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Metabolic Syndrome Disrupts the Association Between Serum Malondialdehyde and Lipoprotein Subclasses

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Abstract

Malondialdehyde (MDA) is a widely used biomarker of lipid peroxidation, yet its relationships with lipoprotein subclasses remain poorly explored, particularly in metabolic syndrome (MS). We investigated associations of free MDA measured by the TBARS assay (MDA-TBARS) and total MDA quantified by high-performance liquid chromatography (MDA-HPLC) with NMR spectroscopy-measured lipoprotein subclasses in 65 healthy volunteers (HV) and 65 patients with MS. MDA-HPLC concentrations were higher than MDA-TBARS, and the two measures were strongly correlated. Free MDA levels were significantly elevated in MS, whereas total MDA did not differ between groups. In HV, MDA-TBARS showed positive relationships with VLDL and IDL, as well as triglyceride enrichment of LDL, IDL, and HDL, and a negative relationship with levels and cholesterol content of small, dense HDL, as well as cholesterol and phospholipid content of LDL subclasses. MDA-HPLC only showed a positive association with VLDL levels in HV. In contrast, no significant associations between MDA and lipoproteins were observed in MS. Multivariable analyses confirmed that lipoprotein profiles were able to discriminate between low and high MDA levels in HV, but not in patients with MS. In conclusion, interactions between oxidative stress and lipoproteins are strongly context dependent, being preserved in health but disrupted in MS, with free MDA emerging as a more informative marker of lipoprotein-associated lipid peroxidation.

Keywords: NMR spectroscopy; lipoprotein profiling; malondialdehyde; metabolic syndrome

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1. Introduction

Malondialdehyde (MDA) is a widely used biomarker of lipid peroxidation (LPO) and oxidative stress (OS). It is produced during oxidative degradation of polyunsaturated fatty acids in plasma membranes and circulating lipoproteins. While a portion of MDA remains free or in labile adducts, a substantial fraction forms relatively stable covalent adducts with amino groups of proteins and other nucleophiles [1].

Lipoprotein particles are heterogeneous in size, density, composition, and atherogenic potential. Subclassification of lipoproteins identifies distinct particle populations of very-low-density lipoproteins (VLDLs), intermediate-density lipoproteins (IDLs), low-density lipoproteins (LDLs), and high-density lipoproteins (HDLs), which differ in susceptibility to oxidative modification and in relationships to cardiometabolic risk [2–4].

Metabolic syndrome (MS) is characterized by the clustering of abdominal obesity, hyperglycemia, hypertension, hypertriglyceridemia, and low HDL-cholesterol, accompanied by chronic low-grade inflammation and increased OS [5]. Oxidative mechanisms in MS include mitochondrial dysfunction, enhanced NADPH-oxidase activity, and impaired antioxidant defenses that together promote LPO and protein modification [6–8].

Because lipid peroxidation products such as MDA can both reflect and mediate vascular and metabolic injury [9–11], understanding how free versus total MDA relate to lipoprotein subclass bioavailability and lipid content may help clarify links among oxidative stress, lipoprotein pathophysiology, and cardiometabolic risk. Accordingly, this study investigates the associations of free MDA measured by the thiobarbituric acid reactive substance (TBARS) assay (MDA-TBARS) and total serum MDA quantified by high-performance liquid chromatography (HPLC) (MDA-HPLC) with nuclear magnetic resonance spectroscopy (NMR)-measured lipoprotein subclasses in healthy volunteers (HVs) and in patients with MS.

2. Materials and Methods

2.1. Study Design, Participants, and Procedures

This study represents a secondary analysis of data from a previously published observational, cross-sectional study [12]. A total of 130 participants, 65 HV and 65 MS patients, aged 45 to 65 years, were enrolled. MS was defined by five internationally unified criteria [5] and diagnosed if at least three criteria were met. Waist circumference thresholds of <102 cm in men and <88 cm in women were considered appropriate for the study population. Participants were asked to attend the study visit after an 8–12 h fast, avoiding both food and caffeinated drinks. They were also instructed to avoid smoking and physical activity and to withhold vasoactive medications and vitamin supplements during the 24 h preceding the visit. The inclusion and exclusion criteria, along with all study procedures, are described in detail in our previous reports [12,13].

The study was approved by the local ethics committees of the Sisters of Charity University Hospital Centre, Zagreb, Croatia (EP 13125/17-4); the University of Zagreb, School of Medicine, Croatia; and the Medical University of Graz, Austria (31-532 ex 18/19). All participants signed an informed consent form, and the study was performed in accordance with the principles of Good Clinical Practice Guidelines and the Declaration of Helsinki [14]. A flowchart of the study is presented in Figure 1.

2.2. Preparation and Handling of Serum

Blood samples were allowed to clot at room temperature for 30 min and then centrifuged at 1800× *g* for 10 min at 4 °C. The resulting serum was divided into 1 mL aliquots and stored at −80 °C. Prior to analysis, aliquots were thawed on ice, transferred to pre-cooled tubes in volumes appropriate for each assay, and returned to −80 °C until use.

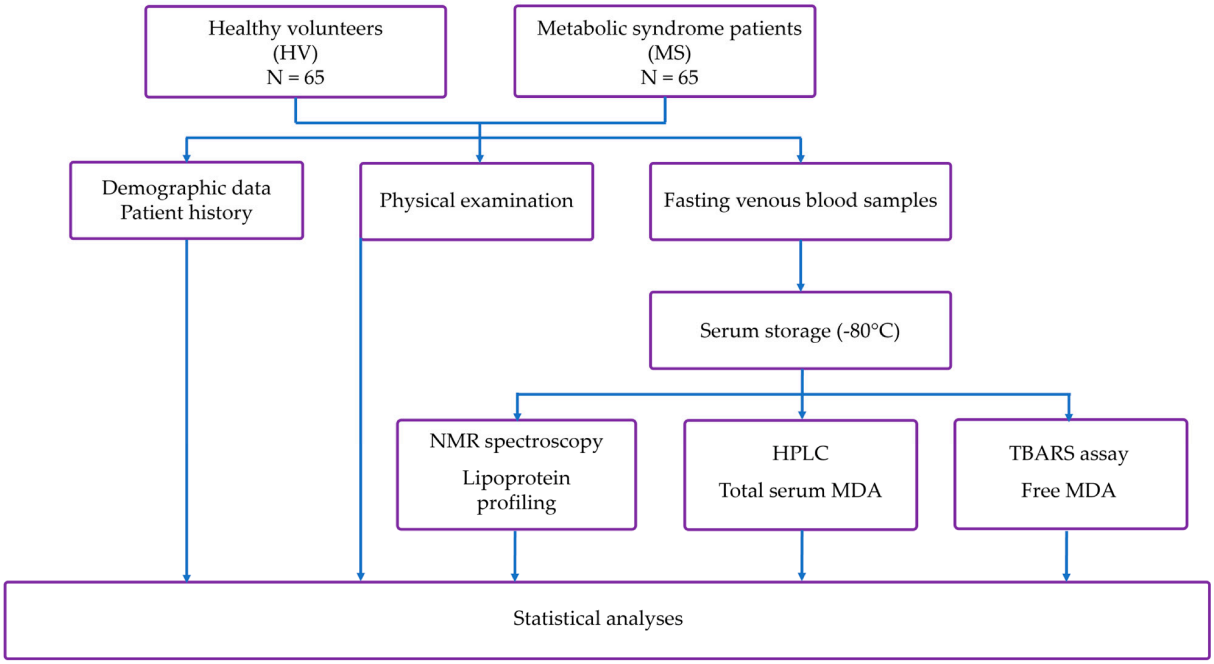


Figure 1. Flowchart of the study. HPLC, high-performance liquid chromatography; HV, healthy volunteer; MDA, malondialdehyde; MS, metabolic syndrome; NMR, nuclear magnetic resonance spectroscopy; TBARS, thiobarbituric acid reactive substances.

