Patients with early rheumatoid arthritis exhibit elevated autoantibody titers against mildly oxidized low-density lipoprotein and exhibit decreased activity of the lipoprotein-associated phospholipase A₂

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Abstract

Rheumatoid arthritis is a chronic inflammatory disease, associated with an excess of cardiovascular morbidity and mortality due to accelerated atherosclerosis. Oxidized low-density lipoprotein (oxLDL), the antibodies against oxLDL and the lipoprotein-associated phospholipase A₂ (Lp-PLA₂) may play important roles in inflammation and atherosclerosis. We investigated the plasma levels of oxLDL and Lp-PLA₂ activity as well as the autoantibody titers against mildly oxLDL in patients with early rheumatoid arthritis (ERA). The long-term effects of immunointervention on these parameters in patients with active disease were also determined. Fifty-eight ERA patients who met the American College of Rheumatology criteria were included in the study. Patients were treated with methotrexate and prednisone. Sixty-three apparently healthy volunteers also participated in the study and served as controls. Three different types of mildly oxLDL were prepared at the end of the lag, propagation and decomposition phases of oxidation. The serum autoantibody titers of the IgG type against all types of oxLDL were determined by an ELISA method. The plasma levels of oxLDL and the Lp-PLA₂ activity were determined by an ELISA method and by the trichloroacetic acid precipitation procedure, respectively. At baseline, ERA patients exhibited elevated autoantibody titers against all types of mildly oxLDL as well as low activity of the total plasma Lp-PLA₂ and the Lp-PLA₂ associated with the high-density lipoprotein, compared with controls. Multivariate regression analysis showed that the elevated autoantibody titers towards oxLDL at the end of the decomposition phase of oxidation and the low plasma Lp-PLA₂ activity are independently associated with ERA. After immunointervention autoantibody titers against all types of oxLDL were decreased in parallel to the increase in high-density lipoprotein-cholesterol and high-density lipoprotein-Lp-PLA₂ activity. We conclude that elevated autoantibody titers against oxLDL at the end of the decomposition phase of oxidation and low plasma Lp-PLA₂ activity are feature characteristics of patients with ERA, suggesting an important role of these parameters in the pathophysiology of ERA as well as in the accelerated atherosclerosis observed in these patients.

Introduction

Rheumatoid arthritis is a chronic inflammatory condition of unknown etiology affecting primarily the synovium, leading to joint damage and bone destruction [1]. Rheumatoid arthritis causes significant morbidity as a result of synovial inflammation, joint destruction and associated disability. Several investigators have reported an excess of cardiovascular morbidity and mortality among rheumatoid arthritis patients. In active
rheumatoid arthritis, the majority of cardiovascular deaths result from accelerated atherosclerosis [2-5].

Oxidative modification of low-density lipoprotein (LDL) is an important event in the development and progression of atherosclerosis. Oxidized low-density lipoprotein (oxLDL) is present in atherosclerotic lesions of humans and animal models, and promotes atherosclerosis by several mechanisms [6-9]. oxLDL has been detected in patients with systemic lupus erythematosus and the antiphospholipid syndrome and also in the synovium and synovial fluids of rheumatoid arthritis patients [10,11].

During LDL oxidation both the lipids and apolipoprotein B-100 (Apo B) undergo a variety of chemical changes via radical-mediated reactions as well as modifications by chemically active products formed on oxLDL particles [12]. An important biochemical change that takes place during LDL oxidation is the hydrolysis of its content in oxidized phospholipids and the production of lysophosphatidylcholine. This reaction is catalyzed by the lipoprotein-associated phospholipase A2 (Lp-PLA2), also known as platelet-activating factor acetylhydrolase [13]. Lp-PLA2 exhibits a Ca2+-independent phospholipase A2 activity and preferentially hydrolyses biologically active phospholipids containing short acyl groups at the sn-2 position, such as platelet-activating factor and oxidized phospholipids [13]; this enzyme therefore plays important roles in inflammatory reactions and atherosclerosis [14]. In human plasma Lp-PLA2 is associated mainly with LDL, whereas a small proportion of circulating enzyme activity is also associated with high-density lipoprotein (HDL) [13,15]. Data from large Caucasian population studies have demonstrated an independent association between plasma Lp-PLA2 (which represents mainly the LDL-associated Lp-PLA2) and the risk of future cardiovascular events [16,17]. In contrast to the total plasma enzyme, several lines of evidence suggest that HDL-associated Lp-PLA2 activity (HDL-Lp-PLA2), although at low levels in plasma, may contribute to the antiatherogenic effects of this lipoprotein [13].

