



Atherogenic lipoprotein phenotype and LDL size and subclasses in drug-naïve patients with early rheumatoid arthritis

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ABSTRACT

Objective: Subjects with rheumatoid arthritis (RA) have increased cardiovascular risk and may show atherogenic forms of dyslipidemia. The present study investigated whether patients with early RA, beyond alterations in plasma lipids, also show lower LDL size and altered LDL subclass distribution.

Design and Methods: We identified 25 subjects with RA (47 ± 8 years, body mass index (BMI) 25 ± 4 kg/m²) by the American College of Rheumatology diagnostic criteria, with a disease durations <1 year and no prior treatment against it. In patients and 22 healthy subjects matched for age and BMI (controls) we measured plasma lipids and LDL size and subclasses by gradient gel electrophoresis.

Results: As compared to controls RA patients had higher plasma triglycerides (1.8 ± 0.5 vs. 1.0 ± 0.5 mmol/L, $p < 0.0001$) and lower HDL-cholesterol concentrations (1.2 ± 0.2 vs. 1.4 ± 0.2 mmol/L, $p = 0.0027$), while total- and LDL-cholesterol concentrations were similar. LDL particle size was lower in RA patients than controls (264 ± 7 vs. 281 ± 9 Å, $p < 0.0001$), due to less LDL-I (31 ± 6 vs. 38 ± 7%, $p = 0.0004$) and LDL-IIA (14 ± 3 vs. 16 ± 3%, $p = 0.0182$), and more LDL-IIIB (7 ± 1 vs. 5 ± 1%), -IVA (11 ± 2 vs. 8 ± 2%) and -IVB particles (12 ± 2 vs. 9 ± 2%,) ($p < 0.0001$ for all). Further, about 1/3 of patients showed the complete “atherogenic-lipoprotein-phenotype” (e.g. the concomitant presence of high triglycerides, low HDL-cholesterol and elevated small, dense LDL).

Conclusions: Beyond plasma lipids, increased levels of small, dense LDL seems to be common in drug-naïve patients with early RA. Yet, whether these findings affect the atherogenic process and the clinical endpoints in these subjects remains to be determined by future prospective studies.

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1. Introduction

Patients with rheumatoid arthritis (RA) have increased cardiovascular morbidity and mortality as compared to the general population [1–3]. Among the traditional cardiovascular risk factors, the lipid profile in RA has often been described as “pro-atherogenic” based on decreased HDL-cholesterol and increased LDL:HDLcholesterol ratio [4,5]. In recent years it has become evident that the lipid triad or “atherogenic lipoprotein phenotype” (ALP), characterized by decreased HDL-cholesterol, moderately raised triglycerides and increased levels of small, dense LDL, is

linked to increased cardiovascular risk, beyond LDL-cholesterol levels [6–8]. In fact, a number of evidences suggest that the quality, and not only the quantity, of LDL exerts a direct influence on cardiovascular risk [9] and the predominance of small dense LDL has been accepted as an emerging cardiovascular risk factor by the National Cholesterol Education Program Adult Treatment Panel III [10].

It has been reported in a previous study that patients with long-term RA may show lower LDL size due to increased levels of small, dense LDL particles [11]. Yet, this has not been further investigated in patients with early RA, without prior treatment for it, which limits the utility of the information we have on atherogenic dyslipidemia in RA. In fact, although drug treatment in RA is in general beneficial on plasma lipids, it seems that long-term anti-tumor necrosis factor- α therapies may induce some pro-atherogenic changes in lipid profile [12–14]. Therefore, we included in the present study a group of patients with early RA, without treatment, as well as a control group of healthy subjects, matched for age and body mass index (BMI) used as controls, in order to

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investigate (1) whether patients with early RA have lower LDL size; (2) whether their LDL subclass distribution is altered (i.e. increased levels of small, dense LDL); (3) the prevalence of ALP.

2. Methods

2.1. Subjects and study protocol

We included in the present study a group of drug-naïve patients with early RA who consecutively underwent a clinical examination at the Department of Rheumatology, Ankara Numune Education and Research Hospital, Ankara, Turkey between January and June 2008. Inclusion criteria were the fulfillment of the American College of Rheumatology diagnostic criteria for RA [15] and a disease durations <1 year. Disease activity was assessed by measuring the 28 joint indices score (DAS-28) [16]. According to these inclusion criteria we have found 144 patients potentially eligible for our study. We then excluded subjects with prior use of any treatment for RA, the presence of abnormal liver, renal or thyroid function, type-2 diabetes, cardiovascular diseases, active neoplasia, chronic systemic or inflammatory diseases, or therapy with drugs known to affect lipid metabolism. According to these exclusion criteria we were able to include in our study 25 drug-naïve patients with early RA. As controls we included 22 healthy subjects with the same exclusion criteria and matched for age and BMI, in order to have a more consistent group with that of patients. Controls were co-workers or family co-workers of the Ankara Numune Education and Research Hospital, Turkey. The study was approved by the Ethics Committee of Ankara Numune Education and Research Hospital, Turkey and each subject gave written informed consent to participate.