2.3. Quantification of Total Serum MDA Using HPLC

Serum levels of total MDA were measured using the HPLC method described by Pilz et al. [15] following derivatization with 2,4-dinitrophenylhydrazine (DNPH). We used minor modifications to the original protocol that have been reported elsewhere [16]. Alkaline hydrolysis of protein-bound MDA was performed by adding 25 µL of 6 mol/L sodium hydroxide to 0.125 mL of each sample (1.5 mL Eppendorf tubes), followed by incubation at 60 °C for 30 min. The hydrolyzed samples were deproteinized with 62.5 µL of 35% (*v*/*v*) perchloric acid. After centrifugation (14,000 g, 2 min), 125 µL of the supernatant was mixed with 12.5 µL DNPH solution and incubated for 10 min. This reaction mixture, along with derivatized standard solutions (0.625–10 µmol/L) and reagent blanks, was injected into the HPLC system (40 µL injection volume). The MDA standard was prepared by dissolving 25 µL of 1,1,3,3-tetramethoxypropane (TMP) in 100 mL of bidistilled water to obtain a 1 mmol/L stock solution. Hydrolysis was carried out using 200 µL of this stock solution in 10 mL of 1% sulfuric acid, incubated for 2 h at room temperature [17]. The resulting 20 µmol/L MDA standard was further diluted in 1% sulfuric acid to the required concentrations. DNPH-derived hydrazones were separated isocratically on a 5 µm ODS Hypersil column (150 × 4.6 mm), protected by a 5 µm ODS Hypersil guard column (10 × 4.6 mm; Uniguard holder). The mobile phase consisted of 0.2% (*v*/*v*) acetic acid in bidistilled water containing 50% acetonitrile (*v*/*v*), delivered at a flow rate of 0.8 mL/min. HPLC analyses were performed using an L-2200 autosampler (40 µL injection volume), an L-2130 HTA pump, and an L-2450 diode-array detector (VWR Hitachi, Vienna, Austria). Absorbance was monitored at 310 nm, and data acquisition and analysis were conducted using EZChrom Elite (VWR).

2.4. Quantification of Free MDA Using TBARS Assay

Serum levels of free MDA were determined in deproteinized serum samples after incubation with thiobarbituric acid at 95 °C for 1 h in a preheated sand bath, followed by absorbance measurement at 532 nm, as described in our previous report [18].

2.5. Lipoprotein Profiling Using Nuclear Magnetic Resonance (NMR) Spectroscopy

Serum lipoprotein subclasses were measured on a Bruker 600 MHz Avance Neo NMR spectrometer (Brucker, Rheinstetten, Germany) using the Bruker IVDr lipoprotein subclass analysis protocol, as described previously [12,13,19,20].

2.6. Calculation of the Lipid Content of Lipoprotein Particles

Lipid content in apoB-containing lipoproteins (VLDL, IDL, and LDL) was calculated as the ratio of the serum concentration of each lipid (mg/dL) to that of apoB (mg/dL). For HDL, lipid content was expressed as the ratio of each lipid to HDL-apoA-I concentration (mg/dL) [12,13,19].

2.7. Statistical Analyses

Qualitative variables are summarized as absolute and relative frequencies, while quantitative variables are described using mean ± standard deviation or median (interquartile range, Q1–Q3), depending on data distribution. Differences between HVs and patients with MS, as well as between HV and MS patients stratified by low and high MDA (based on within-group medians), were assessed using Fisher's exact test, *t*-test, or Mann–Whitney U test, as appropriate. Spearman's correlation coefficients were calculated separately for HV and MS patients. The influence of potential confounders on the associations between MDA and the serum levels and lipid content of lipoprotein subclasses was examined by partial correlation analysis. The following parameters were used for adjustment: age, sex, body mass index (BMI), and C-reactive protein (CRP) or interleukin-6 (IL-6) as covariates. The discriminatory power of lipoproteins to distinguish between individuals with high versus low MDA was assessed separately in HV and MS patients using orthogonal partial least squares discriminant analysis (OPLS-DA) [21]. OPLS-DA, data consistency checks, and 7-fold cross-validation [22] were performed with MetaboAnalyst [23]. A p-value < 0.05 was considered statistically significant for demographic, clinical, and standard laboratory data. For analyses of serum levels and lipid content of lipoprotein subclasses, as well as correlation analyses, Benjamini–Hochberg-corrected (for all n = 100 lipoprotein parameters) p-values < 0.05 were considered significant. All statistical analyses, except OPLS-DA, were conducted using R version 4.1.0.

3. Results

3.1. Demographic and Clinical Characteristics, Standard Laboratory Data, and Lipoprotein Parameters in HV and MS Patients

The demographic and clinical characteristics, the medication and standard laboratory data of the study groups, and differences in lipoprotein parameters between HV and MS patients have been described previously [12,13] and are presented in Tables 1, 2, S1 and S2.

Table 1. Differences in demographic and clinical characteristics between HV and MS patients.

Variable	HV (N = 65)	MS (N = 65)	*p*
Age (years)	56.0 (50.0, 59.0)	57.0 (50.0, 60.0)	0.440
Sex (female)	31 (47.7%)	31 (47.7%)	1.000
Body weight (kg)	77.0 (68.0, 88.0)	98.0 (86.0, 113.5)	**<0.001**
Body height (m)	1.75 ± 0.10	1.73 ± 0.11	0.243
BMI (kg/m^2)	25.1 (23.7, 28.1)	32.6 (29.8, 35.9)	**<0.001**

Table 1. *Cont.*

Variable	HV (N = 65)	MS (N = 65)	*p*
Waist circumference (cm)	92.2 ± 11.6	113.9 ± 13.2	**<0.001**
MAP (mmHg)	88.3 (85.0, 96.7)	98.3 (96.7, 101.7)	**<0.001**
Chronic diseases			
Arterial hypertension	0 (0.0%)	60 (92.3%)	**<0.001**
Diabetes mellitus type 2	0 (0.0%)	27 (41.5%)	**<0.001**
Stable angina pectoris	0 (0.0%)	2 (3.1%)	0.496
Atrial fibrillation	0 (0.0%)	2 (3.1%)	0.496
CVI, TIA	0 (0.0%)	1 (1.5%)	1.000
Intermittent claudication	0 (0.0%)	4 (6.2%)	0.119
Deep venous thrombosis	1 (1.5%)	5 (7.7%)	0.208
Pulmonary embolism	0 (0.0%)	2 (3.1%)	0.496
Functions and habits			
Smoking	16 (24.6%)	18 (27.7%)	0.842
Physical activity (≥3 times/week)	58 (89.2%)	47 (72.3%)	**0.025**
Menstrual cycle (female)	12/31 (38.7%)	6/31 (19.4%)	0.161

Data are presented as N (%), mean ± standard deviation, or median (q1, q3). Differences between HV and MS patients were tested using Fisher's exact test, *t*-test, or Mann–Whitney U test. *p*-values < 0.05 are considered statistically significant and are depicted in bold. BMI, body mass index; cm, centimeter; CVI, cerebrovascular infarction; HV, healthy volunteer; kg, kilogram; m, meter; MAP, mean arterial pressure; MS, metabolic syndrome; N, number; TIA, transient ischemic attack.

