

# Evidence for Immune Activation Against Oxidized Lipoproteins in Inactive Phases of Juvenile Chronic Arthritis

GABRIELE SIMONINI, MARCO MATUCCI CERINIC, ROLANDO CIMAZ, MARIO ANICHINI, SIMONETTA CESARETTI, MASSIMO ZOPPI, SERGIO GENERINI, and FERNANDA FALCINI

**ABSTRACT. Objective.** Oxidative stress contributes to joint inflammation and damage in rheumatoid arthritis. In a mobile inflamed joint, exercise induced multiple cycles of hypoxia-reperfusion injury may lead to the creation of a redox environment in which oxido-reductase systems, by NADPH mechanisms, produce highly reactive chemical species (i.e., oxygen free radicals). We investigated 2 endproducts of lipid peroxidation, malondialdehyde (MDA) and diene conjugates (DC), and the formation of antibodies against oxidized low density lipoproteins (Ab oxLDL) in juvenile chronic arthritis (JCA), and assessed the role of oxidative phenomena in different phases and subsets of this disease.

**Methods.** To assess the role of oxidative stress in JCA, we measured the endproducts of lipid peroxidation, MDA and DC, by the increase of absorbance at 586 nm and 234 nm, respectively, and the levels of Ab oxLDL by ELISA in the sera of 58 patients with JCA and 21 healthy controls. Due to cross-reactivity between Ab oxLDL and anticardiolipin antibodies (aCL), the sera were also tested by a standard ELISA for IgG-aCL. The patients were divided into 3 subsets: 29 with pauciarticular (pauci), 15 with polyarticular (poly), and 14 with systemic (sys) onset disease, and then were subdivided, according to different variables appropriate to each subset, reflecting active and inactive disease, into 30 active (14 pauci, 8 poly, 8 sys) and 28 inactive (15 pauci, 7 poly, 6 sys).

**Results.** Levels of Ab oxLDL were significantly increased in the whole group of patients ( $566.6 \pm 263.0$  vs  $206.6 \pm 136.3$  mU/ml;  $p < 0.001$ ) and in each of the type of onset (pauci  $660.8 \pm 272.1$ ,  $p < 0.001$ ; poly  $341.3 \pm 134.7$ ,  $p < 0.01$ ; sys  $497.8 \pm 114.8$ ,  $p < 0.001$ ) compared to controls. Ab oxLDL were higher in the inactive than in the active group ( $743.5 \pm 231.9$  and  $404.4 \pm 169.9$ ;  $p < 0.001$ ). MDA and DC levels were not increased significantly in patients' sera. No patient was positive for IgG-aCL.

**Conclusion.** These findings suggest that MDA and DC cannot be considered major markers of oxidative stress in JCA and that the Ab oxLDL may represent a delayed sign of oxidative stress previously induced by the inflammatory process in patients with JCA. (J Rheumatol 2001;28:198-203)

## Key Indexing Terms:

JUVENILE CHRONIC ARTHRITIS    LOW DENSITY LIPOPROTEINS    OXIDATIVE STRESS

Juvenile chronic arthritis (JCA) is an inflammatory joint disorder characterized by chronic synovitis and associated with extraarticular manifestations such as fever, lymphadenopathy, pericarditis, and uveitis. The clinical features of arthritis during the first 6 months of illness reveal 3 different subsets of disease onset: pauciarticular (pauci), polyarticular (poly), and systemic (sys) onset<sup>1</sup>. Several mechanisms have been pro-

posed to explain the pathogenesis of synovial inflammation and proliferation.

