Qualitative characteristics of HDL in young patients of an acute myocardial infarction

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A B S T R A C T

Aim: Recently, the concept that high density lipoprotein (HDL) quality is an important parameter for atheroprotection is gaining ground, though little data exists so far to support it. In an attempt to identify measurable qualitative parameters of HDL associated with increased risk for premature myocardial infarction (MI), we studied the structural characteristics of HDL from patients who survived an MI at a young age (≤35 years).

Methods and results: We studied 20 MI patients and 20 healthy control subjects. HDL of patients had reduced apolipoprotein A-I (apoA-I), apolipoprotein M, and paraoxonase 1 levels and significantly elevated apolipoprotein C-III (apoC-III) levels (all p < 0.05). Specifically, the HDL apoA-I/apoC-III ratio was 0.24 ± 0.01 in patients versus 4.88 ± 0.90 in controls (p < 0.001). These structural alterations correlated with increased oxidation potential of HDL of the MI group compared to controls (2.5-fold, p = 0.026). Electron microscopy showed no significant difference in average HDL particle diameter between the two groups though a significant difference existed in HDL diameter distribution, suggesting the presence of different HDL subpopulations in MI and control subjects. Indeed, non-denaturing two-dimensional electrophoresis revealed that MI patients had reduced pre-β1, pre-β1*, and α2, and elevated α1, α1*, and pre-α4 HDL.

Conclusions: Reduction in the HDL apoA-I/apoC-III ratio, changes in the HDL subpopulation distribution and an increase in HDL oxidation potential correlated with the development of MI in young patients. The possibility that such changes may serve as markers for the early identification of young individuals at high risk for an acute coronary event should be further explored.

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1. Introduction

Coronary heart disease (CHD) is triggered by atherosclerosis, a focal disease of the arterial wall that affects large- and medium-sized arteries [1]. Epidemiological studies during the past decades indicated that plasma concentration of high-density lipoprotein (HDL) cholesterol independently predicts one's risk for developing atherosclerosis and CHD [2–7]. Specifically, plasma HDL cholesterol levels correlated inversely with slower progression of atherosclerotic lesions and possible stabilization of unstable atherosclerotic plaques [4,8].

More recently however, the concept that HDL quality is also an important parameter in atheroprotection has emerged, though little data exists so far to support it [9–13]. This new concept adds to the existing view on the relationship between HDL and cardiovascular risk and triggers a discussion as to whether HDL cholesterol levels alone can accurately define one's risk for developing CHD. Unfortunately, the concept of HDL quality offers currently no clear advantage to the patient in the clinic since HDL quality remains poorly defined and under investigation, making its assessment by routine laboratory tests a complicated task.

In the clinic, the vast majority of CHD cases are diagnosed by non-invasive imaging methods when atherosclerosis has already significantly progressed. In these cases atherosclerotic plaques can cause significant occlusion of the arteries (>50%) but are usually stable enough to lead to plaque rupture and embolism [14]. Interestingly, approximately 60% of all CHD-related deaths are reported as “sudden deaths” occurring in asymptomatic patients who have
no detectable atherosclerotic lesions by conventional non-invasive imaging methodologies [15]. This has been attributed to the fact that small plaques that usually go undetected with existing imaging techniques, have a weak fibrous cap that can rupture, allowing the release of plaque core in the circulation and subsequent embolism and heart attack. The identification of prognostic markers that may identify individuals with increased risk towards developing CHD may reduce the number of sudden CHD-related deaths.

In the present study we attempted to identify measurable qualitative structural parameters of HDL associated with premature myocardial infarction (MI). We focused our studies on a group of young patients from Greece who survived their first MI at a young age (≤ 35 years). Biochemical analyses along with advanced analytical methodologies suggested that in addition to reduced HDL quantity, HDL structure and composition are also affected in the patient cohort. Our findings may provide new leads towards the development of clinical laboratory tests for assessing HDL-related risk for an MI.

2. Materials and methods

2.1. Patient Information

We recruited twenty consecutive patients who had survived their first acute MI occurring at ≤ 35 years of age. They had been admitted to the Coronary Care Unit of the University General Hospital “Attikon” between January 2006 and December 2010. The diagnosis of acute MI was based on the presence of ≥ 2 of the following 3 criteria: (1) characteristic chest pain lasting >30 min, (2) ST elevation >0.1 mV on at least 2 adjacent electrocardiographic leads and (3) increase of creatine kinase to peak levels of at least 2-fold the upper limit of normal values.