Table 2. Differences in laboratory data between HVs and patients with MS.

Variable	HV (N = 65)	MS (N = 65)	*p*
Triglycerides (mmol/L)	1.0 (0.8, 1.4)	1.6 (1.1, 2.2)	**<0.001**
Total cholesterol (mmol/L)	5.5 (5.1, 6.0)	5.0 (4.3, 6.2)	0.057
LDL-C (mmol/L)	3.3 (2.8, 3.7)	3.0 (2.3, 3.7)	0.077
HDL-C (mmol/L)	1.6 (1.4, 1.8)	1.2 (1.0, 1.4)	**<0.001**
Glucose (mmol/L)	4.9 (4.8, 5.2)	5.7 (5.3, 6.5)	**<0.001**
Protein (g/L)	72.0 (69.0, 75.0)	75.0 (71.0, 77.0)	**0.002**
Albumin (g/L)	47.0 (46.0, 49.0)	48.0 (45.0, 49.0)	0.465
CRP (µg/mL)	1.2 (0.6, 2.3)	2.4 (1.2, 5.5)	**<0.001**
IL-6 (pg/mL)	2.3 (1.7, 3.0)	4.1 (2.7, 6.8)	**<0.001**
Bilirubin (µmol/L)	9.6 (7.4, 13.3)	7.4 (5.5, 10.4)	**0.012**
AST (U/L)	23.0 (20.0, 25.0)	23.0 (19.0, 32.0)	0.244
ALT (U/L)	22.0 (18.0, 29.0)	30.0 (22.0, 43.0)	**<0.001**
AP (U/L)	60.0 (49.0, 70.0)	65.0 (52.0, 81.0)	0.065
GGT (U/L)	16.0 (13.0, 30.0)	31.0 (21.0, 44.0)	**<0.001**
CK (U/L)	115.0 (81.0, 153.0)	133.0 (86.0, 226.0)	**0.048**
LDH (U/L)	168.0 (147.0, 191.0)	176.0 (158.0, 193.0)	0.365
Urea (mmol/L)	5.0 (4.2, 6.0)	5.6 (4.8, 6.5)	**0.004**

Table 2. *Cont.*

Variable	HV (N = 65)	MS (N = 65)	*p*
Urate (µmol/L)	273.7 (232.0, 327.2)	315.3 (279.7, 362.9)	**<0.001**
Creatinine (µmol/L)	77.9 (69.0, 89.4)	76.6 (65.5, 87.0)	0.414
eGFR (mL/min/1.73 m^2)	87.5 (77.2, 93.6)	88.9 (79.1, 98.0)	0.358
Sodium (mmol/L)	140.0 (138.0, 141.0)	139.0 (138.0, 140.0)	**0.041**
Potassium (mmol/L)	4.3 (4.1, 4.5)	4.2 (4.1, 4.6)	0.703
Chloride (mmol/L)	101.0 (99.0, 103.0)	100.0 (98.0, 101.0)	**0.006**

Data are presented as median (q1, q3). Differences between HV and MS patients were tested using the Mann–Whitney U test. *p*-values < 0.05 are considered statistically significant and are depicted in bold. LDL-C and eGFR data were available for 60 and 64 MS patients, respectively. ALT, alanine aminotransferase; AP, alkaline phosphatase; AST, aspartate aminotransferase; CK, creatine kinase; CRP, C-reactive protein; eGFR, estimated glomerular filtration rate; g, gram; GGT, gamma-glutamyl transpeptidase; HV, healthy volunteer; HDL-C, high-density lipoprotein cholesterol; IL-6, interleukin 6; LDH, lactate dehydrogenase; LDL-C, low-density lipoprotein cholesterol; MS, metabolic syndrome.

3.2. Differences and Correlations Between Serum Levels of MDA-TBARS and MDA-HPLC

MDA-HPLC serum levels were significantly higher compared to MDA-TBARS in both HVs and patients with MS. While MDA-TBARS levels were significantly higher in patients with MS compared to HVs, the levels of MDA-HPLC were not (Figure 1). The serum levels of MDA-TBARS and MDA-HPLC were strongly and significantly correlated in both study groups (r = 0.89; $p < 0.001$) (Figure 2).

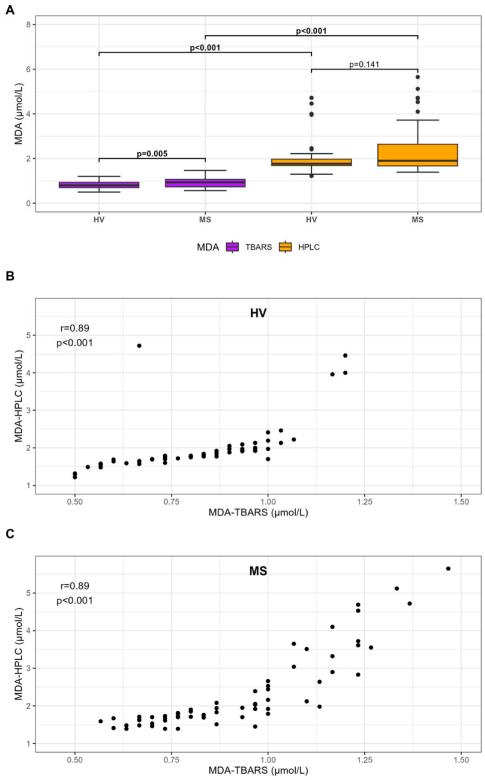


Figure 2. (**A**) Comparison of MDA-TBARS and MDA-HPLC levels within study groups and between HV and MS patients. Group differences were assessed using the Mann–Whitney U test. (**B**,**C**) Spearman correlation analyses between MDA-TBARS and MDA-HPLC in HVs and patients with MS. *p*-values < 0.05 were considered statistically significant. HPLC, high-performance liquid chromatography; HV, healthy volunteer; MDA, malondialdehyde; MS, metabolic syndrome; TBARS, thiobarbituric acid reactive substances.