oxLDL is immunogenic and some of its constituents (oxidized phospholipids, aldehydes and lysophosphatidylcholine) play important roles in the oxLDL antigenicity, participating in the formation of several different epitopes. These epitopes are recognized by specific autoantibodies, which are present in serum of healthy individuals as well as in various pathologic conditions [18]. We recently showed, using various types of mildly oxLDL as antigens, that the extent of LDL oxidation and the levels of LDL-associated Lp-PLA2 activity significantly influence the antibody titers against oxLDL in patients with stable angina [19,20]. Furthermore, we recently showed that the LDL-associated Lp-PLA2 plays an important role in modulating the immune responses against various types of mildly oxLDL observed after an acute coronary syndrome without persistent elevation of the ST segment [21].

The aim of the present study was to investigate the plasma levels of oxLDL and Lp-PLA2 activity as well as the autoantibody titers against various types of mildly oxidized LDL in patients with early rheumatoid arthritis (ERA). The long-term effects of immunointervention on these parameters in patients with active disease were also determined.

Materials and methods

Patients

Fifty-eight consecutive patients with ERA (14 men and 44 women) who met the American College of Rheumatology 1987 criteria for rheumatoid arthritis [22] and 63 apparently healthy nonsmoking volunteers (controls) were investigated. ERA patients were >18 years of age and had early inflammatory disease (disease duration <12 months) without prior use of disease-modifying antirheumatic drugs (DMARDs) and/or corticosteroids. All patients were recruited from the outpatient rheumatology clinic of the University Hospital of Ioannina, Greece. Details on the eligibility criteria for inclusion or exclusion from the study were reported in our previously published prospective, controlled study [23].

ERA patients were treated with methotrexate (0.2 mg/kg/week), and prednisone (7.5 mg/day). The dose of methotrexate remained stable during the study, while the dose of prednisone was tapered to 5 mg/day according to the patients’ clinical response. Disease activity was assessed by measuring the disease activity score for 28 joint indices [24], while the clinical response was evaluated according to the American College of Rheumatology 50% response criteria [25]. All patients were followed up every month for the first 3 months, and every 3 months thereafter. During the follow-up period, a questionnaire concerning changes in dietary habits was carefully completed by all patients. The body weight was also measured appropriately in each visit. Overnight fasting blood samples were obtained at baseline and after 12 months follow-up from both the ERA patients and the control group. The Ethics Committee of the University Hospital of Ioannina approved the study and written informed consent was obtained from each patient and each healthy volunteer.

Measurement of autoantibody titers against oxidized low-density lipoprotein

LDL (density = 1.019–1.063 g/ml) was isolated by sequential ultracentrifugation from pooled fresh plasma [26]. LDL, at a final concentration of 100 μg protein/ml, was oxidized in the presence of 5 μM CuSO4 for up to 3 hours at 37°C under continuous monitoring of the increase in the absorbance at 234 nm, as we recently described [19,20]. Oxidation of LDL was terminated by the addition of 0.01% ethylenediamine tetraacetic acid either at the end of the lag phase (oxLDL0), at the end of the propagation phase (oxLDLp), or during the decomposition phase (oxLDLd), 3 hours after the onset of oxidation [19,20]. The serum autoantibody titers of the IgG type against all types of oxLDL were determined by an ELISA method, as
PLA₂ activity was expressed as nanomoles of 1-O-hexadecyl-2-[3H-acetyl]-sn-glycero-3-phosphocholine degraded per minute of plasma [20,27,28]. The minimum detection limit of the assay is 0.8 nmol/min/ml plasma, whereas the intra-assay and inter-assay coefficients of variation are 3.3–4.2% and 7.1–8.0%, respectively.

**Results**

**Patients’ characteristics and lipid profile**

Fifty-eight patients with ERA and 63 apparently healthy volunteers participated in the study. The clinical and biochemical characteristics of the study population are presented in Table 1. There was no observed difference in sex distribution, age and body mass index between ERA patients and controls. As expected, ERA patients exhibited increased levels of the inflammatory markers CRP and erythrocyte sedimentation rate and had a high disease activity score as measured by the disease activity score for 28 joint indices (Table 1). In addition, ERA patients exhibited a mild dyslipidemia characterized by an increase in the serum levels of total cholesterol, LDL-cholesterol, triglycerides and Apo B as well as by a decrease in the serum levels of HDL-cholesterol and Apo A-I compared with the respective baseline values (Table 1). It should be noted that no female patient was receiving hormone replacement therapy either at baseline or during the follow-up period.