Oxidative stress, sustained by reactive oxygen species (ROS) including oxygen free radicals such as superoxide anion ( $O_2^{\cdot-}$ ) and hydroxyl radicals ( $OH^{\cdot}$ ), may be involved in determining chronic synovitis and cartilage damage as seen in adult rheumatoid arthritis (RA)<sup>2,3</sup>. ROS are highly reactive molecules because of one or more unpaired electrons that, when present in excess, can damage tissues<sup>4</sup>. In an inflamed joint, exercise induced multiple cycles of hypoxia-reperfusion injury may lead to the creation of a redox environment in which oxido-reductase cell systems generate ROS, by nicotinamide adenine dinucleotide phosphate (NADPH) dependent mechanisms<sup>5</sup>, and xanthine-oxidase, an enzyme present in endothelial cells of small synovial blood vessels<sup>6</sup>. Hypoxia has profound effects on the biochemistry and immunobiology of the joint, in which, when the supply of oxygen is restored during the reperfusion phase, these oxido-reductase cell systems produce oxygen free radicals<sup>7,8</sup>.

From the Department of Pediatrics, Rheumatology Unit, and Department of Medicine, Division of Rheumatology, University of Florence, Florence; the Department of Pediatrics, ICP, Milano; and INRCA, Florence, Italy.

G. Simonini, MD, Department of Pediatrics; M. Matucci Cerinic, MD, Associate Professor, Department of Medicine, Division of Rheumatology; M. Zoppi, MD, Associate Professor, Department of Medicine, Division of Rheumatology; S. Generini, MD, Department of Medicine, Division of Rheumatology; F. Falcini, MD, Associate Professor, Department of Pediatrics, Division of Rheumatology, University of Florence; R. Cimaz, MD, Department of Pediatrics, ICP, Milano; M. Anichini, MD, Associate Professor; S. Cesaretti, MD, INRCA, Florence.

Address reprint requests to Dr. F. Falcini, Department of Pediatrics, Via Luca Giordano 13, 50132 Firenze, Italia. E-mail: falcini@cesit1.unifi.it  
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The targets of damage by free radicals are lipids<sup>8</sup>, DNA, proteins, proteoglycans, collagen<sup>2</sup>, and immune cells<sup>9</sup>. Lipid peroxidation of the cell membrane, which contains polyunsaturated fatty acids, is induced by ROS and this may provoke cellular dysfunction and death, yielding a variety of endproducts including aldehydes [as malonildialdehyde (MDA), a thiobarbituric acid reactive substance] and diene conjugates (DC)<sup>10</sup>. Lipid peroxidation endproducts may be cytotoxic and alter T cell/macrophage interactions<sup>9</sup>. Oxidation of polyunsaturated fatty acids in plasma low density lipoproteins (LDL) by endothelial cells and macrophages<sup>11</sup> produces oxidized LDL, and this induces immunogenic epitopes in the LDL molecule with a specific antibody production<sup>12</sup>.

In RA, ROS contribute to bone and cartilage damage, perpetuating the process of chronic synovitis<sup>13,14</sup>. The endproducts MDA and DC as well as antibodies against oxidized LDL (Ab oxLDL), in sera and synovial fluid of RA patients have been proposed as markers of oxidative stress<sup>3</sup>.

The possibility that oxidative stress may also contribute to the pathogenesis of JCA prompted us to investigate, in the 3 subsets and in different phases of the disease, the endproducts of lipid peroxidation, MDA and DC, and the levels of Ab oxLDL.

## MATERIALS AND METHODS

We studied 58 patients (36 girls, 22 boys; mean age  $\pm$  SEM  $8 \pm 3.4$  yrs) with JCA according to EULAR criteria<sup>15</sup>. They were divided into 3 subsets: 29 pauci, 15 poly, and 14 sys onset. These patients were then subdivided as reported<sup>16</sup> according to the clinical findings (fever, rash, joint inflammation) and hematological data, erythrocyte sedimentation rate (ESR), and hemoglobin (Hb) into a group of 30 patients with active disease (14 pauci, 8 poly, 8 sys) and another of 28 patients with inactive disease (15 pauci, 7 poly, 6 sys). Systemic JCA was defined as active in the presence of all the following variables: spiking fever  $> 38.5^{\circ}\text{C}$ , typical rash, joint swelling with reduced range of motion in one or more joints, Hb  $< 10$  g/dl, ESR  $> 25$  mm/h. Polyarticular arthritis was defined as active in the presence of ESR  $> 25$  mm/h and swelling with reduced range of motion in more than 5 joints. Pauciarticular arthritis was defined as active in the presence of joint swelling with reduced range of motion in one to 4 joints.