3.3. Correlation Analyses Between MDA and VLDL Parameters

In HV but not in patients with MS, serum MDA-TBARS levels showed significant positive correlations with several indicators of VLDL abundance, including cholesterol levels in VLDL subclasses 1–3, triglyceride levels in VLDL subclasses 2–4, phospholipid levels in VLDL subclasses 2, 3, and 5, and VLDL-apoB concentrations (Figure 3). In contrast, MDA-HPLC levels were only positively correlated with cholesterol and phospholipid levels in VLDL subclass 5 in HV. Neither MDA-TBARS nor MDA-HPLC were significantly correlated with measures of VLDL lipid content in HV, and no significant associations were observed in patients with MS.

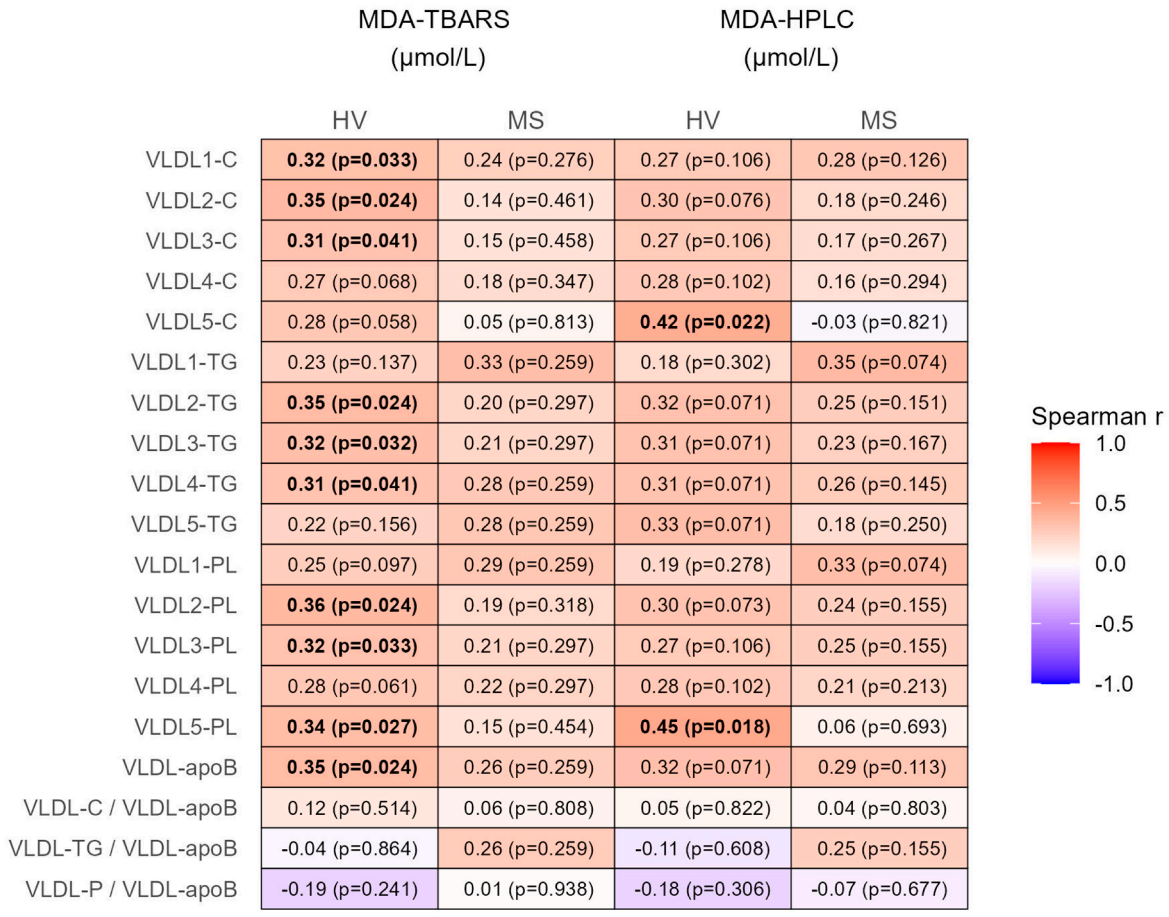


Figure 3. Heatmap showing Spearman correlations between serum MDA-TBARS and MDA-HPLC levels and VLDL subclasses, as well as ratios reflecting VLDL lipid content, analyzed separately in HVs and patients with MS. Serum levels of lipids and apolipoproteins in VLDL subclasses (VLDL1: very large particles; VLDL2: large particles; VLDL3: medium-sized particles; VLDL4: small particles; VLDL5: very small particles) are reported in mg/dL. p-values < 0.05 after Benjamini–Hochberg correction are considered statistically significant. Significant Spearman correlation coefficients and corresponding significant p-values are depicted in bold. ApoB, apolipoprotein B; C, cholesterol; HPLC, high-performance liquid chromatography; HV, healthy volunteer; VLDL, very-low-density lipoprotein; MS, metabolic syndrome; PL, phospholipid; r, Spearman's correlation coefficient; TG, triglyceride; TBARS, thiobarbituric acid reactive substances.

3.4. Correlation Analyses Between MDA and IDL Parameters

Serum levels of MDA-TBARS were significantly positively correlated with the serum levels of triglycerides in IDL and with the triglyceride content of IDL in HV but not in patients with MS (Figure 4). No significant associations were observed in patients with MS.

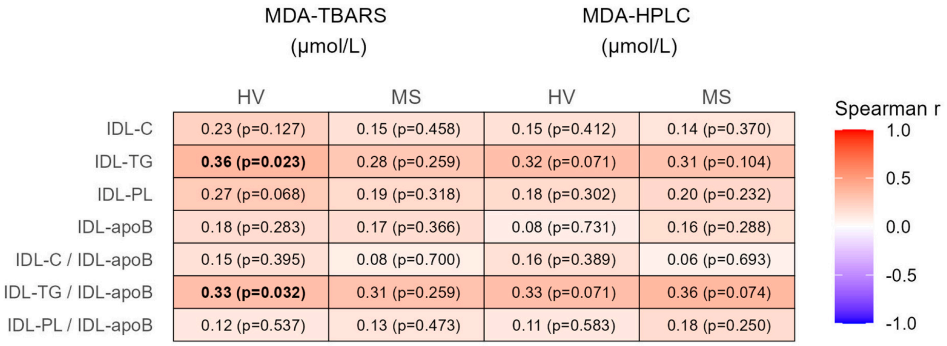


Figure 4. Heatmap showing Spearman correlations between serum MDA-TBARS and MDA-HPLC levels and IDL subclasses, as well as ratios reflecting IDL lipid content, analyzed separately in HVs and patients with MS. Serum levels of lipids and apolipoproteins in IDL are given in mg/dL. *p*-values < 0.05 after Benjamini–Hochberg correction are considered statistically significant. Significant Spearman correlation coefficients and corresponding significant *p*-values are depicted in bold. ApoB, apolipoprotein B; C, cholesterol; HPLC, high-performance liquid chromatography; HV, healthy volunteer; IDL, intermediate-density lipoprotein; MS, metabolic syndrome; PL, phospholipid; r, Spearman's correlation coefficient; TG, triglyceride; TBARS, thiobarbituric acid reactive substance.