All patients had undergone coronary angiography and left ventriculography by the Judkins technique prior to discharge from the hospital. Significant coronary artery stenosis was defined as ≥50% reduction in lumen diameter of any of the three coronary arteries or their primary branches.

Twenty healthy age and sex-matched subjects who had undergone a minor orthopedic intervention in our institution served as the control group. All subjects were free of a personal or family history of cardiovascular, or thromboembolic disease. Both patients and controls were living in the same areas during the time period of the study. Cardiovascular risk factors were recorded in all individuals. The following definitions were used: hypertension, blood pressure ≥140/90 mmHg and/or antihypertensive treatment; hypercholesterolemia, total cholesterol >200 mg/dl (5.7 mmol/l) and/or lipid lowering agents; diabetes mellitus, fasting plasma glucose >125 mg/dl (6.94 mmol/l) and/or glucose lowering treatment. Smokers were defined participants who reported smoking currently and regularly. The study was approved by the ethics committee of Attikon Hospital and all subjects gave their informed consent.

2.2. Blood sampling and plasma lipid determination

Peripheral blood samples were collected from patients and control subjects after overnight fast for assessing plasma levels of lipids. Plasma triglycerides, total cholesterol, free cholesterol, and phospholipids were determined spectrophotometrically, as described previously [16].

2.3. Fractionation of plasma lipoproteins by density gradient ultracentrifugation

One millilitre of pools of plasma from the control or patient cohort were fractionated by KBr density gradient ultracentrifugation (UCF), as described previously [16].

2.4. Western blot analysis of apolipoprotein E and apolipoprotein C-III

Western blot analysis for apolipoprotein E (apoE), and apolipoprotein C-III (apoC-III) was performed as described previously [16], using a goat anti-human apoE antibody (Biodesign International, cat # K45252G) or a goat anti-human apoCIII antibody (Biodesign International, cat # K74140G) as primary. A rabbit anti-goat antibody (Santa-Cruz, cat # sc-2768) was used as secondary. Semi-quantitative determination of relative apoE and apolipoprotein A-I (apoA-I) levels was performed by scanning densitometry analysis of the films.

2.5. Quantification of human apolipoprotein A-I and apolipoprotein C-III by ELISA

Serum apoA-I and apoCIII concentrations were measured by sandwich ELISA as described previously [16]. An anti-human apoA-I antibody (Biodesign International, cat # K45252G) and an anti-human apoCIII antibody (Biodesign International, cat # K74140G) were used for coating microtiter plates. As secondary antibodies we used a polyclonal goat anti-human apoA-I antibody coupled to horse radish peroxidase (Biodesign International, cat # K45252P) and a polyclonal goat anti-human apoCIII antibody coupled to horse radish peroxidase (Biodesign International, cat # K74140P), respectively.

2.6. Two-dimensional denaturing polyacrylamide gel electrophoresis (2D-PAGE) and protein identification by MALDI–MS of HDL fractions