2.2. Biochemistry

A blood sample was collected from each subject after 12–14 h overnight fast in sodium-EDTA tubes and stored at -80°C until analysis. Total cholesterol, triglycerides and HDL-cholesterol were quantified by standard enzymatic-colorimetric methods [17–19]. LDL-cholesterol was calculated using the Friedewald formula. Lipoprotein (Lp) (a) and homocysteine were measured using commercial assays while the rheumatoid factor by ELISA (IgM isotype, positive ≥ 20). C-reactive protein (CRP) was determined by a high-sensitivity nephelometric method [20].

In order to assess the prevalence of each individual component of ALP in both groups of subjects, according the most recent inter-

national guidelines [10,21], we considered low HDL-cholesterol levels those <1.03 mmol/L (i.e. <40 mg/dL) in men and <1.29 (i.e. <50 mg/dL) in women and elevated triglyceride concentrations those >1.69 mmol/L (i.e. >150 mg/dL) [21]. Levels of small, dense LDL (e.g. LDL-IIIa + LDL-IIIb, + LDL-IVa + LDL-IVb, % of LDL stain) were considered to be increased in patients with values greater than mean +2 SD of the values of controls, as already described [22,23]. Further, elevated LDL-cholesterol levels were considered those >4.1 mmol/L (i.e. >160 mg/dL) while high Lp(a) concentrations were considered those >30 mg/dL [10]; elevated triglyceride/HDL-cholesterol ratio was considered as >3.5 [24].

2.3. Nondenaturing polyacrylamide gradient gel electrophoresis

Nondenaturing polyacrylamide gradient gel electrophoresis (GGE) of plasma was performed at University Hospital Zurich, Switzerland in the laboratory of K.B. at $10\text{--}14^{\circ}\text{C}$ in 2–6% polyacrylamide gradient gels. Gels were manufactured by C.B.S. Scientific Company, Solana Beach, CA, USA and subjected to electrophoresis for 24 h at 125 V in tris borate buffer (pH 8.3) as described elsewhere [25]. Gels were fixed and stained for lipids in a solution containing oil red O in 60% ethanol at 55°C . Gels were placed on a light source and photographed using a Luminescent Image Analyzer, LAS-3000 of Fujifilm, detection using white transmitted light source. Migration distance for each absorbance peak was determined and the molecular diameter corresponding to each peak was calculated from a calibration curve generated from the migration distance of size standards of known diameter, which includes carboxylated latex beads (Duke Scientific, Palo Alto, CA), thyroglobulin and apoferritin (HMW Std, Pharmacia, Piscataway, NJ) having molecular diameter of 380 Å, 170 Å and 122 Å, respectively, and lipoprotein calibrators of previously determined particle size. LDL peak particle size was measured as the particle diameter of the predominant peak and LDL subclass distribution (LDL I, IIA, IIB, IIIA, IIIB, IVA and IVB) as percentage of total LDL was calculated as previously described [25].

2.4. Statistical analysis

Statistical analyses were performed using Statview[®] 5.0 (SAS Institute Inc., Cary, NC, USA). Univariate analyses were performed using non-parametric Mann–Whitney test for numeric variables, while the differences in the prevalence for nominal variables were analyzed by chi-square test. Correlation analyses were performed using the Spearman rank correlation method.

Table 1
Clinical and laboratory characteristics in all subjects.

	Controls (n = 22)	p=	Patients (n = 25)
Age (years)	46 ± 4	ns	47 ± 8
Female gender (n) (%)	18 (82)	ns	19 (76)
Body-mass index (kg/m ²)	25 ± 2	ns	25 ± 4
C-reactive protein (mg/L), median (Q1–Q3)	0.35 (0.01–0.91)	<0.0001	1.80 (1.42–2.79)
Total cholesterol (mmol/L)	4.6 ± 0.7	ns	4.8 ± 1.1
Triglycerides (mmol/L)	1.0 ± 0.5	<.0001	1.8 ± 0.5
HDL-cholesterol (mmol/L)	1.4 ± 0.2	0.0027	1.2 ± 0.2
LDL-cholesterol (mmol/L)	2.7 ± 0.7	ns	2.8 ± 0.9
Triglycerides/HDL-cholesterol ratio	1.8 ± 1.0	<0.0001	3.6 ± 1.2
Lp(a) (mg/dL), median (Q1–Q3)	15 (11–21)	ns	25 (11–102)
Homocysteine (mmol/L)	10 ± 4	ns	10 ± 3
LDL size (angstrom)	281 ± 9	<0.0001	264 ± 7
Total small, dense LDL (LDL-III and -IV) (%)	32 ± 5	<0.0001	41 ± 6

Results are shown as mean ± SD, with the exception of the data regarding female gender (expressed as absolute numbers and percentage) and that of CRP and Lp(a) (expressed as median with interquartile range).