3.5. Correlation Analyses Between MDA and LDL Parameters

In HV, but not in patients with MS, MDA-TBARS levels were significantly negatively correlated with the cholesterol content of LDL subclasses 3–6 (medium- to small-sized particles) and with the phospholipid content of subclasses 1–3 (large- to medium-sized particles). In contrast, MDA-TBARS levels showed significant positive correlations with the triglyceride content of LDL subclasses 1–2 (very large and large particles) and 4–5 (small particles) (Figure 4). No significant correlations were observed between MDA-HPLC and indicators of LDL lipid content in either HV or patients with MS (Figure 5). Likewise, no significant correlations were found between serum MDA-TBARS or MDA-HPLC levels and the concentrations of LDL subclasses (Table S3).

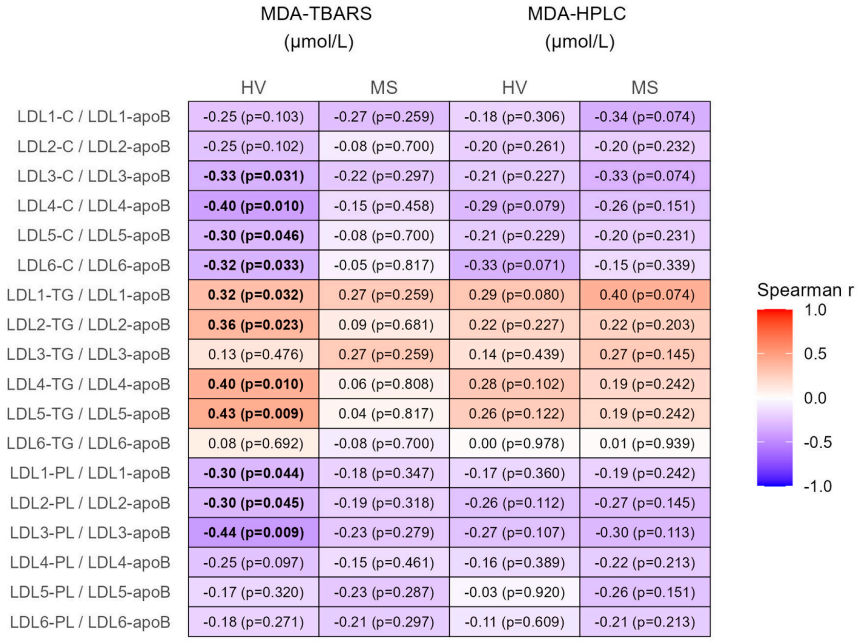


Figure 5. Heatmap showing Spearman correlations between serum MDA-TBARS and MDA-HPLC levels and ratios reflecting lipid content of LDL subclasses, analyzed separately in HVs and patients with MS. *p*-values < 0.05 after Benjamini–Hochberg correction are considered statistically significant. Significant Spearman correlation coefficients and corresponding significant *p*-values are depicted

in bold. ApoB, apolipoprotein B; C, cholesterol; HPLC, high-performance liquid chromatography; HV, healthy volunteer; LDL, low-density lipoprotein; MS, metabolic syndrome; PL, phospholipid; r, Spearman's correlation coefficient; TG, triglyceride; TBARS, thiobarbituric acid reactive substances.

3.6. Correlation Analyses Between MDA and HDL Parameters

In HV, but not in patients with MS, MDA-TBARS levels were significantly negatively correlated with serum cholesterol levels and cholesterol content of small dense HDL subclasses 3–4, as well as with phospholipid, apoA-I, and apoA-II levels in HDL subclass 4 (Figure 6). In addition, significant positive correlations were observed in HV between MDA-TBARS levels and triglyceride concentrations in HDL subclasses 2 and 3, as well as with triglyceride content across HDL subclasses 1–4. Similar associations were observed in HV for MDA measured by HPLC; however, these correlations were weaker and thus not statistically significant.

	MDA-TBARS (µmol/L)		MDA-HPLC (µmol/L)	
	HV	MS	HV	MS
HDL1-C	-0.10 (p=0.611)	0.17 (p=0.366)	-0.08 (p=0.731)	0.19 (p=0.242)
HDL2-C	-0.17 (p=0.299)	0.01 (p=0.945)	-0.11 (p=0.599)	-0.05 (p=0.755)
HDL3-C	**-0.30 (p=0.045)**	-0.11 (p=0.593)	-0.19 (p=0.299)	-0.18 (p=0.246)
HDL4-C	**-0.43 (p=0.009)**	-0.04 (p=0.817)	-0.30 (p=0.072)	-0.18 (p=0.250)
HDL1-TG	0.23 (p=0.127)	0.10 (p=0.662)	0.18 (p=0.303)	0.19 (p=0.242)
HDL2-TG	**0.36 (p=0.024)**	0.05 (p=0.817)	0.31 (p=0.071)	0.13 (p=0.406)
HDL3-TG	**0.33 (p=0.032)**	0.10 (p=0.662)	0.31 (p=0.071)	0.14 (p=0.374)
HDL4-TG	0.21 (p=0.165)	0.17 (p=0.366)	0.23 (p=0.186)	0.20 (p=0.232)
HDL1-PL	-0.07 (p=0.728)	0.07 (p=0.726)	-0.04 (p=0.858)	0.09 (p=0.582)
HDL2-PL	-0.03 (p=0.865)	-0.03 (p=0.892)	0.02 (p=0.964)	-0.06 (p=0.693)
HDL3-PL	-0.16 (p=0.342)	-0.14 (p=0.464)	-0.05 (p=0.837)	-0.21 (p=0.213)
HDL4-PL	**-0.35 (p=0.025)**	-0.12 (p=0.527)	-0.21 (p=0.227)	-0.25 (p=0.155)
HDL1-apoA-I	-0.08 (p=0.708)	0.10 (p=0.662)	-0.04 (p=0.886)	0.14 (p=0.353)
HDL2-apoA-I	-0.11 (p=0.549)	0.01 (p=0.938)	-0.07 (p=0.761)	-0.02 (p=0.882)
HDL3-apoA-I	-0.19 (p=0.222)	0.00 (p=0.990)	-0.08 (p=0.731)	-0.06 (p=0.693)
HDL4-apoA-I	**-0.33 (p=0.031)**	-0.05 (p=0.817)	-0.23 (p=0.197)	-0.16 (p=0.288)
HDL1-apoA-II	0.00 (p=0.998)	0.04 (p=0.817)	-0.02 (p=0.963)	0.05 (p=0.755)
HDL2-apoA-II	0.04 (p=0.859)	0.03 (p=0.853)	0.01 (p=0.964)	0.01 (p=0.939)
HDL3-apoA-II	-0.13 (p=0.480)	0.02 (p=0.911)	-0.11 (p=0.599)	-0.01 (p=0.931)
HDL4-apoA-II	**-0.33 (p=0.032)**	0.02 (p=0.902)	-0.26 (p=0.112)	-0.11 (p=0.456)
HDL1-C / HDL1-apoA-I	-0.08 (p=0.680)	0.12 (p=0.535)	-0.15 (p=0.399)	0.05 (p=0.755)
HDL2-C / HDL2-apoA-I	-0.10 (p=0.595)	-0.04 (p=0.817)	-0.05 (p=0.829)	-0.11 (p=0.467)
HDL3-C / HDL3-apoA-I	**-0.40 (p=0.010)**	-0.21 (p=0.297)	-0.37 (p=0.054)	-0.27 (p=0.145)
HDL4-C / HDL4-apoA-I	**-0.48 (p=0.005)**	-0.07 (p=0.768)	-0.36 (p=0.054)	-0.18 (p=0.250)
HDL1-TG / HDL1-apoA-I	**0.42 (p=0.009)**	0.12 (p=0.535)	0.32 (p=0.071)	0.21 (p=0.213)
HDL2-TG / HDL2-apoA-I	**0.42 (p=0.009)**	0.13 (p=0.485)	0.36 (p=0.054)	0.23 (p=0.168)
HDL3-TG / HDL3-apoA-I	**0.41 (p=0.009)**	0.19 (p=0.318)	0.35 (p=0.068)	0.29 (p=0.113)
HDL4-TG / HDL4-apoA-I	**0.37 (p=0.020)**	0.19 (p=0.318)	0.36 (p=0.054)	0.28 (p=0.126)
HDL1-PL / HDL1-apoA-I	0.07 (p=0.732)	-0.09 (p=0.662)	0.01 (p=0.978)	-0.17 (p=0.270)
HDL2-PL / HDL2-apoA-I	0.11 (p=0.578)	-0.09 (p=0.674)	0.16 (p=0.389)	-0.11 (p=0.463)
HDL3-PL / HDL3-apoA-I	-0.02 (p=0.902)	-0.21 (p=0.297)	0.01 (p=0.964)	-0.25 (p=0.155)
HDL4-PL / HDL4-apoA-I	-0.05 (p=0.804)	-0.26 (p=0.259)	0.06 (p=0.806)	-0.33 (p=0.074)