Given the low levels of HDL in the samples of the diseased subjects and the limited availability of the biological material (due to their rarity samples were collected over a period of several years) we performed MALDI–MS analysis on pools of plasma samples. Despite that CHD is a heterogeneous disease, in the present study each pool of samples was representative of the individual sample in each group since each diseased and control subject had a similar apoA-I, apoC-III, and apoC-I lipoprotein distribution with the corresponding pool of each group. For pooling the samples together, HDL samples containing the same cholesterol content were pooled together and then the protein content of the pool was determined. Samples of the same protein content (1 mg for each pool) were then used for the denaturing 2D analysis and the subsequent MALDI–MS analysis. HDL fractions were diluted 1:3 in a buffer containing 25 mM Tris, 4% CHAPS (w/v), 8 M urea and 2 M thiourea and stored in aliquots at −80 °C until usage. Prior to gel electrophoretic separation proteins were labeled with cyanine dyes (Cy2, Cy3, Cy5) according to the manufacturer’s instructions (GE-Healthcare, Freiburg, Germany) as described previously [17]. For 2D-difference in gel electrophoresis (DIGE) labeled proteins were separated in the first dimension by isoelectric focusing (IEF) using pH 4–7 linear IPG strips performed on a MultiPhor II electrophoresis unit (GE-Healthcare, Freiburg, Germany) and in the second dimension by large format SDS-PAGE (12%). Multifluorescence images of the protein pattern were acquired by laser scanning of the 2D-gels using the three appropriate laser sources on a Typhoon 9400 (GE-Healthcare, Freiburg, Germany) laser scanner and a resolution of 0.1 μm. Detection of protein spots and calculation of relative spot abundances was carried out automatically using ProteinWasher 4.0 image analysis software (BioRad, Munich, Germany). For protein identification gels were re-stained with a ruthenium fluorescent stain. Protein spots exceeding differences in spot intensities of > factor 1.5 were excised from the 2D-gels using a GelPix spot picker (Genetix, Dornach, Germany). After protein digestion with trypsin, extracted peptides were directly applied
to a MALDI Pre-spotted AnchorChip target (Bruker Daltoniks, Bremen, Germany) according to the manufacturer’s instructions. Subsequently, samples were analyzed in a time-of-flight Ultraflex-ToF mass spectrometer (Bruker Daltoniks, Bremen, Germany). Acquired mass spectra were automatically calibrated and annotated using Compass 1.3 software (Bruker Daltoniks, Bremen, Germany). Protein identification via peptide mass fingerprinting (PMF) was performed on the fly engaging Biotools 3.2 (Bruker Daltoniks, Bremen, Germany), searching a human sub-set of Swiss-Prot annotated using Compass 1.3 software (Bruker Daltoniks, Bremen, Germany). Subsequently, samples were analyzed in a time-of-flight UltraflexToF mass spectrometer (Bruker Daltoniks, Bremen, Germany), searching a human sub-set of Swiss-Prot annotated using Compass 1.3 software (Bruker Daltoniks, Bremen, Germany). Protein identification via peptide mass fingerprinting (PMF) was performed on the fly engaging Biotools 3.2 (Bruker Daltoniks, Bremen, Germany), searching a human sub-set of Swiss-Prot annotated using Compass 1.3 software (Bruker Daltoniks, Bremen, Germany).

For verifying the results each protein spot was picked and identified from at least two physically different 2D-gels.

2.7. Oxidation assay

For the analysis of the oxidation potential of HDL we used a modification of the method of Bandoniene et al. [18]. A stock solution of 150 μM of 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Sigma, St. Louis, MO, cat # D-913-2) in 80% methanol was prepared. For the analysis, pools of HDL fractions with identical cholesterol content were concentrated to 2 μl final volume. One microlitre of each HDL sample was dissolved in 9 μl 80% methanol and 190 μl of DPPH solution were added to the sample. As a reference sample we mixed 10 μl 80% methanol with 190 μl of DPPH solution. Following incubation for 30 min in the dark, the optical density at 550 nm for the reference and the test samples was determined. The oxidation potential for each sample was calculated as [(absorbance of reference sample – absorbance of test sample)/absorbance of reference sample] × 100% [19]. Reported values were corrected for the HDL cholesterol content of each test sample. Control was assigned the value 1 and MI values were expressed relatively to the control.

2.8. Electron microscopy analysis of HDL

Pools of HDL fractions isolated from density gradient ultracentrifugation (described above) were dialyzed against ammonium acetate and carbonate buffer, and then stained with sodium phosphotungstate. Particles were visualized in a Phillips CM-120 electron microscope (Phillips Electron Optics, Eindhoven, Netherlands), and photographed as described previously [16]. The photomicrographs were taken at an original 75,000 × magnification and then enlarged three times.

2.9. Statistical analysis of the electron microscopy results

Measurements of area and maximum diameter were performed using Image Pro Plus software. A script was developed for this purpose, which automatically detects the HDL spots against the background and performs the corresponding measurements.

To determine the target spots’ characteristics, we performed a number of tests for preliminary estimations of a typical spot’s area and intensity. In order to facilitate the detection of spots, a low-pass filter was applied, followed by setting an upper limit value for the size of objects under detection. The script was then set to select objects that were within specific limits both with regard to area (in pixels) and image intensity. While it would be possible to estimate the mean spot area and diameter performing measurements manually, the use of a script allowed for a much richer data collection and thus more representative statistics. Diameter measurements were transformed from pixels to nm dividing the measurements by the value of the scaling factor (pixels per nm), derived from the scale imprinted on the image. Due to the generally symmetric (circular) nature of the spots, area measurements were obtained dividing the measured values (pixels) by the square of the above mentioned scaling factor.