One year of therapy with DMARDs in ERA patients resulted in a significant decrease of the inflammatory markers CRP and the erythrocyte sedimentation rate as well as in the reduction of the disease activity score for 28 joint indices (Table 1). In addition, one year of therapy with DMARDs resulted in a significant increase in the serum levels of total cholesterol, LDL-cholesterol and Apo A-I compared with the respective baseline values (Table 1).

**Lipoprotein-associated phospholipase A₂ activity**

At baseline, ERA patients exhibited a significantly lower activity of total plasma Lp-PLA₂ and of HDL-Lp-PLA₂, compared with controls (Table 1). One year of therapy with DMARDs did not influence the total plasma Lp-PLA₂ but it significantly increased the HDL-Lp-PLA₂ activity (Table 1).

**Autoantibody titers against oxidized low-density lipoprotein**

Three types of mildly oxLDL were prepared and used as antigens: oxLDLₐ at the end of the lag phase, oxLDLₚ at the end of the propagation phase and oxLDL₀ at the decomposition phase, 3 hours after the onset of oxidation. As shown in Table 2, ERA patients exhibited higher autoantibody titers against all types of oxLDL at baseline compared with controls. Impor-
tantly, the autoantibody titers against oxLDLP and oxLDLD were inversely correlated with serum HDL-cholesterol levels (Figure 1). In addition, autoantibody titers against oxLDL D were inversely correlated with HDL-Lp-PLA2 activity (Figure 1). One year of therapy with DMARDs resulted in a significant decrease in autoantibody titers against all types of oxLDL in ERA patients compared with the respective baseline values (Table 2).

**Association of autoantibody titers against oxidized LDL and plasma lipoprotein-associated phospholipase A2 with early rheumatoid arthritis**

We initially performed univariate analysis using the lipid parameters that were significant different between ERA patients and controls, the antibody titers against the various types of oxLDL and the Lp-PLA2 activity, in order to evaluate their relationships with the presence of ERA. The results of this analysis showed that only autoantibody titers against all types of oxLDL as well as the low plasma Lp-PLA2 activity are associated with ERA (Table 3). To further identify whether these parameters could independently be associated with ERA, multivariate logistic regression analysis was performed, taking into account all statistically significant factors as they derived from univariate analysis. In the multivariate analysis model we therefore included the autoantibody titers against oxLDL D, oxLDLP, and oxLDLD, and the plasma Lp-PLA2 activity as defined from univariate analysis. In this analysis ERA showed significant associations only with autoantibody titers against oxLDL D and plasma Lp-PLA2 activity (Table 4).

**Discussion**

The present study shows for the first time that ERA patients exhibit low plasma Lp-PLA2 activity and elevated autoantibody titers against mildly oxidized types of LDL (oxLDLP, oxLDLP, and oxLDLD) compared with controls. The low Lp-PLA2 activity is in accordance with previously published data by our group, indicating that patients with active juvenile rheumatoid arthritis presented with lower plasma Lp-PLA2 activity compared with those with inactive disease or to controls [30]. The present study further shows that the low Lp-PLA2 activity is independently associated with ERA. It is well established that the main cellular source of the plasma form of Lp-PLA2 is monocytes, which secrete this enzyme during their differentiation into macrophages [31]. The cellular expression of plasma Lp-PLA2 is regulated by various factors, including the differentiation state of the cell and the degree of activation by proinflammatory mediators [13,32]. Most of the proinflammatory mediators (lipopolysaccharide, tumor necrosis factor alpha, IL-1, IL-8, and interferon gamma) inhibit Lp-PLA2 expression by macrophages in vitro [13]. The reduction in plasma Lp-PLA2 activity found in ERA patients could therefore be attributed to the inflammation-induced decrease in the enzyme expression. According to our previously published results, however, another important determinant of the plasma Lp-PLA2 activity...
is the plasma LDL level [27,28]. Indeed, Lp-PLA₂ in plasma is mainly bound on LDL particles, whereas a small proportion is associated with HDL [13]. We may consequently suggest that the low levels of enzyme activity in the plasma of ERA patients at baseline could be the combined effect of the inflammation-induced reduction of enzyme secretion from macrophages and the expected increase in plasma enzyme levels due to the elevation of LDL-cholesterol in plasma of ERA patients.