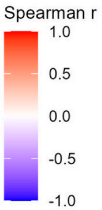


Figure 6. Heatmap showing correlations between serum MDA-TBARS and MDA-HPLC levels and HDL subclasses, as well as ratios reflecting HDL lipid content, analyzed separately in HVs and patients with MS. Serum levels of lipids and apolipoproteins in HDL subclasses are given in mg/dL. *p*-values < 0.05 after Benjamini–Hochberg correction are considered statistically significant. Significant Spearman correlation coefficients and corresponding significant *p*-values are depicted in bold. ApoA-I, apolipoprotein A-I; ApoA-II, apolipoprotein A-II; C, cholesterol; HDL, high-density lipoprotein; HPLC, high-performance liquid chromatography; HV, healthy volunteer; MS, metabolic syndrome; PL, phospholipid; r, Spearman's correlation coefficient; TG, triglyceride; TBARS, thiobarbituric acid reactive substances.

3.7. Adjustment for Patient Characteristics and Inflammatory Parameters

As revealed by partial correlation analyses, all significant correlations presented in Figures 3–6 remained significant after adjustment for age, sex, BMI, and CRP or IL-6 (Table S4).

3.8. Associations of Lipoprotein Parameters with Low and High MDA-TBARS and MDA-HPLC in HV and MS Patients

We also performed multivariate analyses using OPLS-DA to investigate the relative contributions of lipoprotein parameters to low and high MDA-TBARS and MDA-HPLC levels in HVs and patients with MS. Low and high values were defined as below or above (≥) the respective group median. For MDA-TBARS, the median values were 0.80 µmol/L for HV and 0.93 µmol/L for MS, and for MDA-HPLC, the median values were 1.77 µmol/L for HV and 1.90 µmol/L for MS, respectively. Differences in serum levels and lipid content of lipoproteins between participants with low and high MDA-TBARS and MDA-HPLC levels, analyzed separately for HV and MS patients, are summarized in Tables S5 and S6.

OPLS-DA modeling revealed that lipoprotein profiles are associated with MDA levels in HVs but not in patients with MS. For MDA-TBARS, the model explained 29.5% of variance in HVs ($R^2Y = 0.295$, $p = 0.001$) with modest predictive ability ($Q^2 = 0.188$, $p = 0.001$), while in patients with MS, no significant association was found ($R^2Y = 0.175$, $p = 0.4$; $Q^2 = -0.054$, $p = 0.11$). MDA-HPLC showed a non-significant association in HVs ($R^2Y = 0.247$, $p = 0.07$) with minimal predictive power ($Q^2 = 0.030$, $p = 0.03$) and no significant link in patients with MS ($R^2Y = 0.149$, $p = 0.64$; $Q^2 = -0.047$, $p = 0.14$).

OPLS-DA plots show the following patterns: in HVs, low-MDA samples (red) are clustered on the left and high-MDA samples (green) on the right along the predictive component, with some overlap, whereas MS samples showed weaker separation with dominant orthogonal variation (Figure 7).

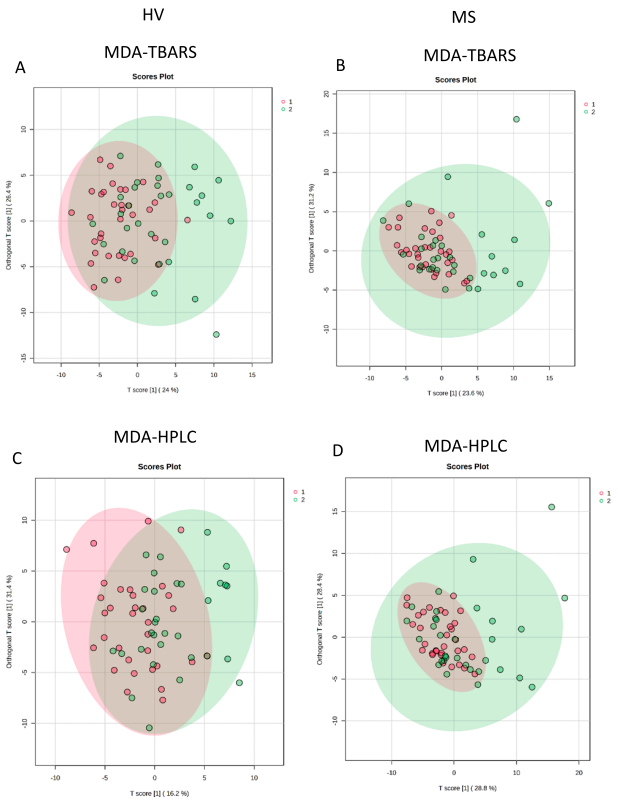


Figure 7. OPLS-DA score plots of lipoprotein parameters by MDA levels. Low-MDA samples are shown in red and high-MDA samples in green: (**A**) low MDA-TBARS < 0.80 mmol/mL, high MDA-

TBARS ≥ 0.80 µmol/L; (**B**) low MDA-TBARS < 0.93 µmol/L, high MDA-TBARS ≥ 0.93 µmol/L; (**C**) low MDA-HPLC < 1.77 µmol/L, high MDA-HPLC ≥ 1.77 µmol/L; (**D**) low MDA-HPLC < 1.90 µmol/L, high MDA-HPLC ≥ 1.90 µmol/L. HPLC, high-performance liquid chromatography; HV, healthy volunteer; MDA, malondialdehyde; MS, metabolic syndrome; OPLS-DA, orthogonal partial least squares discriminant analysis; TBARS, thiobarbituric acid reactive substances.