CHD, coronary heart disease; LDL, low density lipoprotein; HDL, high density lipoprotein.

In the remainder of the manuscript, the terms diameter and area refer to variables measured in nm and nm², respectively. Statistical analysis of diameter and area was performed with SPSS v.17 (SPSS Inc., 2009, Chicago, IL).

2.10. Nondenaturing two-dimensional electrophoresis analysis of HDL fractions

The analysis of HDL subpopulations in the plasma of patients and control subjects by non-denaturing two-dimensional electrophoresis was performed by a modification of the method described previously [20]. Specifically, in the first dimension, 4 μl of HDL isolated by density gradient UCF were separated on a 0.75% agarose gel at 4 °C with a non-barbital running buffer [21], until the bromophenol blue marker had migrated 5.5 cm into the gel. Agarose gel strips (~3 mm in width) containing the separated lipoproteins were then transferred to a 4 – 20% non-denaturing polyacrylamide gradient gel (Biorad, CA) and the second dimension electrophoresis was performed at 90 V, for 2–3 h, at 4 °C. The separated proteins were transferred to a PVDF membrane and apoA-I was detected by using a goat polyclonal anti-human apoA-I antibody (Biodesign International, cat # K45252G). The assignment of mobility for the various HDL subpopulations is based on the work of Asztalos and Schaefer [20].

2.11. Statistical analysis

Comparison of data from the two groups was performed using the Student t-test. Where more than a two-group comparison was required the results were analyzed using ANOVA. Data are reported as mean ± standard error of the mean. A p value <0.05 was considered significant.

3. Results

3.1. Clinical characteristics and lipid levels

The mean time of blood collection and patients’ interview after the occurrence of MI was 6.1 ± 3.2 months. Table 1 shows the clinical characteristics and lipid levels of patients and controls. The vast majority of patients were males, and almost half of them continued smoking after the MI. In terms of plasma lipids, there was a trend for higher triglycerides levels while HDL cholesterol levels were significantly lower in patients with premature MI compared to controls. Regarding drug treatment, all patients were taking antiplatelets, 17 (85%) were on lipid lowering medication (all on statins), 17 (85%) were taking beta-blockers and 13 (65%) were on angiotensin-converting enzyme inhibitors. Coronary angiogram showed multivessel CHD in 5 (25%), one vessel disease in 11 (55%) and “normal” coronary arteries in 4 (20%) patients.

Table 1 Clinical characteristics and lipid levels in young survivors of myocardial infarction and control subjects.

<table>
<thead>
<tr>
<th></th>
<th>Patients (n=20)</th>
<th>Controls (n=20)</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>32.4 ± 3.3</td>
<td>31.3 ± 2.5</td>
<td>0.232</td>
</tr>
<tr>
<td>Males (%)</td>
<td>85</td>
<td>90</td>
<td>0.633</td>
</tr>
<tr>
<td>Current smokers (%)</td>
<td>45</td>
<td>50</td>
<td>0.752</td>
</tr>
<tr>
<td>Hypertension (%)</td>
<td>15</td>
<td>5</td>
<td>0.292</td>
</tr>
<tr>
<td>Family history of CHD (%)</td>
<td>45</td>
<td>0</td>
<td>0.001</td>
</tr>
<tr>
<td>Cholesterol (mg/dl)</td>
<td>171.1 ± 59.8</td>
<td>190.2 ± 31.3</td>
<td>0.223</td>
</tr>
<tr>
<td>Triglycerides (mg/dl)</td>
<td>142.9 ± 67.6</td>
<td>103.6 ± 50.1</td>
<td>0.047</td>
</tr>
<tr>
<td>HDL cholesterol (mg/dl)</td>
<td>125.1 ± 53.3</td>
<td>128.8 ± 26.2</td>
<td>0.758</td>
</tr>
<tr>
<td>HDL cholesterol mass index (kg/m²)</td>
<td>36.3 ± 8.6</td>
<td>50.8 ± 11.7</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LDL cholesterol mass index (kg/m²)</td>
<td>28.2 ± 4.3</td>
<td>24.2 ± 3.1</td>
<td>0.002</td>
</tr>
</tbody>
</table>