The basic treatment in children with systemic JCA consisted of steroids and nonsteroidal antiinflammatory drugs (NSAID), while in the poly subset, methotrexate represented the second-line drug used in association with NSAID. All patients in the pauci onset group were given only NSAID.

Twenty-one healthy subjects (14 girls and 7 boys, mean age  $\pm$  SEM  $10 \pm 2.1$  yrs) acted as controls. None had had chronic illnesses or any infection or other clinical abnormalities in the 4 weeks before blood samples were taken.

Informed consent was obtained from parents of all subjects.

**Assessment of lipid peroxidation.** Sera were taken from the antecubital vein during routine laboratory tests. Before storage, butylated hydroxytoluene was added (final concentration  $20 \mu\text{M}$ ) as a chain-breaking antioxidant. Serum samples were stored at  $-80^{\circ}\text{C}$  and analyzed within 6 weeks. The addition of an antioxidant to samples and a short storage time are required to minimize spontaneous lipid autooxidation.

**Diene conjugates and malonildialdehyde.** DC were measured in serum with a spectrophotometer by the increase of the absorbance at  $234 \text{ nm}$ ; MDA was measured by condensation of one molecule with 2 molecules of  $10.3 \text{ nM}$  N-methyl-2-phenylindole in acetonitrile and by measuring the absorbance at  $586 \text{ nm}$ <sup>17,18</sup>.

**Antibodies against oxidized LDL.** Autoantibody titers of antioxidantized LDL

were quantified by an enzyme catalyzed color change detectable on a standard ELISA reader. Antigens for this assay included native LDL, protected against oxidation by  $0.27 \text{ mM}$  edetic acid, and butylated hydroxytoluene in phosphate buffered saline (PBS,  $10 \text{ mM}$  sodium phosphate,  $\text{pH } 7.2$ ) and oxidized LDL, obtained after  $18 \text{ h}$  oxidation with  $2 \mu\text{M}$   $\text{CuSO}_4$ .

The wells were incubated (coated) with  $50 \mu\text{l}$  of native LDL and oxidized LDL antigen ( $5 \mu\text{g/ml}$ ) in PBS for  $16 \text{ h}$  at  $4^{\circ}\text{C}$ . After removal of the unbound antigen and washing of the wells (with PBS containing  $0.05\%$  Tween 20, then with distilled water), the remaining nonspecific binding sites were saturated using  $2\%$  bovine serum albumin in PBS. The wells were washed, and  $50 \mu\text{l}$  of serum sample (diluted to  $1:20$  and  $1:50$ ) were added to wells coated with native LDL and oxidized LDL, and incubated overnight at  $4^{\circ}\text{C}$ . Antibodies, if present in prediluted serum, bind specifically to the antigen.

After incubation, the wells were aspirated and washed, a washing step that consists of 4 washings with  $300 \mu\text{l}$  diluted washing buffer, before an appropriate IgG peroxidase conjugated rabbit anti-human monoclonal antibody was added to each well ( $50 \mu\text{l}$ ) to detect the presence of bound antibodies. After incubation ( $4 \text{ h}$  at  $4^{\circ}\text{C}$ ) and after removal of unbound conjugate through washing,  $50 \mu\text{l}$  of freshly prepared substrate ( $0.4 \text{ mg/ml}$  o-phenylenediamine and  $0.045\%$   $\text{H}_2\text{O}_2$  in  $100 \text{ mM}$  acetate buffer,  $\text{pH } 5.4$ ) was added to the wells as a nontoxic chromogenic substrate and incubated for  $5 \text{ min}$  at room temperature. The enzyme reaction was terminated by addition of  $50 \mu\text{l}$  of  $2 \text{ M}$   $\text{H}_2\text{SO}_4$ .