DAS-28: disease activity for 28 joint indices score [16]. Q: quartile.

Italic values show the statistical data.

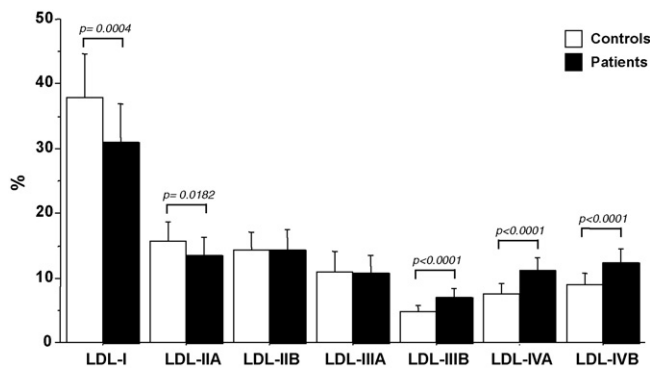


Fig. 1. LDL subclasses in patients with early RA and controls, as assessed by whole plasma nondenaturing polyacrylamide gradient gel electrophoresis. Results are presented as mean + SD.

3. Results

All patients with early RA were rheumatoid factor positive (rheumatoid factor: 279 ± 116 IU/mL) and displayed high disease activity scores (DAS-28: 6.2 ± 1.6) (data not shown). According to the inclusion criteria, patients with early RA and controls were matched for age and BMI (Table 1). Expectedly, patients had higher CRP concentrations than controls (median 1.80 vs. 0.35 mg/L, $p < 0.0001$), triglyceride levels were also higher (1.8 ± 0.5 vs. 1.0 ± 0.5 mmol/L, $p < 0.0001$) and HDL-cholesterol concentrations lower (1.2 ± 0.2 vs. 1.4 ± 0.2 mmol/L, $p = 0.0027$) as was LDL particle size (264 ± 7 vs. 281 ± 9 Å, $p < 0.0001$), due to reduced LDL-I (31 ± 6 vs. $38 \pm 7\%$, $p = 0.0004$) and LDL-IIA (14 ± 3 vs. $16 \pm 3\%$, $p = 0.0182$) and increased LDL-IIIB (7 ± 1 vs. $5 \pm 1\%$), -IVA (11 ± 2 vs. $8 \pm 2\%$) and -IVB particles (12 ± 2 vs. $9 \pm 2\%$) ($p < 0.0001$ for all, see Fig. 1); therefore, levels of small, dense LDL were increased in patients vs. controls (41 ± 6 vs. $32 \pm 6\%$, $p < 0.0001$).

High concentrations of small, dense LDL with normal triglyceride and HDL-cholesterol concentrations were found in two RA patients (8%). In addition, we have found that the triglyceride/HDL-cholesterol ratio was increased in patients (3.6 ± 1.2 vs. 1.8 ± 1.0 , $p < 0.0001$) and a high ratio was found in 15 patients (68%) vs. 3 controls (14%) ($p = 0.0011$). Yet, the concordance between elevated small, dense LDL and high ratio was low (60%) and the correlation between the two parameters was not significant in both patients and controls (data not shown). We also calculated the prevalence of different forms of atherogenic dyslipidemia in patients vs. controls (see Table 2), with significant differences in the prevalence of high triglycerides (64 vs. 5%, $p < 0.0001$), low HDL-cholesterol (68 vs. 23%, $p = 0.0019$), elevated small, dense LDL (40 vs. 0%, $p = 0.0005$) and high Lp(a) (32 vs. 5%, $p = 0.0112$). Eight patients vs. none in the control group ($p = 0.0036$) showed the complete form of ALP, i.e. the

Table 2

Prevalence of different forms of atherogenic dyslipidemia in all subjects.