LDL, low density lipoprotein; HDL, high density lipoprotein.
HDL from MI patients has a higher oxidation potential than HDL. The ratio of HDL [apoA-I]/[apoC-III] was significantly lower in the coronary patients (apoA-I level of 3.3 mg/dl and apoC-III level of 13.85 mg/dl). Thus, the oxidant potential of HDL from control subjects had HDL apoA-I level of 25.9 mg/dl and apoC-III was distributed mainly in lighter HDL fractions compared to control subjects (apoA-I level of 25.9 mg/dl and apoC-III level of 13.85 mg/dl). Therefore, in the next set of experiments isolated from control subjects. To test this hypothesis we performed a classical (DPPH) assay as described in Section 2. As shown in Fig. 3, we found that HDL isolated from coronary patients had a much higher oxidation potential compared to the HDL isolated from control subjects. Specifically the oxidation potential of the HDL from MI patients was approximately 2.5 times higher than that of the control subjects (p = 0.026).

3.4. Electron microscopy analysis of HDL particles

In humans, HDL may be found in either spherical or discoidal conformations and the esterification of HDL cholesterol by plasma enzyme Lecithin:Cholesterol Acyl Transferase (LCAT) is essential for the conversion of discoidal into spherical HDL [23–27]. To determine potential differences in the geometry and size distribution of HDL particles from our control subjects and MI patients we performed a qualitative negative staining electron microscopy (EM) analysis of HDL particles isolated from pools of plasma from both groups following KBr density gradient UCF fractionation [16]. As shown in Fig. 4A, this analysis revealed that HDL particles from the control and the patient groups exhibited a similar spherical geometry while no discoidal HDL particles could be detected in either HDL preparation.

Following the application of upper limits for the size of HDL particles (<15 nm), 7169 HDL particles from the control group and 3293 HDL particles from the MI group entered the analysis. By visual inspection, the distributions of HDL particle diameter and area for controls and MI patients were evidently skewed. The deviation from normality was confirmed using the Kolmogorov–Smirnov and the Shapiro–Wilk tests, both showing (p < 0.001) that our measurements should be treated with non-parametric tests. A logarithmic transformation of the values did not alter this result. The Brown–Forsythe test for homogeneity of variances (based on median) did not reveal significant inhomogeneities between controls and coronary patients in diameter (p = 0.068) or area (p = 0.106). Fig. 4B presents the descriptive statistics of the distributions. No significant differences, either in diameter or area, between controls and patients with MI were identified by either the Mann–Whitney or the independent samples median test.

We further investigated the possible differences between controls and MI patients after grouping particle diameter into 4 groups: smaller than 8 nm, between 8 and 10 nm, between 10 and 12 nm and larger than 12 nm (Fig. 4C).

It was found that the distribution of diameter values is significantly different between control subjects and MI patients (p = 0.0026, chi-squared test). Particles between 8 and 10 nm in diameter are significantly more frequent among patients with MI (p = 0.0002, z-test for proportions), while particles between 10 and 12 nm in diameter are significantly less frequent among coronary patients (p = 0.0151, z-test for proportions). No significant differences were observed in small (<8 nm) or large (>12 nm) HDL particles.

Taken together, our data showed that plasma from patients with MI contains only spherical HDL though it suggested a significant difference in the HDL subpopulations present in the MI group compared to the control group. No significant difference in average HDL diameter was observed between groups indicating that average HDL diameter cannot serve as a prognostic marker for life-threatening MI.

3.5. Nondenaturing two-dimensional electrophoresis analysis of HDL subpopulations

Our EM analysis raised the possibility that significant difference in the HDL subpopulation distribution exists between control subjects and patients with MI. Therefore, in the next set of experiments...
we sought to determine the HDL subpopulations that were present in the control subjects and patients with MI. Pools of plasma from both groups were analyzed by a classical two dimensional electrophoresis [20], as described in Section 2. This analysis revealed significant differences in the distribution of HDL among different subpopulations between the two groups (Fig. 5), confirming the results of the EM analysis. The assignment of mobility to the various HDL subpopulations was based on the work of Asztalos and Schaefer [20]. Control subjects had approximately equal amounts of pre-β1a and pre-β1b particles, while the majority of HDL was α-HDL that was almost equally distributed between α2 and pre-α3 subpopulations (Fig. 5A). In MI patients, there were very low levels of pre-β1a and pre-β1b present (mainly pre-β1b) and most of the HDL was α-HDL mainly distributed in pre-α3, pre-α4, α2 and α3 HDL (Fig. 5B). In comparison to control subjects, coronary patients had significantly lower levels of pre-β1a and pre-β1b, reduced α3 levels, while pre-α3 levels were comparable between the two groups. In addition, HDL from MI patients contained α1, α3, and some pre-α4 particles that were absent from the HDL of the control group.

