assessed in this study (ADMA and adiponectin), we did not find significant differences between SS and NASH subjects. Among NASH subjects, adiponectin and ADMA were inversely correlated, while we did not find any other significant correlations between the evaluated parameters.

We wish to highlight that the subjects included in this pilot study were young men and most of them were overweight or obese, potentially limiting the generalizability of our data. However, BMI was not statistically different between two groups. Therefore, the participants were young and without chronic metabolic disorders and this may be the reason why differences between several biomarkers associated with inflammation did not attain statistical significance. Strengths of our study include the strict exclusion criteria as well as the blinded measurements of biochemical parameters. We used high-quality methodology to assess the full spectrum of LDL and HDL subclasses. A Limitation of this study is the relatively small number of subjects included.

CONCLUSION

To our knowledge, this is the first study showing the full lipoprotein profile in NASH and

SS subjects comparing directly these two phenotypes of NAFLD. Our study suggests that NASH subjects have increased levels of sdLDL that may contribute to an increased risk for atherosclerosis and CVD compared to subjects with SS. *However, due to the limitations of the relatively small number of patients enrolled in this study, these findings should be considered with caution until additional larger studies can be performed.*

CONFLICT OF INTEREST

The authors did not receive financial or professional help with the preparation of the manuscript.

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Highlights

- NAFLD is associated with changes in the LDL profile.
- Subjects with NASH, had increased small dense LDL (sdLDL) particles.
- sdLDL correlate with triglycerides (positively) and with HDL-C (inversely).
- The young age and the early stage of NASH may explain some no significant changes.
- Increased sdLDL may aggravate the liver disease and increase CVD risk.