	Controls (n = 22)	p =	Patients (n = 25)
High LDL-cholesterol (>4.1 mmol/L) (n) (%)	1 (5)	ns	3 (12)
High triglycerides (n) (%)	1 (5)	<0.0001	16 (64)
Low HDL-cholesterol (n) (%)	5 (23)	0.0019	17 (68)
High small, dense LDL particles (n) (%)	0 (0)	0.0005	10 (40)
High triglycerides/HDL-cholesterol ratio (n) (%)	3 (14)	0.0011	15 (68)
Elevated Lp(a) (>30 mg/dL) (n) (%)	1 (5)	0.0112	8 (32)
Atherogenic lipoprotein phenotype (n) (%)	0 (0)	0.0036	8 (32)

Italic values show the statistical data.

concomitant presence of high triglycerides, low HDL-cholesterol and elevated small, dense LDL.

Spearman correlation analysis was performed to assess potential correlations between LDL size and subclasses and age, BMI and biochemical parameters in all subjects (see Table 3). CRP and triglycerides were inversely correlated with LDL size and LDL-I and positively with LDL-IIIB, -IVA and -IVB; by contrast, HDL-cholesterol was positively correlated with LDL size and LDL-I and inversely with LDL-IIIB, -IVA and -IVB. These correlations remained significant after adjustment for age and BMI (data not shown). No significant correlations were found with age, BMI, total- and LDL-cholesterol, Lp(a) and homocysteine. We have also performed correlation analysis separately for the groups of patients and controls (data not shown) and found the following significant associations in the group of patients only: LDL size with triglycerides ($r = -.585$, $p = 0.0042$) and LDL size with CRP ($r = -.612$, $p = 0.0032$).

4. Discussion

It has been shown that subjects with early RA may have an atherogenic lipid profile, with increased LDL-cholesterol and LDL:HDLcholesterol ratio [5]. In the present study we have extended such preliminary observation investigating in this category of patients LDL size and all seven LDL subclasses, as well as the full ALP. In fact, LDL are very heterogeneous particles and comprise multiple distinct subclasses that differ in size, density, physicochemical composition, metabolic behaviour and atherogenicity, with at least four major subspecies: large LDL-I, medium LDL-II, small LDL-III and very small LDL-IV [26]. The predominance of small, dense LDL has been associated with up to a seven fold increased risk for coronary artery disease [7] and several reasons have been suggested for the atherogenicity of small dense LDL. In relation to larger, more buoyant LDL, small dense LDL are taken up more easily by arterial tissue, have decreased sialic acid content and receptor-mediated uptake, as well as increased oxidative susceptibility and reduced antioxidant concentrations [9]. Therefore, screening for the presence of

Table 3

(Online Supplementary Material Only). Spearman correlations between LDL size and subclasses and age, BMI and biochemical parameters in all subjects.

	Age	BMI	C-reactive protein	Total cholesterol	Triglycerides	HDL-cholesterol	LDL-cholesterol	Lp (a)	Homocysteine
LDL size	-.008	-.151	-.645 ^a	-.102	-.664 ^a	.457 [†]	-.012	-.234	.014
LDL-I	.079	-.241	-.429 [†]	.161	-.396 [†]	.450 [†]	.181	-.104	-.063
LDL-IIA	.007	-.030	.012	.156	-.154	.221	.162	-.067	-.198
LDL-IIB	-.185	.130	.241	.071	.107	-.139	.087	.092	-.123
LDL-IIIA	-.245	.036	.011	-.206	.141	-.148	-.238	-.056	.073
LDL-IIIB	.078	.169	.380 [†]	-.217	.381 [†]	-.437 [†]	-.243	.170	.193
LDL-IVA	.110	.191	.377 [†]	-.205	.399 [†]	-.481 [†]	-.222	.154	.173
LDL-IVB	.092	.227	.316 [†]	-.156	.383 [†]	-.456 [†]	-.168	.075	.182

In bold the relationships that reached the statistical significance.

^a <0.0005.

[†] <0.05.

[†] <0.005.

small, dense LDL may potentially identify subjects with higher vascular risk and may contribute in directing specific interventions of cardiovascular prevention

Patients with RA have a higher cardiovascular risk than the general population and lipid and lipoproteins alterations are common and usually include decreased HDL-cholesterol and increased LDL:HDLcholesterol ratio [4,5]. In the present study patients with early RA showed increased triglyceride concentrations and reduced HDL-cholesterol levels than controls, while total- and LDL-cholesterol did not differ between the two groups. Elevated triglycerides and reduced HDL-cholesterol represents two lipid abnormalities usually accompanied by increased levels of small, dense LDL in the ALP. We found that patients with early RA had lower LDL size than controls and LDL subclass analysis revealed that this was due to changes in distinct LDL subspecies, with reduced LDL-I and -IIA subclasses and increased LDL-IIIB, -IVA and -IVB particles. Interestingly, 40% of patients with early RA showed elevated levels of small, dense LDL, though we recognize the limitation of analyzing % data, which is that they must sum to 100, so technically there can be at most only $n-1$ different fractions.