The optical density was then measured spectrophotometrically at  $492 \text{ nm}$ . To calculate the antibody titer, we used the ratio of the corresponding spectrophotometric reading of antioxidantized LDL and anti-native LDL wells from the same serum sample. Using this approach, the spectrophotometric readings of anti-native LDL wells represent the corresponding blanks of antioxidantized LDL wells and reduce the possible detection of false positive values. The assay is standardized with defined amounts of Ab oxLDL in a serum matrix. The IgG concentration in the samples is quantified in mU based on a characterized serum with high titers of Ab oxLDL titer and a low cross-reactivity with native LDL ( $< 5\%$ )<sup>19-21</sup>.

**Anticardiolipin antibodies.** Since cross-reactivity between Ab oxLDL and anticardiolipin antibody (aCL, one of the antiphospholipid antibodies associated with the antiphospholipid syndrome) has been observed in systemic lupus erythematosus (SLE)<sup>22</sup>, the sera were also tested for IgG-aCL. IgG-aCL, binding to solid phase cardiolipin, were detected in serum samples by a standard aCL ELISA: levels of IgG antibodies above  $20 \text{ GPL}$  units were considered positive<sup>23</sup>.

**Statistical analysis.** Results are expressed as mean  $\pm$  standard deviation. Assessment of significant variation between the control group and patients with JCA was by nonparametric Mann-Whitney U tests or unpaired 2 tailed Student's t test, if appropriate; differences between disease groups were examined using one-way ANOVA for comparison of more than 2 groups. Nonparametric tests were used, where necessary, in univariate analysis due to the small size of our groups and to the skewness of our data. P values  $< 0.05$  were considered statistically significant. All analyses were performed on the SPSS package.

## RESULTS

**Antibodies against oxidized LDL.** Serum Ab oxLDL levels above the normal range were detected in 50 of all 58 patients with JCA ( $86.2\%$ ), while only 2 of 21 healthy controls ( $9.5\%$ ) showed higher values than the cutoff point. In the whole group of patients, the levels of Ab oxLDL ( $566.6 \pm 263.0 \text{ mU/ml}$ ) were significantly ( $p < 0.001$ ) increased compared to controls ( $206.6 \pm 136.3 \text{ mU/ml}$ ), and were also significantly higher than in healthy subjects in each subset (pauci  $660.8 \pm 272.1$ ,  $p < 0.001$ ; poly  $341.3 \pm 134.7$ ,  $p < 0.01$ ; sys  $497.8 \pm 114.8$ ,  $p < 0.001$ ) (Figure 1A, Table 1). The antibody levels in the pauciarticular subset were higher than in polyarticular