The dependence of the plasma Lp-PLA₂ levels from the LDL-cholesterol levels could also explain our results showing that therapy with DMARDs did not affect either the plasma LDL-cholesterol levels or the plasma Lp-PLA₂ activity. A factor that could also influence the plasma Lp-PLA₂ levels in ERA patients is Lp(a). Indeed, we [33] and others [34] have previously shown that Lp(a) contains several-fold greater Lp-PLA₂ activity

### Table 2

| Antigen                               | Controls (n = 63) | Early rheumatoid arthritis patients |
|---------------------------------------|------------------|-------------------------------------|
|                                       | Baseline (n = 58) | Post-treatment (n = 56)             |
| oxLDL in the lag phase                | 0.919 ± 0.271    | 1.131 ± 0.229*                      |
|                                       |                  | 0.872 ± 0.198†                      |
| oxLDL in the propagation phase        | 0.962 ± 0.289    | 1.223 ± 0.278*                      |
|                                       |                  | 1.045 ± 0.300‡                      |
| oxLDL in the decomposition phase      | 0.985 ± 0.376    | 1.375 ± 0.327*                      |
|                                       |                  | 1.144 ± 0.247‡                      |

Data presented as the mean ± standard deviation. *P < 0.0001 compared with controls, †P < 0.001, ‡P < 0.01 and **P < 0.05 compared with baseline values.

### Figure 1

Correlation between serum levels of high-density lipoprotein-cholesterol and autoantibody titers against oxidized low-density lipoprotein. Correlation between serum levels of high-density lipoprotein (HDL)-cholesterol and autoantibody titers against (a) oxidized low-density lipoprotein in the propagation phase (oxLDLₚ) and (b) oxidized low-density lipoprotein in the decomposition phase (oxLDLₜ) in early rheumatoid arthritis patients at baseline. (c) Correlation between HDL-associated lipoprotein-associated phospholipase A₂ (HDL-Lp-PLA₂) activity and autoantibody titers against oxLDLₜ in early rheumatoid arthritis patients at baseline.
compared with LDL when assayed at equimolar protein concentrations. Importantly, recent results have provided evidence that the Lp(a)-associated Lp-PLA2 may play an important role by degrading oxidized phospholipids that are preferentially sequestered on Lp(a) [35]. It is unlikely, however, that the Lp(a)-associated Lp-PLA2 activity might have influenced the plasma levels of this enzyme in ERA patients since the mean serum levels of Lp(a) in our patients as well as in controls are between 8.6 and 11.2 mg/dl – according to our previously published results, the plasma levels as well as the distribution of Lp-PLA2 between LDL and HDL can be influenced by the presence of Lp(a) only when plasma levels of this lipoprotein exceed 30 mg/dl [33].

An important observation of the present study is that ERA patients exhibited higher autoantibody titers against all types of mildly oxLDL (oxLDL_L, oxLDL_P and oxLDL_D) at baseline compared with controls. One year of therapy with DMARDs resulted in a significant decrease of autoantibody titers against all types of oxLDL compared with the respective baseline values, a finding that could be attributed, at least partially, to the repression of the immune system activation due to immunointervention. Importantly, the antibody titers against oxLDL_0 are independently associated with ERA, thus providing evidence that such types of mildly oxLDL may be implicated in the pathophysiology of ERA. Indeed, previously published results showed that modified LDL with characteristics of minimally modified LDL, but not extensively oxidized LDL, is present in the synovial fluid of patients with rheumatoid arthritis [36].

Another important finding of the present study is that ERA patients exhibit low plasma HDL-cholesterol levels at baseline. According to our previously published results, this phenomenon could be at least partially attributed to the increased activity of the cholesterol ester transferring protein observed in plasma of ERA patients [23]. The present study further shows

| Table 3 | Univariate logistic regression analysis of factors associated with the presence of early rheumatoid arthritis |
|---------|--------------------------------------------------------------------------------------------------|
|         | Odds ratio | 95% confidence interval  | P value |
| Age     | 1.009      | 0.982–1.036             | Not significant |
| Female gender | 1.014      | 0.965–1.123             | Not significant |
| Total-cholesterol | 0.998      | 0.990–1.006             | Not significant |
| Low-density lipoprotein-cholesterol | 0.960      | 0.991–1.010             | Not significant |
| High-density lipoprotein-cholesterol | 0.969      | 0.931–1.008             | Not significant |
| Triglycerides | 0.998      | 0.991–1.006             | Not significant |
| Apolipoprotein B-100 | 1.012      | 0.934–1.134             | Not significant |
| Apolipoprotein A-I | 0.985      | 0.876–1.078             | Not significant |
| Antibodies against oxidized low-density lipoprotein in the lag phase | 0.027      | 0.004–0.177             | 0.000 |
| Antibodies against oxidized low-density lipoprotein in the propagation phase | 0.029      | 0.005–0.171             | 0.000 |
| Antibodies against oxidized low-density lipoprotein in the decomposition phase | 0.034      | 0.008–0.152             | 0.000 |
| Plasma lipoprotein phospholipase A2 activity | 1.038      | 1.012–1.066             | 0.004 |
| High-density lipoprotein-associated lipoprotein phospholipase A2 activity | 1.021      | 0.897–1.245             | Not significant |