Fig. 2. ApoA-I, apoC-III, and apoE distribution in the lipoprotein fractions of control subjects (A, C, E), and patients with premature myocardial infarction (MI) (B, D, F). Pools of plasma from each group were analyzed by density gradient ultracentrifugation (UCF) and various density fractions were collected and analyzed by SDS-PAGE (A, B) and Western blot analysis (C–F) for apoA-I, apoC-III and apoE, as indicated. Panels G–I represent a semi-quantitative determination by scanning densitometry of apoA-I (G), apoC-III (H) and apoE (I) levels in the HDL density fractions (UCF fractions # 5–8). Panel J indicates the statistical significance of the differences in apoA-I, apoCIII, and apoE levels between control and MI groups, shown in the bar graphs of panels G–I. * indicates p < 0.05.
Control MI

0.0
0.5
1.0
1.5
2.0
2.5
3.0
relative oxidation potential (arbitrary units)

* p=0.026

Fig. 3. Relative oxidation potential of high-density lipoprotein isolated from controls and patients with premature myocardial infarction. Oxidation potential was measured using the classical DPPH assay as described in Section 2. Oxidation potential for control subjects was assigned the value 1 and the oxidation potential of MI patients was reported in relation to the controls.

4. Discussion

Recently it has been proposed that in addition to HDL quantity, HDL quality is also a very important parameter in atheroprotection. Though extensive research in the literature has focused on the identification of the atheroprotective properties of HDL [22], the principle of HDL quality remains vaguely defined and offers no true benefit to the patient. Furthermore, there are no standardized routine laboratory tests for the assessment of HDL quality in the clinic.

Acute MI is rare in young individuals. It is estimated that approximately 2% of all MIs occur in patients at an age ≤35 years [28,29]. Young survivors of MI are characterized by a high proportion of heavy smoking and family history of CHD and a low proportion of hypertension and diabetes mellitus [30–32]. It has also been...
reported that reduction of HDL cholesterol is the most common lipid abnormality followed by elevation of triglycerides, LDL cholesterol and lipoprotein (a) [33]. In the present study we sought to identify potential measurable structural characteristics of the HDL of these patients that may relate to its quality and functions. It is conceivable that by recruiting very young survivors of MI, we exclude alterations in HDL structure associated with aging and potentially other chronic accompanying diseases.

Up until recently, it was common belief that lipid poor apoCIII associates randomly in the circulation with existing classical apoE- and apoA-I-containing HDL [34]. However, using animal models recently we established that the accumulation of apoCIII in HDL density fractions in vivo is not simply the result of a random association of plasma apoCIII with pre-existing HDL, rather this process is dependent on the action of the lipid transporter ABCA1 [16] in a fashion similar to the biogenesis of apoA-I- and apoE-containing HDL. Furthermore, those findings indicated that apoCIII-containing HDL particles are distinct from apoA-I containing HDL particles. In the present study, isolation of plasma lipoproteins by UCF followed by SDS-PAGE and Western blot analysis showed that the HDL of MI patients had reduced apoA-I levels and increased apoC-III levels. ApoE levels remained unchanged though apoE distribution among HDL density fractions differed between the two groups. Previous studies in animal models [16] have shown that in addition to other atheroprotective properties, HDL may also act as a buffer that prevents accumulation of excess plasma apolipoproteins (such as apoCIII and possibly other small exchangeable apolipoproteins) on VLDL. Given that total plasma apoCIII levels in the diseased subjects were 19.67 ± 1.48 mg/dl compared to 10.9 ± 0.8 mg/dl in the control group, it is possible that increased levels of apoC-III HDL in the MI patients represents a protective response aiming at keeping excess apoC-III from accumulating on chylomicrons and VLDL, where it could interfere with lipolysis of triglycerides by plasma lipoprotein lipase.