The relative contributions of lipoprotein parameters to the separation of low and high MDA-TBARS and MDA-HPLC samples were assessed using Variable Importance in Projection (VIP) scores. VIP scores are shown only for HV (Figure 8), as the non-significant models in MS patients preclude meaningful interpretation.

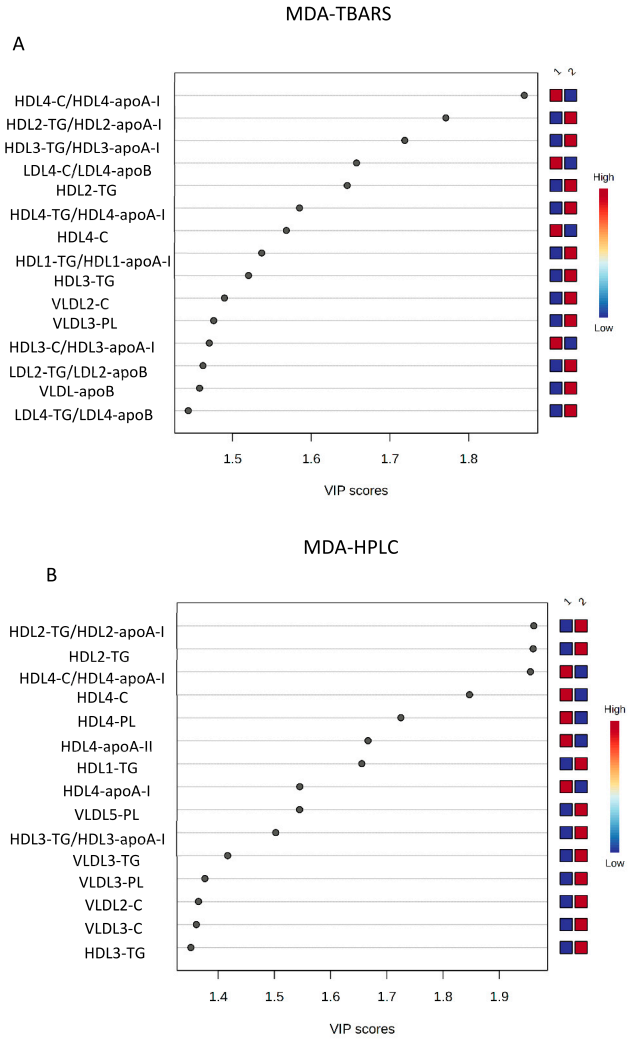


Figure 8. VIP scores of lipoprotein parameters in HV. Top 15 VIP scores reflecting the contribution of the parameters to the separation of low and high (**A**) MDA-TBARS and (**B**) MDA-HPLC samples. In the image, 1 and 2 indicate low or high MDA-TBARS or MDA-HPLC levels, respectively. ApoA-I, apolipoprotein A-I; ApoA-II, apolipoprotein A-II; apoB, apolipoprotein B; C, cholesterol; HDL, high-density lipoprotein; HPLC, high-performance liquid chromatography; HV, healthy volunteer; LDL, low-density lipoprotein; MDA, malondialdehyde; PL, phospholipid; TBARS, thiobarbituric acid reactive substances; TG, triglyceride; VIP, variable importance in projection; VLDL, very-low-density lipoprotein.

Among the top 15 lipoprotein parameters with the highest VIP scores, eight were shared between MDA-TBARS and MDA-HPLC. These included cholesterol content in HDL subclass 4 (HDL4-C/HDL4-apoA-I), triglyceride content in HDL subclass 2 (HDL2-TG/HDL2-apoA-I) and HDL subclass 3 (HDL3-TG/HDL3-apoA-I), serum triglyceride levels in HDL

subclasses 2 (HDL2-TG) and 3 (HDL3-TG), serum cholesterol levels in HDL subclass 4 (HDL4-C) and VLDL subclass 2 (VLDL2-C), and serum phospholipid levels in VLDL subclass 3 (VLDL3-PL) (Figure 8A,B).

4. Discussion

Associations between circulating MDA levels and lipoprotein subclasses have remained largely unexplored. In this study, we therefore investigated the relationships between two analytically distinct measures of MDA, free MDA assessed in deproteinized serum using the TBARS assay and total MDA quantified by HPLC after release of protein-bound MDA, and NMR-based lipoprotein subclasses in HVs and patients with MS. The principal finding is that lipoprotein subclass composition and lipid content are strongly associated with circulating levels of free MDA in healthy individuals but not in patients with MS, despite significantly higher free MDA levels in the latter group. These results suggest that the interactions between OS and lipoproteins differ fundamentally between physiological and metabolically dysregulated states such as MS, which is characterized by increased OS, perturbed lipid metabolism, impaired antioxidative functions of HDL, and persistent low-grade inflammation [5,6,24].

MDA-HPLC concentrations were markedly higher than MDA-TBARS levels in both study groups, consistent with previous reports showing that total MDA largely reflects protein-bound or otherwise derivatized MDA species, whereas TBARS predominantly captures free or labile MDA fractions [1,9,25]. The strong correlations between MDA-TBARS and MDA-HPLC in both HV and MS patients indicate that these measures reflect related oxidative processes while representing partially distinct biochemical pools of lipid peroxidation products.

Importantly, free MDA (MDA-TBARS) was significantly elevated in patients with MS, whereas total MDA (MDA-HPLC) did not differ between groups. This dissociation suggests that MS is characterized by an increased burden of circulating reactive aldehydes rather than an accumulation of stable MDA adducts. Such a pattern may reflect enhanced lipid peroxidation rates associated with the chronic OS and low-grade inflammation typical of MS [6,24]. The majority of observed associations between MDA and lipoprotein subclasses were stronger for MDA-TBARS than for MDA-HPLC. It remains unclear whether this reflects methodological differences between the assays or physiological mechanisms that weaken associations between protein-bound MDA species and circulating lipoproteins.

One of the most striking findings was the consistent association between MDA-TBARS and HDL subclass levels and lipid composition in HV. In line with the established antioxidative capacity of small, dense HDL particles, conferred by specific proteins, enzymes, and lipid constituents [24,26–28], higher free MDA levels were associated with lower serum concentrations of cholesterol, phospholipids, apoA-I, and apoA-II, as well as reduced cholesterol content in smaller, denser HDL subclasses, together with increased triglyceride content across HDL subclasses. This pattern is indicative of HDL triglyceride enrichment and functional impairment, consistent with results of experimental studies demonstrating that triglyceride enrichment negatively affects and cholesterol enrichment positively affects HDL antioxidative activity [24,29–31]. These findings suggest that even in a healthy metabolic context, increasing OS is associated with compositional HDL alterations that may compromise its protective functions. The stronger associations observed for MDA-TBARS compared with MDA-HPLC further underscore the relevance of free MDA in modulating the structure and function of HDL, or the generation of free MDA upon oxidation of HDL-associated lipids. Notably, these associations remained robust after adjustment for age, sex, BMI, and inflammatory markers, which are known modulators of the composition and function of HDL [32–35].