| Table 4 | Multivariate logistic regression analysis of factors associated with the presence of early rheumatoid arthritis |
|---------|--------------------------------------------------------------------------------------------------|
|         | Odds ratio | 95% confidence interval (β) | P value |
| Antibodies against oxidized low-density lipoprotein in the lag phase | 0.431      | 0.023–7.945             | Not significant |
| Antibodies against oxidized low-density lipoprotein in the propagation phase | 0.155      | 0.010–2.295             | Not significant |
| Antibodies against oxidized low-density lipoprotein in the decomposition phase | 0.047      | 0.008–0.282             | 0.001 |
| Plasma lipoprotein phospholipase A2 activity | 1.044      | 1.013–1.077             | 0.006 |

The model includes antibodies against oxidized low-density lipoprotein in the lag phase, the propagation phase and the decomposition phase, and plasma lipoprotein phospholipase A2 activity as defined from univariate analysis after adjustment for age and female gender.
that HDL-cholesterol levels are inversely correlated with autoantibody titers against oxLDLp and oxLDLd. Furthermore, autoantibody titers against oxLDLd at baseline are inversely correlated with HDL-Lp-PLA2. Several studies over the past years have demonstrated that HDL exerts potent anti-inflammatory, antioxidant and antiatherogenic effects through its constituents. Among these constituents, the enzyme Lp-PLA2 may have a prominent role by degrading proinflammatory oxidized phospholipids formed on LDL during oxidation, thus limiting their accumulation on oxLDL [13]. The negative correlation between HDL-Lp-PLA2 activity and antibodies against oxLDLd found in the present study could therefore be attributed to the fact that oxLDLd compared with the other types of oxLDL is enriched in oxidized phospholipids that significantly contribute to the antigenicity of this type of oxLDL [37]. These phospholipids are substrates for HDL-Lp-PLA2; consequently the HDL-Lp-PLA2 activity could significantly lower the levels of oxidized phospholipids formed on oxLDLd, thus diminishing the antigenicity of this type of oxLDL. In addition to the HDL-Lp-PLA2, the Apo A-I content of HDL can bind oxidized lipids and remove them from LDL, therefore significantly contributing to the HDL-mediated retardation of LDL oxidation and thus the prevention of oxLDL proinflammatory activities [38].

According to our results, the low baseline levels of HDL-cholesterol and HDL-Lp-PLA2 activity in ERA patients are significantly increased after immunointervention, a phenomenon that could be at least partially attributed to the immunointervention-induced reduction in cholesterol ester transferring protein activity [23]. The elevation of HDL-cholesterol and HDL-Lp-PLA2 activity in ERA patients after immunointervention is associated with a reduction in the autoantibody titers against oxLDL. We may consequently suggest that the immunointervention-induced reduction in the autoantibody titers against oxLDL could be attributed not only to the repression of the immune system activation, but also to the increase in plasma HDL-cholesterol and HDL-Lp-PLA2 levels. Furthermore, this action of DMARDs may represent a potentially antiatherogenic effect of these drugs.

Conclusion
The present study shows for the first time that ERA patients exhibit low plasma Lp-PLA2 and HDL-Lp-PLA2 activities and elevated autoantibody titers against mildly oxLDL. The low plasma Lp-PLA2 activity and the increased titers against oxLDLd are independently associated with ERA, suggesting an important role of these parameters in the pathophysiology of ERA. This hypothesis needs to be further supported by large-scale clinical studies.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
ESL wrote the paper and performed the biochemical measurements. ANG participated in the selection of the patients and therapy. ECP contributed to the biochemical measurements and to writing the paper. AIP participated in the statistical analysis and in writing the paper. AAD participated in the selection of the patients and therapy. ADT conceived the idea for the study, participated in its design and coordination, and helped to draft the manuscript. All authors read and approved the final manuscript.

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