MALDI–TOF mass spectrometry analysis revealed that HDL from MI patients has reduced apoM and PON1 levels.apoM is a member of the lipocalin protein superfamily, secreted primarily by the liver in a lipid free form, and associates in the circulation with pre-existing HDL lipoproteins [35]. ApoM is believed to be atheroprotective while it has been suggested that it contributes to the antioxidant potential of HDL [36]. Similarly, the presence of antioxidant enzymes on HDL, such as PON1, prevents the oxidation of LDL in in vitro studies [37]. PON1 is an enzyme with lactonase and esterase activities, and in humans, it is synthesized mainly by the liver [38]. It hydrolyses lipid peroxides, and circulates in plasma bound to HDL particles. In our study, the reduced apoM and PON1 content of HDL in the patient group correlated with dysfunctional HDL, since a classical DPPH assay showed that HDL from patients had approximately 2.5 times higher oxidation potential than the HDL of the control subjects.

In humans and in experimental mouse models, HDL may assume either a spherical conformation (mature HDL) with varying diameters or a discoidal conformation (immature HDL) [39–41]. The conversion of discoidal HDL into spherical requires the action of the plasma enzyme LCAT that catalyzes the esterification of free cholesterol of HDL and VLDL lipoproteins by transferring a fatty acyl group from the C-2 position of lecithin to the 3-hydroxy group of cholesterol [26]. Our qualitative negative staining EM analysis of HDL particles revealed that both MI patients and control subjects contained only spherical HDL particles, while statistical analysis did not reveal significant differences in mean area and mean diameter between the HDL of the two groups. However, there was a significant difference in the HDL diameter distribution between the two groups, raising the possibility that there is a difference in HDL subpopulation distribution between the two groups. To define the HDL subpopulations present in the control and MI groups, non-denaturing two-dimensional electrophoresis was performed. It was found that in comparison to controls, coronary patients had significantly lower levels of pre-β1, pre-β1, and α2 HDL. In addition, HDL from MI subjects contained α1, α3 and some pre-α4 particles that were absent from the HDL of the control group.

Interestingly, previous studies suggested that increased α3 HDL and reduced PON1 levels are associated with non-atheroprotective HDL [22].

Though statin therapy is very effective in reducing LDL cholesterol, a significant risk for life-threatening MI events persists among statin-treated patients, a phenomenon also known as “residual risk” [42]. As described in Section 2, plasma samples from our patients were collected approximately 6 months following their acute MI, during which most of them were treated with statins. The impact of statin treatment on cholesterol levels is reflected by the fact that their total cholesterol levels were comparable to those of the control group. It is conceivable that the reported cholesterol levels of patients were approximately 35–50% lower than their pretreatment levels. On the contrary, the mild raising effect of statins on HDL cholesterol levels (5–10%) could only partially blunt the difference in HDL cholesterol levels between patients and controls. In our study, HDL particles from patients possessed a significantly higher oxidation potential and contained reduced apoM and PON1 levels compared to the control subjects. Furthermore, significant structural differences existed between the HDL of the two groups (subpopulation distribution, etc.). It is possible that some of the observed changes in the HDL structure and function might have been underestimated in our study due to the statin treatment that most of our patients received [43]. In particular, it has been reported that statins have antioxidant properties, mainly by increasing PON1 activity [43,44]. It would be very interesting to evaluate in future studies the potential contribution of these qualitative differences in HDL to the “residual risk” associated with statin therapy.

In summary, here we report marked differences in HDL structure and functionality between the MI patients and the control group, further supporting the recent idea that changes in HDL quality may be an important parameter in atheroprotection. Despite that the results of the present study are mainly qualitative, our data suggest that measurable alterations in the ratio of plasma apoA-I concentration over plasma apoC-III concentration ([apoA-I]/[apoC-III] ratio), in the HDL subpopulation distribution and in the oxidation potential of HDL between the two groups were identified in our analysis. These changes could be a cause or an effect of pathological conditions leading to an early acute MI. The possibility that such changes may serve as markers for the early identification of young individuals at high risk for a life-threatening acute MI should be further explored in a prospective epidemiological study. Interestingly, in our study the average HDL diameter did not differ between the two groups analyzed, suggesting that this parameter has little, if any, value in the prognosis of MI.

**Conflict of interest**

The authors have no financial conflicting interests to disclose.

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