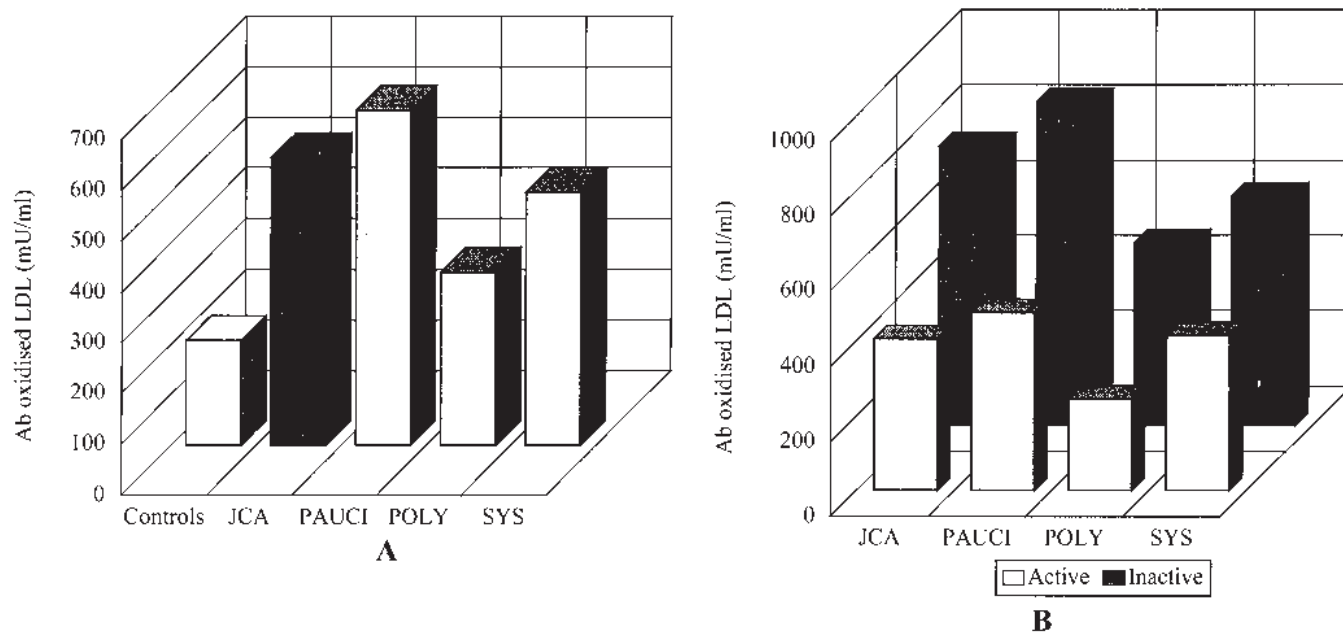


Figure 1. A. Antibodies against oxidized LDL in controls, in the whole group of patients with JCA, and in the different types of onset, pauciarticular (PAUCI), polyarticular (POLY), and systemic (SYS). B. Serum levels of antibodies against oxidized LDL in JCA and in the 3 types of onset, pauciarticular (PAUCI), polyarticular (POLY), and systemic (SYS) in the different phases of disease (active and inactive).

Table 1. Serum levels of diene conjugates (DC), malonildialdehyde (MDA), and antibodies against oxidized LDL (Ab oxLDL) in juvenile chronic arthritis (JCA), in the different types of onset and in healthy controls. Values are mean ( $\pm$  SD).

Oxidative Stress Products (reference values)	Controls, n = 21	JCA, n = 58	Pauci, n = 29	Poly, n = 15	Systemic, n = 14
DC, (80 $\pm$ 20 $\mu$ M/l)	70.6 ( $\pm$ 24.0)	69.3 ( $\pm$ 24.6)	71.8 ( $\pm$ 27.9)	63.3 ( $\pm$ 23.9)	61.5 ( $\pm$ 24.6)
MDA, (< 7.5 $\mu$ M/l)	4.5 ( $\pm$ 1.0)	4.9 ( $\pm$ 2.1)	4.6 ( $\pm$ 1.6)	6.3 ( $\pm$ 3.5)	4.4 ( $\pm$ 0.9)
Ab oxLDL, (119 $\pm$ 4 mU/ml)	206.6 ( $\pm$ 136.3)	566.6** ( $\pm$ 263.0)	660.8** ( $\pm$ 272.1)	341.3* ( $\pm$ 134.7)	497.8** ( $\pm$ 114.8)

\*\*p < 0.001, \*p < 0.01, significant vs controls.

JCA (p < 0.001) and systemic JCA (p < 0.01). Moreover, Ab oxLDL were higher in systemic JCA than in polyarticular JCA (p < 0.05) (Figure 1A).

Ab oxLDL were higher in the group with inactive than active disease (743.5  $\pm$  231.9 and 404.4  $\pm$  169.9; p < 0.001), and this difference was also present within the 3 individual subsets (Figure 1B, Table 2).

In the group with inactive disease, Ab oxLDL were significantly higher in the pauciarticular than in the polyarticular subset (p < 0.001) and the systemic subset (p < 0.001). In inactive systemic JCA, Ab oxLDL were significantly higher than in inactive polyarticular JCA (p < 0.05) (Figure 1B).