These findings are somewhat consistent with what previously reported by Hurt-Camejo et al. [11]. These authors interestingly found that patients with long-term RA showed lower LDL size due to increased levels of small, dense LDL particles; yet, in this study, about 90% of patients received therapies for the disease and this potentially limits the utility of the information we have on atherogenic dyslipidemia in RA. In fact, although drug treatment in RA is in general beneficial on plasma lipids, it seems that long-term anti-tumor necrosis factor- α therapies may induce some pro-atherogenic changes in lipid profile [12–14]. Since the triglycerides/HDL ratio has been proposed as a simple way to identify subjects with a predominance of small, dense LDL [24,27], we further assessed the relationships between these two lipid parameters. The concordance between elevated small, dense LDL and high triglycerides/HDL ratio was only 60% and this was confirmed by the non-significant correlation between the two parameters, supporting the concept that lipid ratios cannot represent fully reliable markers of lipoprotein abnormalities [10].

We have found by correlation analysis that such alterations in LDL subclasses were strongly associated with the extent of inflammation, as indirectly measured by CRP levels, in all subjects; further, in the group of RA patients, despite the small cohort, there was a significant inverse correlation between LDL size and CRP concentrations. Interestingly, significant associations between markers of inflammation and LDL subclasses have been reported in another rheumatic disease, i.e. the systemic lupus erythematosus [28]. Besides correlation analysis, we directly investigated the prevalence of complete and incomplete forms of ALP in patients and controls; we found that elevated triglycerides, low HDL-cholesterol and high small, dense LDL are very common in patients with early RA and the complete form of ALP was found in 1/3 of patients. Overall, our findings suggest that more than the traditional lipids should be potentially taken into account in patients with early RA for a better management of their disease. Since the therapeutic modulation of ALP and particularly of distinct LDL subspecies (such as small, dense LDL particles) is of great benefit in reducing cardiovascular risk [8,29–30], their measurement should be potentially extended to this category of patients.

In fact, increasing evidence suggest that treatment of atherogenic dyslipidemia should be considered as a part of cardiovascular risk management in RA [31]; in addition, recent studies have shown the beneficial effects of statins in such patients, particularly on disease activity, swollen joint count and endothelial dysfunction (as reviewed in [32]). Since statin use in RA is now suggested, it should be highlighted that statins have shown differential effects on the “quantity” and the “quality” of LDL [30,33] and, therefore, the

analysis of a more detailed lipid profile may contribute to assess a personalized therapy, choosing the best statin to be used.

Yet, it is still questioned whether atherogenic dyslipidemia precedes or follows onset of RA [34]. Van Halm et al. [35] have interestingly determined plasma lipids and lipoproteins in blood donors who later developed RA vs. age- and gender-matched controls: they found that future RA patients had higher levels of total-cholesterol, triglycerides and apoproteinB as well as reduced HDL-cholesterol concentrations at least ten years before the onset of symptoms. Since other authors have shown an active modulating role of lipids in inflammation [36,37], on the basis of available evidence it cannot be excluded that atherogenic dyslipidemia may precede the onset of RA.

A potential limitation of the present study is the relative small cohorts of patients and controls. Yet, it should be noted that despite the small numbers, the observation appears to be solid, as we found highly significant differences in several parameters, including LDL size and small, dense LDL subclasses. Another potential limitation of the present study is represented by the lack of analysis of apolipoproteins (such as A1 and B) or phospholipase A2; yet, we reasoned not to assess such parameters as they have already been analyzed in patients with early RA [5,38]. Finally, although we have assessed (by correlation analysis) the associations between LDL size and subclasses with clinical and biochemical parameters, we did not assess independent predictors of small, dense LDL (by multivariate analyses): such analyses were beyond the specific aim of the present study and the number of patients had to be extended considerably.

In conclusion, we found in the present study that drug-naïve patients with early RA (i.e. who had a disease durations <1 year and not prior treatment for it), in relation to age- and BMI-matched controls showed reduced LDL size due to changes in their LDL subclass distribution, with a strong reduction in larger particles and a concomitant increase in the smallest, most dense LDL. Further, we found that 40% of patients with early RA showed elevated levels of small, dense LDL and about 1/3 the complete form of ALP. Yet, whether these findings affect the atherogenic process and the clinical endpoints in this category of subjects remains to be determined by future prospective studies.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.atherosclerosis.2009.07.015.

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