In HVs, MDA-TBARS showed strong positive associations with multiple VLDL subclasses, particularly with cholesterol, triglyceride, phospholipid, and apoB concentrations. These findings suggest that triglyceride-rich lipoproteins are major contributors to, or targets of, lipid peroxidation under physiological conditions. The lack of associations with per-particle lipid content implies that MDA levels are more closely related to particle abundance than to compositional remodeling at the particle level. Similarly, positive associations between MDA-TBARS and IDL triglyceride levels and content further support a link between OS and the metabolism of apoB-containing lipoproteins. These associations were weaker or absent for MDA-HPLC, again suggesting that free MDA more sensitively reflects dynamic LPO processes occurring in circulating lipoproteins. Collectively, these findings align with the established pro-atherogenic and pro-inflammatory properties of triglyceride-rich lipoproteins and their remnants and their causal roles in cardiometabolic disease [2,36,37].

In HVs, higher MDA-TBARS levels were also associated with lower cholesterol and phospholipid content and higher triglyceride content across several LDL subclasses. These lipid components, together with antioxidants such as ubiquinol-10 and α-tocopherol and enrichment with polyunsaturated fatty acids, are known determinants of the oxidative susceptibility or resistance of LDL [38–40]. The absence of associations between MDA levels and LDL particle concentrations either suggests that oxidative stress primarily affects LDL composition rather than particle number or, vice versa, that LDL composition rather than abundance affects LPO. Notably, these relationships were absent in MS patients, indicating that LDL particles in MS may already be extensively remodeled or oxidatively modified, thereby obscuring associations between circulating MDA and LDL subclass characteristics.

A central finding of this study is the loss of associations between MDA measures and lipoprotein subclass parameters in patients with MS, despite elevated free MDA levels. This dissociation may partly reflect differences in the degree and duration of OS between patients with MS and HV. In HV, lower and potentially shorter-term OS may result in a broader range of OS levels and greater interindividual variability, allowing associations between MDA and lipoprotein parameters to be detected. In contrast, persistent and more pronounced OS in MS may lead to extensive oxidative modification of circulating lipoproteins, potentially reducing interindividual variability and weakening these associations. Other mechanisms may also contribute to this dissociation. Accelerated clearance or sequestration of oxidized lipoproteins by macrophages may disrupt the relationships between circulating MDA and lipoprotein subclasses, while altered antioxidant defenses in MS may further uncouple these associations [6,41].

Multivariate OPLS-DA analyses reinforced these univariate findings, demonstrating that lipoprotein profiles discriminated between low and high MDA-TBARS levels in HV but not in MS patients. HDL- and VLDL-related parameters dominated the results, particularly those reflecting triglyceride enrichment and cholesterol depletion of HDL particles. The overlap in VIP-ranked variables between MDA-TBARS and MDA-HPLC suggests a shared underlying biology, although free MDA appears to be the more informative marker of lipoprotein-associated lipid peroxidation. The lack of discrimination between high and low MDA values in MS highlights the disruption of the relationship between MDA and lipoprotein metabolism in this condition, limiting the utility of circulating MDA as a marker of lipoprotein-specific oxidative damage in this pathophysiological state.

Recently, we demonstrated associations between advanced oxidation protein products, markers of protein oxidation, and serum lipoproteins in both HV and MS patients [42]. However, the strength of these associations and the relative contributions of individual lipoprotein subclasses differed markedly between groups. Together with the absence of an association between MDA and lipoprotein parameters observed in MS in the present study,

these findings suggest that different mechanisms underlie protein versus lipid oxidation and that the impact of the MS pathophysiology on these oxidative processes differs.

Strengths of this study include the use of complementary MDA assays, high-resolution NMR-based lipoprotein profiling, and comprehensive multivariate analyses. However, the cross-sectional design precludes causal inference. Because MDA was not quantified in isolated lipoprotein fractions, it remains unclear whether the observed relationships reflect lipoproteins as sources of MDA or their influence on MDA generation through peroxidation of non-lipoprotein-associated lipids. Furthermore, TBARS-based measurements may be influenced by other reactive aldehydes [1]. Nevertheless, the strong correlation between MDA-TBARS and MDA-HPLC and their similar associations with serum glucose and albumin (Table S7) argue that MDA-TBARS primarily reflects free circulating MDA. In addition, the lack of detailed information on chronic antioxidant use and dietary habits, as well as on the doses, duration, and combinations of chronic medication, prevented us from assessing the potential effects of these factors on MDA levels. Finally, the relatively small sample size precluded reliable subgroup analyses according to menstrual cycle status or MS severity. These factors may have influenced the results and warrant further investigation in larger studies.

5. Conclusions

In conclusion, free circulating MDA is closely linked to lipoprotein subclass levels and composition in healthy individuals, whereas these relationships are disrupted in MS. These findings indicate that interactions between LPO and lipoproteins are highly context-dependent and prone to dysregulation under metabolic stress. Improved understanding of the mechanisms underlying these interactions in health and disease may help guide the rational use of oxidative stress biomarkers and inform lifestyle-based or pharmacological strategies aimed at reducing OS.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/antiox15111443/s1, Table S1: Chronic medication in patients with MS; Table S2: Differences in serum levels of lipoproteins between HVs and patients with MS; Table S3: Correlations between serum MDA-TBARS and MDA-HPLC levels and LDL subclasses, analyzed separately in HVs and patients with MS; Table S4: Partial correlation analyses between the serum MDA-TBARS and MDA-HPLC levels and serum levels and lipid content of lipoproteins in HVs; Table S5: Differences in serum levels of lipoprotein subclasses between HVs and patients with MS with low and high MDA-TBARS; Table S6: Differences in serum levels of lipoprotein subclasses between HVs and patients with MS with low and high MDA-HPLC levels; Table S7: Correlation analyses between serum levels of MDA-TBARS and MDA-HPLC and clinical and laboratory parameters.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the ethics committees of the University Hospital Centre Sisters of Charity, Zagreb, Croatia (EP 13125/17-4; date of approval 7 September 2017); the University of Zagreb, School of Medicine, Croatia; and the Medical University of Graz, Austria (31-532 ex 18/19; date of first approval 17 September 2020).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The raw data supporting the conclusions of this article will be made available by the authors upon request.

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Abbreviations

The following abbreviations are used in this manuscript:

MDA	Malondialdehyde
TBARS	Thiobarbituric acid reactive substances
HPLC	High-performance liquid chromatography
OPLS-DA	Orthogonal partial least squares discriminant analysis
OS	Oxidative stress
LPO	Lipid peroxidation

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