In the group with active disease, the levels of Ab oxLDL were significantly higher in the pauciarticular than in the polyarticular subset (p < 0.001), while in systemic onset JCA levels were higher compared to polyarticular JCA (p < 0.01). No difference was found between systemic and pauciarticular JCA (Figure 1B).

Table 2. Comparison in the 3 types of onset disease between antibodies against oxidized LDL (Ab oxLDL) in active and inactive juvenile chronic arthritis (JCA). Values are mean ( $\pm$  SD).

Type of Onset	JCA Active	JCA Inactive	p
Pauciarticular	473.9 ( $\pm$ 175.9)	861.1 ( $\pm$ 206.2)	< 0.001
Polyarticular	245.3 ( $\pm$ 60.2)	485.3 ( $\pm$ 48.5)	< 0.001
Systemic	412.85 ( $\pm$ 30.28)	611.1 ( $\pm$ 66.79)	< 0.05

*Diene conjugates and malonildialdehyde.* We found no significant differences in MDA and DC levels between patients and controls, or within the different groups of patients (Table 1). The levels of MDA in active and in inactive JCA showed no statistically significant differences (4.8  $\pm$  1.6 vs 5.1  $\pm$  2.1  $\mu$ M/l); as well no difference in DC values was found between active and inactive disease (68.4  $\pm$  22.1 vs 71.5  $\pm$  24.6  $\mu$ M/l).

*Anticardiolipin antibodies.* None of 58 samples was found positive for IgG-aCL.

## DISCUSSION

Increased oxidative damage occurs in several human diseases, in adults as well as in children. However, ROS have been shown to play a role in pathogenesis only in some of them, such as atherosclerosis, idiopathic pulmonary fibrosis, early brain injury<sup>24</sup>, or cystic fibrosis<sup>25</sup> or in different autoimmune disorders such as RA<sup>14</sup>, diabetes mellitus type I<sup>26</sup>, Kawasaki disease<sup>27</sup>, SLE<sup>28</sup>, and Behçet's disease<sup>29</sup>. It is well known that oxidative stress can damage proteins, DNA, and lipids. Because ROS have a short lifespan, it is very difficult to evaluate the levels of oxidative stress by a direct measurement of free radicals and to quantify the oxidative dependent damage. It is easier, instead, to assess lipid peroxidation that often occurs late in the injury process. For this reason endproducts of lipid peroxidation, such as MDA and DC, can be used as markers of oxidative stress, due to their longer lifespan and since they are easily detectable in serum samples<sup>3</sup>.

This tenet is not confirmed by our findings, which show that MDA and DC are not at significant levels in JCA, in active or in inactive disease; this datum prevents us considering these 2 lipid peroxidation products as useful markers of oxidative stress and disease activity. These results are in agreement with those of Michel, *et al*<sup>30</sup>, who found that serum MDA levels in children with SLE and with localized scleroderma were similar to controls. Moreover, in RA and SLE, Suryaprabha, *et al* observed an increased production of free radicals using the nitroblue-tetrazolium test and hydrogen peroxide production in stimulated polymorphonuclear leukocytes, but could not find elevated MDA levels<sup>31</sup>.

Oxidized low density lipoproteins (LDL) have chemotactic properties for monocytes, are cytotoxic for endothelial and smooth muscle cells<sup>32</sup>, bind to collagen<sup>33</sup>, and stimulate connective tissue formation<sup>34</sup> and monocyte-endothelial cell interactions<sup>35</sup>.

While native LDL have no cytotoxic effect, their oxidation by free radicals during atherosclerotic and ischemic conditions generates a molecule that alters the cell calcium pathway and, ultimately, the function and viability of different kinds of cells<sup>36</sup>.

Ab oxLDL have been detected in sera of patients with chronic periaortitis<sup>37</sup> and reported to be predictive of myocardial infarction<sup>38</sup> and of progression of atherosclerosis<sup>39</sup>. In patients with atherosclerosis, LDL oxidation takes place *in vivo* and plays a critical role in the development of atheromatous plaque<sup>40</sup>. In patients with JCA, elevated Ab oxLDL levels have been detected in significantly higher levels than in controls, in accord with our findings; moreover, in the same study, a fairly good correlation between Ab oxLDL and aCL was observed, suggesting that in JCA, as in SLE, these 2 moieties are in part directed against shared antigenic epitopes<sup>41</sup>.

In our patients no evidence for cross-reactivity between Ab

oxLDL and IgG-aCL was found, ruling out the possibility that Ab oxLDL do not arise on the basis of cross-reactivity with IgG-aCL, at least in JCA. Except for very rare cases, children do not yet have atherosclerosis, and it is possible that Ab oxLDL in patients with JCA arise as a secondary manifestation of lipid peroxidation in the inflamed joints.

The high levels of Ab oxLDL detected in our children may be evidence of enhanced oxidation, which can play a significant role in tissue damage and in perpetuating inflammation not only in RA but also in JCA. Thus, the use of antioxidants in JCA may be suggested, in order to limit the development of a redox environment in inflamed tissues.

Moreover, our data show that Ab oxLDL levels are higher in the pauciarticular group than in other subsets, in active and inactive disease. Pauciarticular arthritis is usually characterized by involvement of lower limb joints, such as knees or ankles; these are large joints, where the amount of hypoxia-reperfusion injury induced by exercise is greater than in small joints of the hands and feet that are more commonly involved in polyarticular or systemic onset disease.

In our study, in all subsets of JCA, the immune response against the products of lipid peroxidation was greater during the inactive than the active phase of the disease. This may suggest that oxidative damage takes place in the active phase of the disease, while an immune activation against oxidized LDL, represented by IgG class antibodies, arises gradually while the disease activity is diminishing. ROS are highly reactive molecules that quickly impair tissue and cellular functions. When the disease is active and the first articular damage probably occurs, it is reasonable to speculate that the immune system may react against the free radical damage by the fastest immune response: the T cell response. Antibody response is a slow immune activity, and antibody titers rise gradually, after amplification of B cell clone. The free radical sensitization may take place during the active phases, and elevated Ab oxLDL may reach detectable levels in the inactive phases of disease, when the inflammation process and free radical attacks are fading.

Ab oxLDL in serum may represent only a marker of oxidized LDL generation, evidence for immune activation against an oxidative phenomenon that occurs in inflamed joints; moreover, the pathogenetic role of these antibodies cannot be excluded. It has been hypothesized that oxidized LDL may combine with Ab oxLDL, leading to the formation of immune complexes, and the uptake of these by Fc receptors on macrophages occurs in synergy with but faster than the other antioxidant scavenger pathways<sup>42,43</sup>. An *in vitro* study showed that the uptake of radiolabelled oxidized LDL by a monocyte/macrophage-like cell line was more rapid in the presence of Ab oxLDL than the uptake of oxidized LDL alone<sup>43</sup>. This mechanism may contribute to the elimination of excess of oxidized LDL produced by free radical action. When this scavenger pathway is saturated, immune complexes composed of oxidized LDL and Ab oxLDL could accumu-

late in the synovial membrane, leading to a circulating immune complex-like disease. The activation of complement by immune complex deposits can subsequently lead to the generation of chemotactic and vasoactive factors, resulting in the influx of neutrophils into the joint. Phagocytosis of immune complexes by polymorphonuclear cells results in the release of lysosomal enzymes and ROS, producing tissue injury. Ab oxLDL and oxidative stress products may therefore induce ROS generation themselves, thus taking part in a vicious cycle of oxidative stress capable of perpetuating the process of chronic synovitis.

Our data suggest that the IgG class Ab oxLDL could represent a delayed sign of oxidative stress, previously induced by the inflammatory process in JCA; a longitudinal study might clarify if changes in Ab oxLDL levels could mirror the progressive passage from the active to the inactive phase of the disease. Further studies are needed to confirm our findings and to verify the exact role of these antibodies.

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